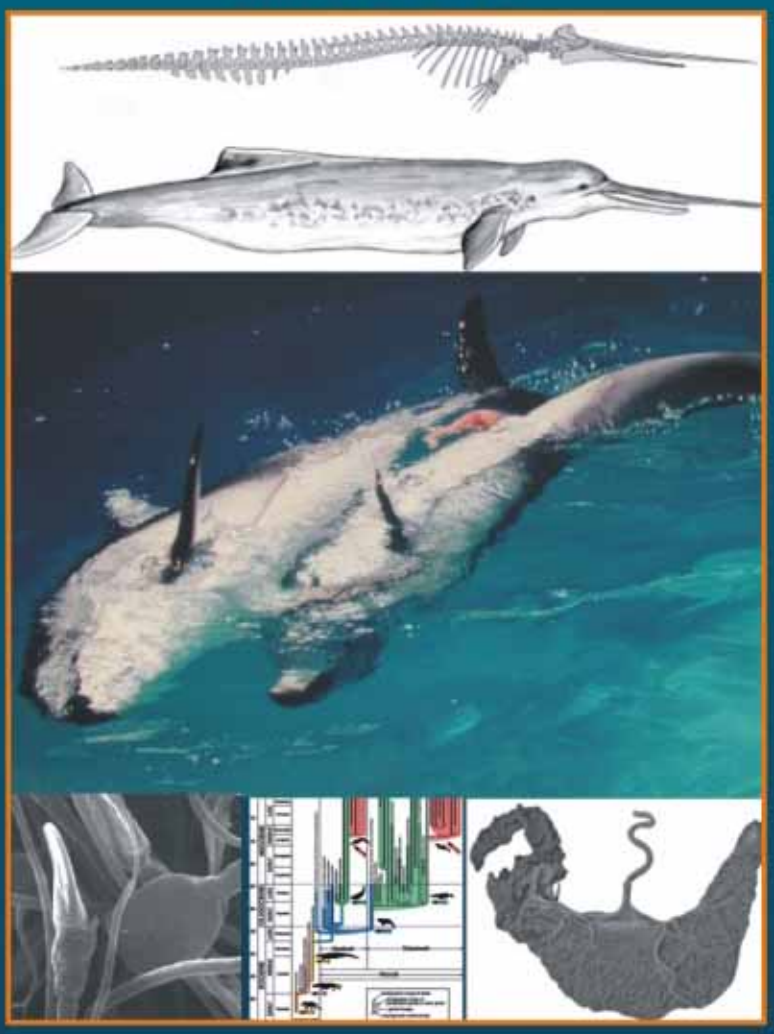
Reproductive Biology and Phylogeny of Cetacea

Whales, Dolphins and Porpoises

Volume edited by
Debra L. Miller



Volume 7 of Series:
Reproductive Biology and Phylogeny

Series edited by
BARRIE G.M. JAMIESON

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Vol. 7: Reproductive Biology and Phylogeny of Cetacea
(Volume Editor: Debra L. Miller)

Reproductive Biology and Phylogeny of Cetacea

Whales, Dolphins and Porpoises

Volume edited by
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Preface to the Series

This series was founded by the present series editor, Barrie Jamieson, in consultation with Science Publishers, in 2001 and bears the title '*Reproductive Biology and Phylogeny*', followed in each volume with the name of the taxonomic group which is the subject of the volume. Each publication has one or more invited volume editors (sometimes the series editor) and a large number of authors of international repute. The level of the taxonomic group which is the subject of each volume varies according, largely, to the amount of information available on the group, the advice of proposed volume editors, and the interest expressed by the zoological community in the proposed work. The order of publication of taxonomic groups reflects these concerns, and the availability of authors for the various chapters, and it is not proposed to proceed serially through the animal kingdom in a presumed "ladder of life" sequence. A second aspect of the series is coverage of the phylogeny and classification of the group, as a necessary framework for an understanding of reproductive biology. Evidence for relationships from molecular studies is an important aspect of the chapter on phylogeny and classification. Other chapters may or may not have phylogenetic themes, according to the interests of the authors.

It is not claimed that a single volume can, in fact, cover the entire gamut of reproductive topics for a given group but it is believed that the series gives an unsurpassed coverage of reproduction and provides a general text rather than being a mere collection of research papers on the subject. Coverage in different volumes varies in terms of topics, though it is clear from the first volumes that the standard of the contributions by the authors will be uniformly high. The stress varies from group to group; for instance, modes of external fertilization or vocalization, important in one group, might be inapplicable in another.

The first six volumes on Urodela, edited by Professor David Sever, Anura, edited by myself, Chondrichthyes, edited by Professor William Hamlett, Annelida, edited by Professors Greg Rouse and Fredrik Pleijel, Gymnophiona, edited by Professor Jean-Marie Exbrayat, and Birds (in two parts) edited by myself, reflected the above exacting criteria and the interests of certain research teams. This, the seventh volume, arises from the ever burgeoning interest in Cetacea. The controversial issue of whaling has barely been

touched upon but I look forward to the day when cetaceans are no longer exploited by man.

My thanks are due to the School of Integrative Biology, University of Queensland, for facilities, and especially to the Executive Dean of the Faculty of Biological and Chemical Sciences, Professor Mick McManus, for his continuing encouragement. I am everlastingly indebted to Sheila Jamieson, who has supported me indirectly in so many ways in this work. I and, I am sure, the scientific community are grateful to the publishers for their support and high standards in producing this series. Sincere thanks must be given to the volume editors and the authors, who have freely contributed their chapters, in very full schedules. Dr. Debra Miller is most gratefully thanked for her boundless enthusiasm, unfailing courtesy, and careful shepherding of the volume in the chief stages of editing. The editors and publishers are gratified that the enthusiasm and expertise of these contributors have been reflected by the reception of the series by our readers.

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

14 August 2006

Barrie G.M. Jamieson
School of Integrative Biology
University of Queensland

Preface to this Volume

This volume is dedicated to those amazing creatures we know as "cetaceans" in the hope that by learning about them through purposeful research, opportunistic observation, or fortuitous happenstance, we may gain the wisdom to share this world harmoniously with our fellow inhabitants.

The order Cetacea is composed of some amazing species, representing some of the most evolved creatures that inhabit this earth. Yet, they also represent a group of species for which much remains unknown; perhaps due to the difficulty of studying cetaceans within their natural environment or perhaps due to lack of available funding emanating from public indifference. Regardless, with the passing years has come increased public awareness of these fascinating creatures and advanced technology to make possible studies that once were impossible.

There are over 80 species of cetaceans composed of porpoises, dolphins and whales. This volume represents the latest of published and previously unpublished information regarding cetacean reproductive biology and phylogeny with data being added even just prior to press. Further, the information presented in these pages includes that gained through various means and under various conditions. Often data was obtained purposefully, either via planning and implementation of fact-finding missions or research. In other cases, data were obtained by chance, through unfortunate or untimely deaths. In yet other cases, data were obtained opportunistically in situations that often may be termed controversial, even by the scientists collecting the data. Obviously, a conflict-free world does not exist; yet we strive to reach that harmonious state of being. Ironically, it may be out of our fortuitously and often controversially obtained data, that we speed our progression toward a harmonious existence and in a backward sort of way render the respect due the cetaceans that provided us that information. As scientists we fit together pieces of a puzzle with multiple investigators working in unison. Perhaps we come from various scientific realms but still we add our valuable piece of data working toward the common goal of helping species survive.

Between the covers of this volume is a compilation of a diverse group of authorities from around the world. Each author presents their chapter in their own personal style. We start with the historical overview of Cetacea, provided

by Drs. Bianucci and Landini. This chapter represents a unique introduction to these amazing creatures following the historical accounts of facts and folklore, and I might add, making for an interesting read. It brings to light the fact that cetaceans have been part of our history from its conception and explores the many facets of humankind's treatment of these glorious creatures.

The search for the origin of any species, including our own, is an expedition of great undertaking. Fossil discovery along with the latest of molecular technology allows us to build more precise timelines than ever before. In chapters 2 and 3 of this volume the reader will find revelations that often correct or fine-tune what once we thought about cetacean origin. Bianucci and Landini follow the fossil history from the earliest discovery of the presumed origin of Cetacea in the early Eocene to the more recent Holocene, which has the occasional advantage of recorded history. Montgelard, Douzery and Michaux use molecular technology to classify cetaceans and then combined their findings with fossil and morphological data to provide us a phylogenetic understanding of the evolution of Cetacea.

Cetacean reproduction largely remains a mystery. We have only dented the surface toward understanding female reproductive anatomy and physiology and, for males, we have only scratched the surface. The chapters on anatomy offer us an overview of the cetacean reproductive system. Rommel, Pabst and McLellen provide us a tour through cetacean functional anatomy. They do this in a unique approach by comparison to the domestic dog. You will recognize Dr. Rommel's attention to detail and illustrative representations of the vascular structures. This is followed by Plön and Bernard's chapter on descriptive anatomy which historically has been provided only as fragments of partially described or sometimes poorly interpreted recordings gleaned from a spattering of necropsy specimens. In their chapter, Rommel *et al.* concentrate on the female but emphasize the importance of making use of specimens that were collected for other purposes so as to maximize the amount of information obtained from each valuable specimen.

From the hormonal influences of reproduction to courtship and mating rituals, and from spermatogenesis and oogenesis to fertilization, there have been concentrated studies and applications of techniques that once were applied only to humans. The authors covering these topics detail the intense investigation and experimentation that has been done to provide us knowledge of the factors influencing cetacean reproduction. Atkinson and Yoshioka provide us with knowledge of cetacean reproductive cycles that can be used to guide our understanding of their relationship to their marine environment. Great advances in our understanding of fertilization and ovarian development have been made through application of techniques that once were reserved only for humans. In his chapters, Fukui presents these applications and the current and potential value of this knowledge. Plön and Bernard and Miller, Styer, Kita and Menchaca provide us the current knowledge of the testicular cycles and unique features of spermatozoa from various cetacean species. Finally, Schaeff presents detailed accounts of the

unique mating strategies used by some species and provides interpretations in terms of possible benefits gained.

Probably one of the most fascinating facts that children learn (after they learn that whales are not fish!) is that most cetacean calves are born tail first and often there is another female present to help the newborn reach the surface for its first breath. Unfortunately, our knowledge of fetal development is limited but in the last decade great progress has been made, thanks, in part, to ultrasonographic studies on captive pregnant cetaceans. I still remember the first time that I heard Dr. Fiona Brook speak. I was fascinated by the wealth of information that she was able to glean from the seemingly simple and non-invasive procedure. The authors of the placental structure chapter, offer comments on the promise of this technique for expanding our understanding of fetal development. Likewise, there has been a recent thrust in the study of embryogenesis. Thewissen and Heyning take us on an excursion of embryogenesis based on museum collections and introduce us to the first-stage findings of a large project designed to document cetacean development. This study brings hope to expanding and elucidating the mysteries of early cetacean development.

Concurrent with study of the developing fetus is study of the placenta. Unfortunately, collections of well-preserved placentas have historically been rare, even in captive environments. The chapter on placental structure offers an introduction to macro-, micro- and ultra-structure from purposeful post-expulsion placental collections by trainers and veterinarians. These descriptions are compared to previous reports by fortunate researchers who had the unexpected circumstance of placental discoveries.

Ultimately, the knowledge gained from reproductive and phylogenetic studies will be combined with biological and ecological studies to better manage free-ranging cetacean populations. This concept is brought to light in the chapters on conservation and commercial exploitation by Hohn, Ewing and Zaias and life histories and population genetics by O'Corry-Crowe. Here too, we are reminded of the importance of making full use of collected specimens. Regardless of the tissue collected or the purpose of that collection, many additional bits of knowledge may be gained from that same specimen with additional testing. Such data could have profound impacts for future management of these species.

As with any project of this undertaking, this venture represents immense dedication by many individuals. First and foremost, this volume represents great effort by a group of dedicated scientists. The authors of the various chapters possess a passion for knowledge that is nothing but amazing. Their passion drives their respective quest as, earnestly, they seek to share with the world what they have discovered. True, the process of discovery often is ambiguous, but in the end, the product is knowledge and eventually, understanding.

In addition to the authors, many individuals helped behind the scenes and lent both proactive and retroactive advice and expertise. I would like to thank

the series editor, Dr. Barrie Jamieson for offering me this valuable opportunity and providing me support and guidance whenever I asked for it. Each chapter was read and reread by multiple individuals and I would like to thank them and specifically thank Dr Eloise Styer and my dear long time friend, Dr. Victoria Woshner, for their editorial assistance and expertise. Dr. Woshner's knowledge of Cetacea and good humor were helpful on more than one occasion. When one takes on a project such as this, they tend to take for granted the enormous amount of computer time, literature searching and printer usage that is necessary to complete the task, I would like to acknowledge the University of Georgia, especially Dr. Charles (Sandy) Baldwin, for supporting me in this venture, and Ms Krista Mattocks and Mr Ken West for technical assistance. Finally, many investigators were unfortunately not able to contribute as authors due to professional or personal conflicts or in some cases, nature made the decision for them, as with the 2005 hurricane season. Yet, those individuals were still supportive of this work and in some cases (Thanks Dr. Todd Robeck!) provided some of the latest information to be included in appropriate chapters. That was a wonderful gesture and is the mark of a true scientist who recognizes the need to share their information with the scientific community.

Because science is my passion and my life, I tend to shy away from insights into my personal life but in this case, I have decided to stray from that path and add a personal note. During production of this volume I and many of the authors were challenged with family emergencies and other 'life' events, the kind of things that force us to reflect on our own lives. My challenges left me feeling extremely grateful to be blessed with great parents (Jeanette and Ray Miller) that are still with me and remain strong with life even after their battles. Family and friends surround each of us and whether we like it or not, they have a major impact on our lives and often initiate or perhaps fine-tune our professional pathway. But for each of us, there tends to be one individual who is the most influential and shares our particular passion and compliments our life. With that said I would be remiss to not thank the one who is by my side providing me with endless moral support and inspiration and most importantly shares my passion for science and compliments my life….thank you Dr. Matthew Gray.

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Tifton, 14 August 2006

Contents

CHAPTER 1

Cetacea: An Historical Overview

Giovanni Bianucci and Walter Landini

1.1 INTRODUCTION

Myths, legends, hunting, and natural history, having a common and often mixed origin, provide the evidence that allows us to investigate the past relationships between man and cetaceans. This contribution is not meant to be an exhaustive analysis. Rather, it is intended as an integrated approach to elucidate the reasons for and the nature of these extraordinary relationships.

Different methodological approaches have been adopted by writers describing, time after time, the real and/or the fantasy world evoked by these animals. Among the poignant stories, myths, and legends included in this chapter, we recognize shared elements among oral testimonies and written documentation from different geographic areas, group them in homogenous classes and, when possible, follow their historical trajectory. It is interesting to note that the interactions between man and dolphins, or other small cetaceans, are accorded mutual respect worldwide. Less defined and less universally shared is the role of baleen whales and other large cetaceans: monsters of the abysses in the western cultures and good giants of the sea in the holistic and subsistence cultures of the Pacific and North America.

To describe the hunting history, we chose a comparative approach. Legends, intriguing stories and ancient traditions, some still in existence, tell us about these delicate and often difficult relations. Even if the aim of all fishermen is the capture of the prey and its alimentary use, there is no relationship between the subsistence whaling, managed by need, and the trade-industrial whaling, dominated by profit. For this reason, we prefer to separate these whaling practices. The same comparative and integrated method is the best approach to describe the different and complicated traditions and rituals that govern simple subsistence whaling activities; however, to describe industrial hunting we followed a chronological order. In

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fact, the sequence of the technological innovations is, in this case, the keystone to describe the rapid development of hunting activities and their effects on cetacean communities. Travel diaries, fishermen's and naturalists' stories, economic and scientific papers, and regulatory laws constitute the immense amount of literature produced in this field in the last centuries. We have reported only the part we considered useful to document the process in its chronological development, without pretending to be exhaustive.

At a minimum we analyzed the development of scientific studies. The transition from the informal to the scientific approach is neither linear nor sequential. In some cases, the informal approach never disappeared but remains even today. This is the case for the first scientific studies begun in the middle of the "Myth Ages." It seemed suitable to emphasize the ancient studies because they represent the basis of scientific thought and because they are easily delimited. With the development of cetology as a science, the quality and amount of contributions is so great that, in the economy of this chapter, it is impossible to supply an exhaustive picture of these studies.

In addition to several ancient works cited in the text, our principal sources are some recent contributions that deal with all or a part of the theses here presented. In particular, legends and stories related to cetaceans were reported by Thompson (1988), Constantine (2002), and Slijper (1979). Supplementary data are available in many web sites, such as that by Cressey (2000). Some classical papers about whaling, such as those by Tonneson and Johnsen (1982), Stoett (1997), and Ellis (1999), deserve to be cited. A more concise and general resume on whaling was made by Harrison (1988). Specific aspects of whaling are reported in several articles in journals or book chapters by many authors, such as Clapham and Baker (2002), Ellis (2002a, b), Kasuya (2002), MacLean *et al.* (2002). The history of whale research has been previously summarized by Slijper (1979), Matthews (1978), Berta and Sumich (1999), and Würsing (2002).

1.2 MYTHS, LEGENDS AND OTHER STORIES ON CETACEANS

Ancient traces revealing a direct knowledge of cetaceans go back to Prehistoric time. Neolithic engravings, such as those discovered inside Norwegian, Dutch and Italian caves, and on South Korean cliffs (Fig. 1.1), reveal a well refined artistic sense, while whale bones, found in the dumps of Danish villages, indicate an alimentary use both of hunted and casually stranded whales. During the Bronze Age, in some populations living in the Orkney Islands off the coast of Scotland, hunting was recognizably a very well developed and practical way of life. In fact, they used whale bones as beams for their buildings.

Cetaceans represent more than an important source of food for these ancient human economies. The peculiar behaviors of these marine creatures, so different from other animals, as well as their often imposing dimensions, generated curiosity and amazement, or evoked great fear. Traces of these

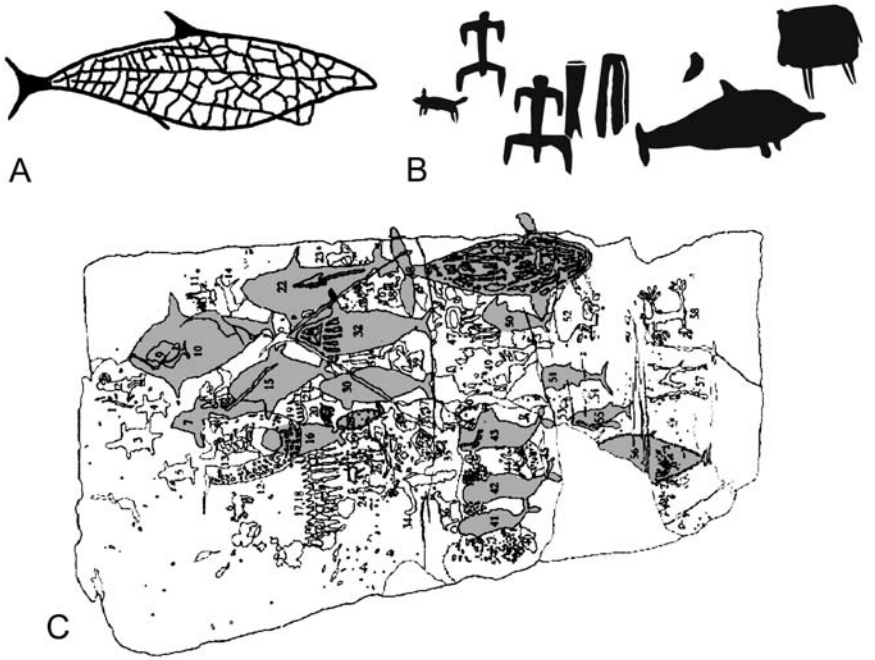

Fig. 1.1 Prehistoric rock engraving of whales. **A**. mummified dolphin, Åskollen, Vestfold, Norway. **B**. men, dolphin and other animals, "Grotta del Genovese," Egadi islands, Italy. **C**. Several whales (some highlighted in gray) and other figures, Bangu-Dae, South Korea. A. From Shäfer 1972. Ecology and palaeontology of marine environments. The University of Chicago Press, Chicago, 568 pp., Fig. 9 (modified). B. Original drawing. C. From Lee and Robineau 2004. L'anthropologie 108: 137-151, Fig. 2 (modified).

archaic traditions are still recognizable in some fishing rituals worldwide. It was, however, in the ancient Mediterranean culture that cetaceans enriched the mythological imagery with thought provoking legends. A number of attractive pictures coming from the Minoan and the ancient Greek world (Fig. 1.2) are tangible evidence of these legends.

Among different myths, legends, and true stories, cetaceans are described in four main ways indicative of their relationships with man over time: human metamorphosis and reincarnations, helpers of shipwrecked people and fishermen, riders of the sea, and carriers of ships and souls.

1.2.1 Metamorphosis and Reincarnations

1.2.1.1 Mediterranean sea stories

The dolphin-man metamorphosis is one of the most enduring themes in cetacean mythology and it can be seen as a return to a former condition, from which it is possible to emerge renewed. Some Greek deities simultaneously had human appearances and supernatural powers and often assumed

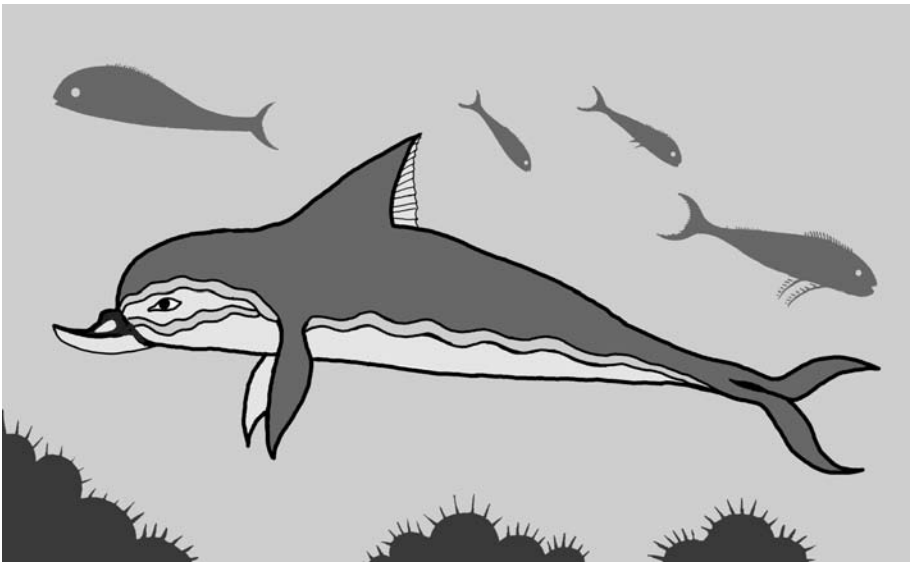

Fig. 1.2 Dolphin fresco in Queen's Megaron, Knossos, Crete (ca 1600 BC). Original.

dolphin appearances too**.** In Greek mythology dolphins were symbols of both the feminine element and womb; in fact, the Greek word "*delphis*" (dolphin) is closely related to "*delphys*" (uterus, womb). In addition, the idea of dolphins as a living womb of the generative water is often in opposition to, or identified with the other generative force: the sun. One of the most important legends connected to the mythological cycle of Apollo tells about these dualistic forces: water and sun. Apollo – god of the Sun – struggles against Delphyne, the dolphin-womb monster. He wins and founds Delphi (the town of dolphins), and after taking on the title of Delphinios, which means god-dolphin, he is able to control the generative womb. Throughout the Mediterranean Sea, Apollo, with dolphin features, looks for priests to honor his cult. He follows a Cretan merchant ship direct to Pilo and hijacks it to Crisa where he reveals his divine nature, changing himself into a young, handsome man and choosing the sailors of that ship as ministers of his temple. According to another mythological version, the founder of Delphi is Apollo's son Ikadios. He shipwrecks during a journey around the sea, but a dolphin draws him in safe near Mount Parnassus. There he founds Delphi, in honor of the dolphin which saved him. Another legend tells that Poseidon, god of the sea, assumes aspects of a dolphin. He does this to seduce Melantho, Deucalion's daughter. Their son is called Delphus, after whom Delphi was named.

The contradictory and uninhibited Greek Pantheon reserved for a special animal, the dolphin, an equally special ancestor: man. This legend goes back to 1500 BC, when poets and philosophers considered dolphins and whales as divine creatures or human soul reincarnations representing the vital force of

the sea. In fact, dolphins, reporting to Poseidon about the rescue of his son the poet Arion, said: "*Don't be astonished Poseidon at these ours good actions: we were men, before being fishes*..."

Dolphins as "remorseful men" are described in another well known legend. Dionysus, with human appearance, was captured by Etruscan pirates who wanted to sell him as a slave in Egypt or Cyprus. During the navigation the god revealed his real nature: invisible flutes began to play, the chains binding him fell from his body, paddles were transformed into snakes, bunches of grapes and ivy shoots covered sails and trees. Finally, the god transformed himself into a ferocious lion. The dismayed pirates jumped overboard in their terror and were already floating when Dionysus transformed them into dolphins. So, with this new aspect, the "remorseful pirates" became the sailors' rescuers, as the legend tells.

Also, in a Middle East legend we can recognize sexual implications; in fact, in this area, the Nabatean goddess, Galenaia was the object of a fervent cult. She represented the physical love born from the sea and she was usually associated with dolphins. Probably, this divinity derived from the fusion of the two older elements: the goddess Dolphin, announcing good weather, and the goddess Fish, associated with fertility.

1.2.1.2 Austral sea stories

From Greece to the Pacific lands, several common themes appear in ancient mythology. In addition to philological and etymological relationships or shared sexual implications, austral sea legends spread the belief in the instinctive and extraordinary ability of cetaceans to communicate with man. It is just for this reason that, in mythology, these animals play the intriguing role of reincarnations of human souls, representing the life force of the sea.

The Australian coastline is considered a holy place due to the presence of dolphins and whales. In fact, the local tribal names for many mainland places mean "dolphin dreaming sites." Moreover, some tribal people of southeastern Australia regard the dolphin as a sacred symbol or totem. This view resulted in some tribes historically engaging in a sort of cooperative fishing effort aided by the dolphins. It always has been forbidden to hunt or kill dolphins because dead souls are believed to inhabit dolphin bodies and remain offshore, helping and guiding human beings to land.

An aboriginal tribe of northern Australia believed their medicine men to be in telepathic communication with Bottlenose dolphins (*Tursiops* spp.), and only if these communications were maintained, were fortunes and happiness ensured. Dolphins and whales commonly appear in stories about the birth or creation of some tribes. In northern Australia, the origin of Groote Island's natives is celebrated in cave paintings dating back millennia. In the early days of the Dreamtime lived a very arrogant creature called Indjbena, the dolphin. Its unpleasant nature prompted small shellfish (Yakunas) to ask for help from Mana, the Tiger shark (*Galeocerdo cuvieri*). Eventually, the entire population of dolphins was killed and their souls left their bodies to become human beings

on land. Only a pregnancy female dolphin was spared, and her son, named Dinginjabana. Dinginjabana was the first of the friendly, intelligent dolphins we know today. The story tells that one day Dinginjabana's mother was swimming in the waters when she met Dinginjabana's father and they were both transformed into human beings. Later on, they had many children, who became the "Dolphin Tribe" of Groote Island.

Similarly, large whales play an intriguing role in aboriginal beliefs. For coastal tribes they are, like snakes, associated with fire, earth energy, wind, water, the sun, and the moon. To these "people of the whale," blowholes and caves are sacred because those were the apertures through which whale ancestors, coming from the Milky Way, made their first appearance on Earth.

People living in New Guinea tell the legend of Dudugera, which translates into English as "The Leg Child." The story deals with the son of a god and a woman. One day the woman was swimming in the sea and the god appeared to her with the aspect of a dolphin who brushed against her skin. He went between her legs, making her magically pregnancy and when the child was born, he was named Dudugera to underline his singular birth. When the boy grew up, he was mocked by people because of his origin, so he promised to destroy the world he was from, setting it on fire. One day, Dudugera flew beyond the sky and started to throw flames and, in doing so, he became the sun. His mother, fearing for her safety, found shelter in a cave. To save herself and the village, she threw mud toward him. This created the first clouds and darkened the sun, but at the same time, pacified the anger of her unhappy son.

1.2.1.3 Other stories around the world

Along the Amazon River, many people believe river dolphins (*Inia geoffrensis*) are able to transform into young men. This belief has been so strong that some children were thought to have been generated by these pink dolphins. Consequently it is taboo to hurt these revered creatures. Not all Amazonian people share this belief, however, and in Brazil these dolphins, called botos, are objects of black market trade.

From the equator to the Arctic, the myths go on. In northwestern North America some native people tell stories about the origin of Killer whales (*Orcinus orca*). *Orcinus orca* images occur in their masks, totems, carvings, blankets, and house screens (Fig. 1.3). In particular, Tlingit people of southeastern Alaska believe *Orcinus orca* was carved from wood by a man from the mythical seal people. Only the cetaceans that this man carved from yellow cedar were able to swim, and it is believed that he taught them to hunt but not hurt people. For this reason, the Tlingit do not hunt *O. orca* and believe the whales to be their guardians.

1.2.2 Cetaceans as Helpers

1.2.2.1 Helpers of shipwrecked people

Every myth includes real elements and, in the case of dolphins, their innate ability to communicate and their physical appearance are recognizable in

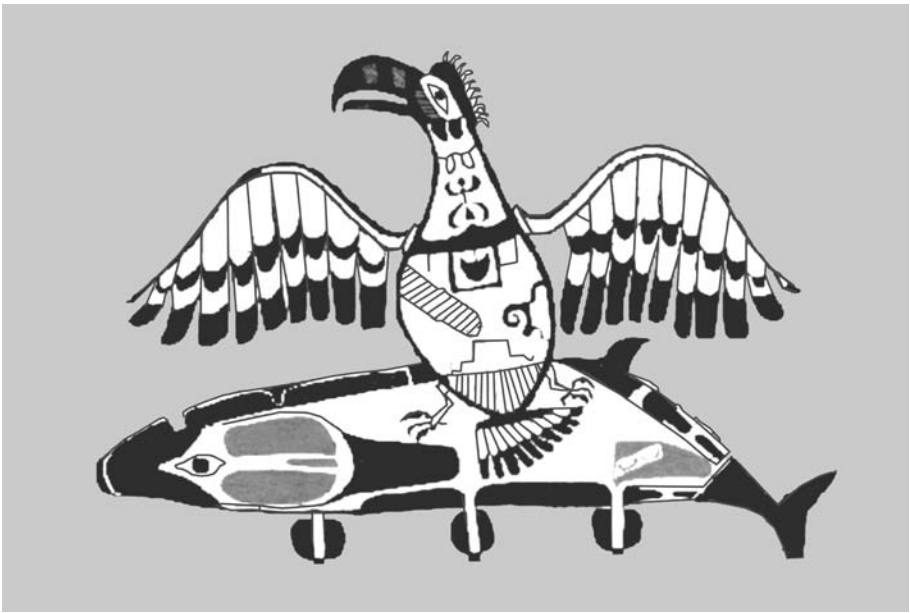

Fig. 1.3 Thunderbird carrying a whale from a painted house screen of Nookta people, Vancouver Island (late XIX century). Original.

some of the myths regarding the magical foundations of coastal towns, seaports, and sanctuaries. In all of these cases dolphins become a metaphor for friendly divine powers. They were considered by ancient Mediterranean people as fish of the calm sea able to save shipwrecked sailors and to be good friends to the seafaring people.

Greek mythology tells about rescue episodes and the more or less affectionate relationships between dolphin and man. Poseidon, who is always represented with dolphins, took advantage of their innate abilities as hounds and messengers. This Greek god fell in love with Nereus' beautiful daughter, Amphitrite, and abducted the woman to the island of Naxos. She succeeded in escaping and found a refuge on Atlas but a dolphin – sent by Poseidon to search for the nymph – persuaded her to marry the god. In return, Poseidon immortalized the dolphin in the heavens among the constellations.

From hounds to rescuers of shipwrecked people, the dolphin's mythological story goes on. A legend tells about Taras, another of the many sons of Poseidon, born from Poseidon's relationship with Minos' daughter, Satyria. After a shipwreck, Taras was saved by a dolphin and transported onto the coast of Italy, where he founded the town of Taranto. The image of the man riding a dolphin, which is reproduced on ancient coins, recalls this legend (Fig. 1.4). Pausanias (ca. 110-180 AD) described the same scene with a different protagonist in his *Description of Greece*. The Spartan, Phalantus, who was saved by a dolphin during a shipwreck and was taken to the coast of Italy, founded Taranto. Also Telemachus was saved by a dolphin and to

Fig. 1.4 Several ancient Mediterranean peoples reproduced dolphins on coins, both for their reputation as rescuers and as a symbol of equilibrium of forces. Some of these coins are shown in this original drawing. **A**. Olbia, Sarmatia (V-II century BC) bronze coin cast in the shape of dolphin. **B**. Calabria, South Italy (212-209 BC) Taras on dolphin and eagle. **C**. Roman denarius (I century BC) Taras on dolphin. **D**. Syracuse, Sicily (480-400 BC) Arethusa surrounded by four dolphins. **E**. Macedon (410-357 BC) dolphin. **F**. Syracuse, Sicily (IV century BC) dolphins. **G**. Syracuse, Sicily (344-336 BC) Pegasus and dolphins. **H**. Istros, Thrace (400-350 BC) sea eagle attacking a dolphin. **I**. Roman denarius (69 AD) tripod with a dolphin above and a raven below.

express his gratitude his father Ulysses engraved a dolphin on his ring and emblazoned one on his shield.

Another famous legend of the Mediterranean tells about Poseidon's son Arion, a poet and a very well-known lyre player. During his homeward journey from Sicily to Corinth, the sailors decided to throw him in the sea, in order to steal his fortune. Arion's last wish was to play a song and he threw himself into the sea after he finished. The dolphins, attracted by his enchanting song, saved him and carried him safely to Corinth. Since then, Arion and his lyre took their places among the constellations. Even if the poet Arion seems to have existed, this story probably has been invented to emphasize the figure of the Greek god, Melicertus, who, according to the myth, came to Corinth riding on a dolphin.

In other religions, dolphins are positive symbols. In Mithraism, an ancient Iranian religion, they are associated with Mithras, while in the Celtic religion they are the symbol of water's power. The special saving power of dolphins seems a firm attribute throughout the centuries. With the spread of Christianity, Jesus was represented under dolphin features as a symbol of the Resurrection. Dolphins are carved on christening fonts to represent Christ protecting men in the turbulent waters of life and leading them towards the shore, finally purified of their sins.

Cetaceans appear in hagiographic legends too: two dolphins took Saint Callistratus to shore when Diocletian ordered him thrown into the sea. A dolphin transported the body of Saint Lucian of Antioch and Saint Martinianus escaped lustful temptations by riding on a dolphin. This theme is reproduced in the mosaic pavement of the cathedral of Otranto, Italy. Nevertheless, in the Middle Ages the prohibition against eating dolphin meat during Lent was not connected with Christian symbology. Even if dolphins were considered fish, their fatty meat and warm blood were much too similar to "real" meat.

In every legend there are always some elements of truth. On the basis of these legends describing the rescue behavior of dolphins, we would expect a well-developed instinct for holding injured or sick companions at the surface. In fact, in particular cases, their instinctive behavior contributes to rescuing humans, because they treat people as if they were dolphins. In his *History of Animals*, for example, the Greek philosopher, Aristotle (384-322 BC), reports dolphins looking after young bathers to avoid misfortune or assisting sea victims.

Even now this helping behavior of dolphins is well known; in fact, nobody was astonished at the particular adventure experienced by a woman along the coast of Florida in 1943. According to a witness, she was floating but still alive when a dolphin took her ashore. An alternative possible interpretation is that the animal was just playing; in fact taking floating objects and unloading them on the beach is a well known preferred dolphin activity.

The famous Greek historian, Plutarch (ca. 46-120 AD), said in his *Moralia*: "T*o the dolphin alone, beyond all other, nature has granted what the best philosophers seek: friendship for no advantage."* Nowadays, it is known that dolphins have healing qualities to cure autism or psychosomatic diseases. In 1978, Dr. David Nathanson started a dolphin-human therapy program at Ocean World in Florida (Nathanson 1998). The results were startling. Children with Down's syndrome retained more and learned four times faster. Many therapists believe this was related to the dolphin's sonar which causes a phenomenon inside the soft body tissue of the human body called cavitation.

1.2.2.2 Helpers of fishermen

Pliny the Elder (23-79 AD), Pliny the Younger (61-112 AD), Plutarch, and other Roman and Greek writers, philosophers, and travelers described the

special friendship between men and dolphins. They described not only joyful meetings, but also mutually beneficial actions between dolphins and fishermen. From Nimes to Halicarnassus along the ancient Mediterranean coasts, dolphins helped fishermen to capture mullet and fishermen shared the harvest with them.

Pliny the Elder, in particular, in his *Naturalis Historia* tells us the way in which dolphins and men communicated with each other to catch fish in the ponds of Languedoc and how fishermen used to call dolphins with the name of Simon, derived from the Latin word "*simus*" that means snub-nosed. When fishermen called them, dolphins swam up and pushed the shoals of fish toward the nets, swimming around them to prevent their dispersal. At the end, the dolphins were rewarded with part of the catch.

Nowadays, this co-operative fishing continues on in some parts of the world, such as Brazil, Australia and Mauritania. In some Australian aboriginal communities, this apparently selfless assistance found a very intriguing connection with religious beliefs. A tribe living on Stradbroke Island, Australia, believed it shared a common ancestor with dolphins. This hero, a man named Gowonda, was transformed into a dolphin and thereafter helped his people with fishing. According to this legend, Gowonda was recognizable by his white fin, and this characteristic passed down to his descendants as a mark of the dolphin leader. During fishing, the tribesmen on the beach called each dolphin by name, communicating by special sounds and whistles. Dolphins drove the fish towards the nets and were rewarded for their help with part of the prey, for which they waited patiently in the fishing area. Unfortunately, when Europeans arrived in this territory, they learned the aboriginal whistles and sounds and used them for killing and eating these beautiful creatures.

1.2.3 Dolphin Riders

The stories about dolphin riders, so frequent in ancient legends, contain true elements, as more recent stories show us. Eros rode dolphin-back across the sea and Orion was carried to the sky riding a dolphin, when the gods rewarded him with three stars: the Orion's Belt. But the most famous story dealing with people riding on dolphins among the waves is the legend of Iasus. This unhappy story, set in the II century BC, deals with the love between a dolphin and a young man. Every day the boy rode on the dolphin in the waters, but one day he fell off the dolphin back and died when he was accidentally hit by the dorsal fin. The animal carried the boy's body onto the beach and died as well. The place was named Gulf of Iasus.

Besides these extraordinary sea-legends, the past gives us many real ancient chronicles that testify to the strong bond between men and dolphins. In spite of his skepticism about myths, Pausanias tells about the friendship between a dolphin, hurt by fishermen, and the boy who saved him. In this case, the dolphin not only followed the boy tamely, but also let the boy climb upon his back.

Another story about the friendship between a dolphin and a young boy is told by Pliny the Elder (*Naturalis Historia*). The dolphin arrived at the lake of Lucrino near Naples and every day he brought the boy on his back across the lake. Their friendship was so strong that, when the young boy got ill and died, for a long time the dolphin carried on searching for its young friend, until it died of a broken heart. This image of dolphins being ridden by young men was spread everywhere and in every time, always with the same pathos.

Not only dolphins offered their friendship to men, but other cetaceans did the same. Generally speaking, in our common imagery, baleen whales, *Physeter macrocephalus* (Sperm whale), and *Orcinus orca* caused unfounded fears, but in some cultures (Australian aborigines, Maori, and Arctic native peoples) they played a positive role similar to dolphins. Scenes, actors, and places change, but the ritual of these relationships is the same.

In northwestern North America, the Haida people tell about a wicked ocean people using *Orcinus orca* as canoes. One of the Haida chiefs was turned into *O. orca*. Thenceforth, they believed this cetacean protected them from ocean peoples' attacks.

Maori people believe their ancestors were carried safely on whales' backs across the Pacific to New Zealand. *Physeter macrocephalus* off the coast of the South Island are considered by the Ngai Tabu Maori as "taonga" (treasures). When a whale strands, they pray that its spirit returns to Tangaroa, the Maori Sea-god, and then they remove the lower jaw-bone and place it in the tribe's traditional temple "marae," for ceremonial carving. Another Polynesian legend describes the friendship between a Maori woman, Putu, and her two daughters with a *Physeter macrocephalus*, named Tokama, and its two young sons. This friendship caused the jealousy of the evil Kae, who killed Putu. Kae was captured by Putu's daughters, riding the two young dolphins, and then given to the priests to be condemned to death. Like other legends, this story tells about a world of harmony disrupted by human wickedness.

A sad story similar to Tokama's legend, but in modern and real terms, comes from New Zealand. In the early summer of 1955 in the Hokianga Harbour, a *Tursiops* spp. became a favorite, first of the local Opononi community, and then of its vacationing visitors. Known as Opo, the female dolphin reacted well to everyone she came in contact with, being particularly careful and gentle when surrounded by children. Thousands of visitors began to arrive every day on the beach of Opononi to see the shows Opo put on for them. Some people worried for their safety and the government passed a law limiting human interaction with dolphins. Only a few people agreed with the law, mainly fishermen blaming Opo for their empty nets. Like the Maori Tokama legend, this idyllic relationship between dolphins and men was interrupted by a wicked action. The day after the law was passed, Opo was found dead. During the night, a fisherman had blown her up with gelignite. The whole nation was devastated and the local community gave her a public funeral and erected a statue as a memorial of her loving spirit.

1.2.4 Carriers of Souls and Ships

Stories of the roles and attributes of these extraordinary animals abound in ancient Mediterranean lore with its multiplicity of gods. Their swimming, their flashes, and their disappearance into the deep sea seemed to ancient sailors an invitation to visit and to penetrate the secrets of a sunken kingdom. A legend says that Glaucus, a Greek sponge fisherman, disappeared after joining a merry group of dolphins while Theseus, guest of Amphitrite on the sea bed, received a gold crown surrounded by dolphins as is represented on Euphronius's cup (dating back to V century BC).

The ancient Mediterranean peoples gave dolphins the delicate role of carrying souls to their new life after death. The attribute of "carriers of souls," (psychopomp) given to these creatures is probably connected with their instinctive tendancy to help and rescue men at sea. In the Egyptian culture, the dolphin was an attribute of Isis, protectress of the dead and able to resuscitate the dead. The ancient Cretans believed their dead to reach the "Blessed Island," at the limits of the world, riding on dolphin back. Also, in Etruscan sepulchral art, dolphins are represented as carriers of souls to the "Blessed Island." A tradition, still current in some Greek villages, dictates that a coin with a dolphin image be put in the right hand of a dead person to ensure him a "safe journey" into the next world. Similarly, a Jewish sarcophagus of the II century BC, found in Beit Shearim near Haifa, was decorated with dolphins.

That these myths are simultaneously so ancient and so contemporary, can be understood because of the dolphin's innate ability to interact with man. This keystone remains valid as we consider the passage from myth to reality. An intriguing story from New Zealand demonstrates this particular ability. A Risso's dolphin (*Grampus griseus*), named Pelorus Jack, used to lead ships through the French Pass, a channel through the D'Urville Islands at the top of South Island. This dangerous channel, full of rocks and with strong currents, has been the site of many shipwrecks but none occurred when Pelorus Jack was at work. He began to lead ships through this narrow and dangerous channel in 1888, continuing for many years until a passenger of a ship called the "Penguin" took out a gun and shot at him. Despite this encounter, the Risso's dolphin reappeared and once again began to guide ship after ship through the channel, except for the "Penguin." When the "Penguin" appeared, the dolphin would immediately disappear.

1.2.5 Premonitors of Events

Stranded whales are reported in many medieval chronicles and generally looked upon as portents of positive or negative events. For example, Albert Krantz (1448-1517) reported that a young whale captured near Lùbeck in 1333 presaged the war between England and France, which broke out soon afterwards. Also, the sudden Swedish invasion of Holstein (1643) was foretold by the stranding of two *Orcinus orca*. On the other hand, Procopius of Cesarea (ca. 500-565 AD), the most important of the Byzantine historians, in

his *Bellum Gothicum,* looked upon the capture of a large whale near Byzantium as an omen portending the end of the Gothic war.

The Roman historian, Titus Livius (59 BC-17 AD), in his *Ab urbe condita libri,* narrates that during the Second Punic War between the Roman republic and Carthage, extraordinary natural events took place and were considered by Romans as premonitory of good luck. Among these, he reports that snakes of admirable dimensions danced on the sea, like joking fishes. These snakes could have been shoals of dolphins preceding the passage of large cetaceans. Still today, in fact, whales pass through these waters following the favorable currents.

1.2.6 The Abyss Bestiary: The Other Cetacean Face

The sea, with its mysterious and impenetrable abysses and with its furious storms, aroused fear and terror in all marine populations. In order to justify these ancestral fears derived from an environment known only by its surface, sailors and fishermen imagined a new enemy: the monster. They create the rich abyss bestiary, describing how monstrous were the marine animals fished, met, or just seen. Many archaic and mythological symbols and creatures took shape, and eventually the waters were filled with monsters. From *Physeter macrocephalus* and *Orcinus orca* to baleen whales fate could not reserve these mysterious symbols of the sea anything but the roles of monsters.

The ancient Mediterranean people believed cetaceans to be the mysterious abyss-keepers. The most feared keeper was the Leviathàn. The keepers were believed to have the power to change good days into unfavorable ones and to cause eclipses.

Mythology reserved a place of honor to Ketos (Latin: *cetus* = whale), the marine monster that Perseus killed to free Andromeda (Fig. 1.5). In fact,

Fig. 1.5 Perseus and Andromeda from Piero di Cosimo (ca. 1515).

ancient astronomers named some of the sky constellations after the characters of this legend. Those characters with named constellations include Cetus (Fig. 1.6A), Cepheus, Cassiopeia, Perseus, Andromeda, and Pegasus.

It was during the Middle Ages and the Renaissance that new monsters appeared in the seas. Olaus Magnus (1490-1558) in *Historia de Gentibus Septentrionalibus* dedicated a volume to the North Sea monsters, getting information from sagas and medieval folklore. These monstrous "fishes" (actually cetaceans) had horrible features and aroused fear with their thorns and the long horns over their head. They sank ships by hitting them with all their weight on the bow and on the stern. Scandinavian sailors believed that the fierce "Springhuals" would attack ships to feed on human meat. In addition, they believed "Physeter" to be able to stand on the waves and overturn ships. These two monsters would be identified respectively as *Orcinus orca* and *Physeter macrocephalus*. Olaus Magnus' whale illustrations inspired other contemporary renaissance scholars such as Conradus Lycosthenes (1518-1561), Conrad Gesner (1516-1565), and Ulisse Aldovrandi (1522-1605) (Fig. 1.6).

In the human imagination, there is a close relation between monstrosity and size. On land, ogres and dragons were gigantic, so in the sea the abyss monsters had to be huge and some cetaceans were suited to play this role. The big mouth and the half-surfacing back gave birth to two types of legends that we can define as: the "**Swallowing Mouth**" and the "**Monster-Island.**"

1.2.6.1 The swallowing mouth

The echo of legends about the mouth-that-swallows comes from the Book of Jonah, one of the Old Testament books bearing the name of a minor prophet. In Jonah (Bible in Basic English version) it is written: "And the Lord sent out a great wind on to the sea and there was a violent storm in the sea, so that the ship seemed in danger of being broken" (1,4). Then Jonah said to the sailors: "Take me up and put me into the sea, and the sea will become calm for you: for I am certain that because of me this great storm has come on you" (1,12). "So they took Jonah up and put him into the sea: and the sea was no longer angry" (1,15). "And the Lord made ready a large fish to take Jonah into its mouth; and Jonah was inside the fish for three days and three nights" (1,17). "Then Jonah made prayer to the Lord his God from the inside of the fish" (2,1). "And at the Lord's order, the fish sent Jonah out of its mouth on to the

Fig. 1.6 Whale illustrations from 1500s-1600s. **A**. Cetus constellation from Jan Hevelius's *Firmamentum Sobiescianum* (1690). **B**. Sperm whale (*Physeter macrocephalus*) rising above a ship from Olaus Magnus's *Historia de Gentibus Septentrionalibus* (1555). **C**. Whales attacking a ship from Conradus Lycosthenes's *Prodigorum ac ostentorum chronicon* (1557). **D**. "Ziphius" (probably *Orcinus orca*) devouring a seal from Conrad Gesner's *Historia Animalium* (1551-1558). **E**. "Aper marinus" (a whale with paws) from Ulisse Aldovrandi's *Mostrorum Historia* (1642).

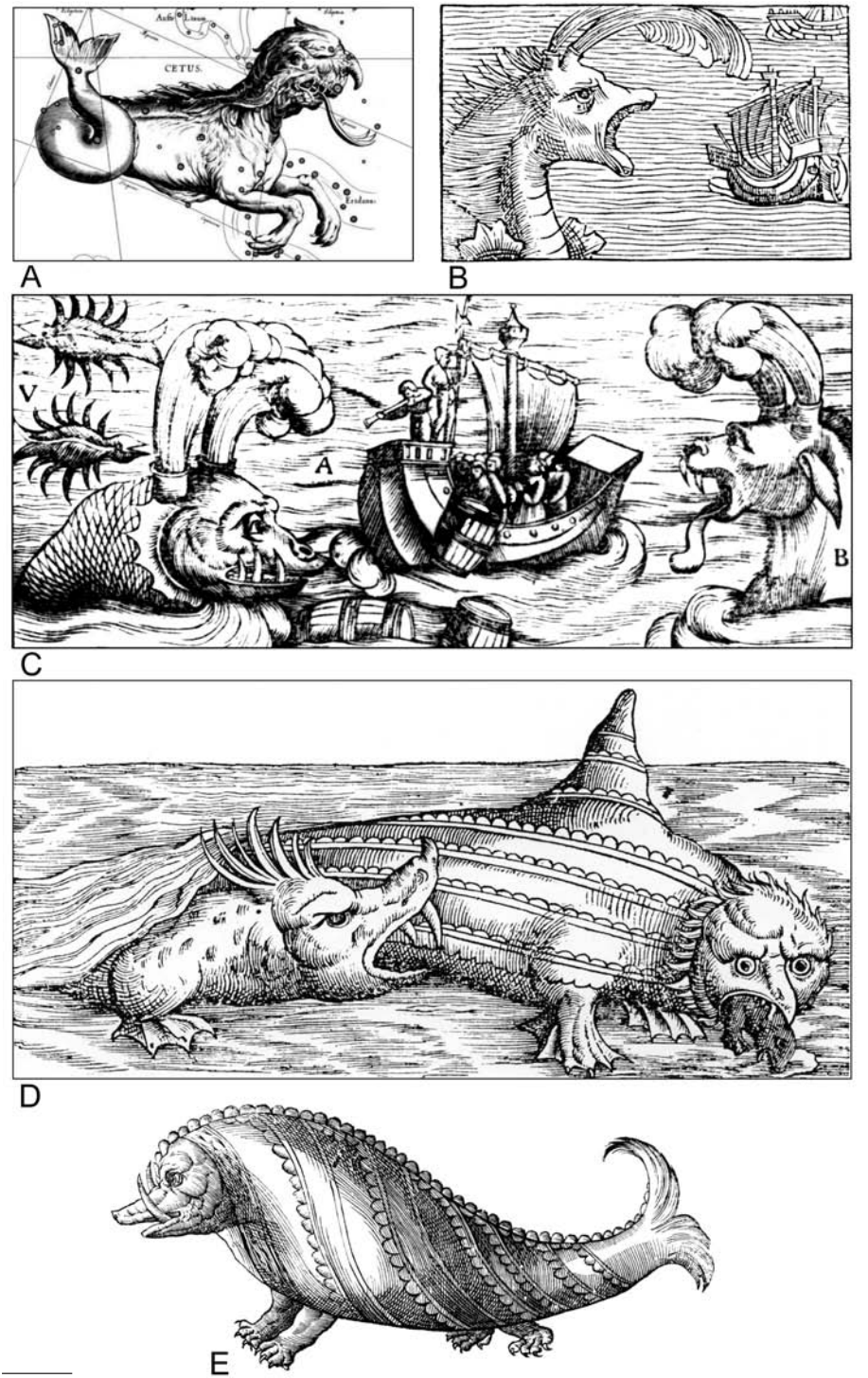

Fig. 1.6

dry land" (2,12). Medieval and renaissance iconography represented this big fish as a whale (Fig. 1.7).

Fig. 1.7 Jonah and the whale from Johann Dietenberger's *Die Katholische Bibel* (1534).

Lucian of Samosata (ca. 120-180 BC) in his *True Story* carries this type of legend to its logical extreme. The mouth of the monster is described like a wide and deep cave able to contain a town of ten thousand people. Inside, there is an island with marine birds, gulls, and kingfishes where men live together with the other savage and monstrous inhabitants.

Olaus Magnus, in his *Historia de Gentibus Septentrionalibus*, told about a large whale that swallowed and ejected torrents of water. Even cannonballs rebounded from its skin. But, like all big monsters, it had its Achilles' heel. Its eardrums were delicate and the sound of bells was sufficient to force it to flee.

1.2.6.2 The monster-island

The stories about Sinbad the sailor of the *Arabian Nights*, are fantastic tales of voyages. During his first voyage, Sinbad meets a whale-island. In the English translation by Sir Richard Francis Burton, we read "One day – tells Sinbad the Sailor – the captain dropped anchor near a beautiful island and we went ashore. We had hardly lit the fires to cook our meal when the captain suddenly shouted: Quick! Get away! This is not an island. It's a huge fish that's been sleeping on the waves so long that trees have grown on it. The heat from the fires is wakening it. It will dive to the deep immediately. Back to the ship! Drop everything! Many managed to climb aboard again, but I was

too far away and ended up at sea. Luckily, I found a floating empty barrel. Climbing to this and drifting with the winds and currents, I reached an island." Analogous to Sinbad's story is the seafaring legend of San Brandish, reported in *Navigatio Sancti Brandani* (Anonymous, IX-X century), that tells about a group of monks who went through the Atlantic Ocean, even before the Vikings, and landed on the back of a sleeping whale. Similar medieval chronicles tell of marine monsters on whose skin brush-woods grew up so that sailors mistook them for islands and docked their boats and lit fires. In doing so, the heat from the fires woke the animal-island, which submerged into the deep water, sinking or damaging the boats.

In modern literature, the role of these sea giants is not resolved. In Herman Melville's story *Moby Dick* (1851), *Physeter macrocephalus* represents different symbols to each character. For captain Ahab, who lost his leg hunting this animal, the cetacean is the personification of evil; for Father Mapple, it represents the biblical monster; and for Ishmael, the whale is at the same time, favorable and wicked, beautiful and horrible, vulnerable and immortal.

The Scottish "Nessie" is indubitably the most famous lake monster in the world, but Ogopogo's story, from the Canadian Okanagan Lake, also is surprising. The name Ogopogo is derived from a song, but Indians use the word "N'ha-a-itk," which means Lake Demon. Ogopogo sightings date back to the early XIX century and have been reported until the present. The monster is described as 15-20 feet long, with a horse or goat-like head. Some cryptozoologists have affirmed this creature to possibly be a primitive extinct whale (*Basilosaurus*).

1.3 CETACEAN HUNTING

It is important to note that not all contacts between cetaceans and ancient tribes were based on mutual friendship and respect. Scottish and Greenland ancient villages, built with whalebones, testify that the populations living in the North Atlantic or on the northern Pacific coasts (Eskimo, Aleut, Tlingit, Haida, etc.) acquired not only the main part of their food from cetaceans, but also the raw materials used in their daily life (skins, bones, fats, etc.). At the beginning, they exploited only cetaceans casually stranded on the coast, but then the high value of this prey induced the populations to hunt these animals. In the ancient Mediterranean Sea, so rich in traditions and myths on cetaceans, stories about hunting activities are quite incomplete. Certainly, whaling was practiced by Phoenicians, although ancient Greeks and Romans did not undertake it.

Subsistence hunting has been conducted for centuries at various latitudes with essentially unchanging techniques, until the XIV-XV centuries, when the first whalers started the whaling industry and the intensive exploitation of the mammalian communities. Subsistence hunting may be symbolized by the use of harpoons, the main and the easiest way for catching these sea creatures, a technique still surviving nowadays in some subsistence cultures.

On fragile boats, each man, by himself or in a group, faces the cetaceans directly, with a harpoon in his hand. More indirect methods also are used by these cultures to catch and kill whales, and it also is common to perform curious and intriguing rituals to show the particular bond between the tribe and these extraordinary animals. The XVI century practice of killing dolphins swimming near boats with harquebuses (primitive smoothbore matchlock guns) or cross-bows cannot be considered subsistence hunting, but only barbarous slaughter.

1.3.1 Harpoons

Since the Neolithic Age (ca. 6000 years ago), harpoon whaling has been practiced by different populations of the North Atlantic and North Pacific Oceans. For ancient people, cetacean hunting may have had a social role, as depicted in the engravings found in archeological sites, such as those of Norway and South Korea (Fig. 1.8A, B). Bangu-Dae (South Korea) engravings testify to the use of boats, ropes and harpoons to hunt baleen whales, sperm whales, and *Orcinus orca*.

Ancient Scandinavians theorized that the same harpoons used for catching reindeer could be used for cetaceans. The Inuit began to hunt cetaceans as soon as they learned to make harpoons that were reusable, i.e., that could be retrieved if they missed the prey. Smaller cetaceans, like *Monodon monoceros* (Narwhale), were hunted from small skin boats, called *kayak,* that carried one or two people using harpoons with buoys and floating anchors. Conversely, large whales were hunted by a very well organized crew on large skin boats called *umiak.*

The change from subsistence to commercial whaling began in northern France in the VII century, after the Norman invasion and the development of monasteries. In fact, for monks, *Eubalaena glacialis* (Right whale) represented a source of food, oil for lamps, and fat for lubrication. The specifics of whaling remain unclear until the XII century, when the Basques started to hunt *Eubalaena glacialis*, which was common in the Bay of Biscay. Harpoons and fish-spears were manually thrown to capture these mammals. The floating bodies of the killed whales were recovered easily using ships, called whalers. This hunting practice, based on traditional rules, respect for the prey, and the solidarity between man and the sea, still survives from the Azores to the Tonga islands, where whaling maintains an ancient tradition. As soon as a whale is sighted, it is approached by multiple canoes and, when its back emerges, a man jumps on it and strikes the whale with his harpoon. As the animal is dying, all the canoes are rapidly tied to the rope of the harpoon to provide resistance and prevent the escape of the wounded animal.

1.3.2 Like a Trap

Aristotle tells that the barbarians, whom he considered to be all non-Greek people, used to trap and catch dolphins by making a great noise. Recent

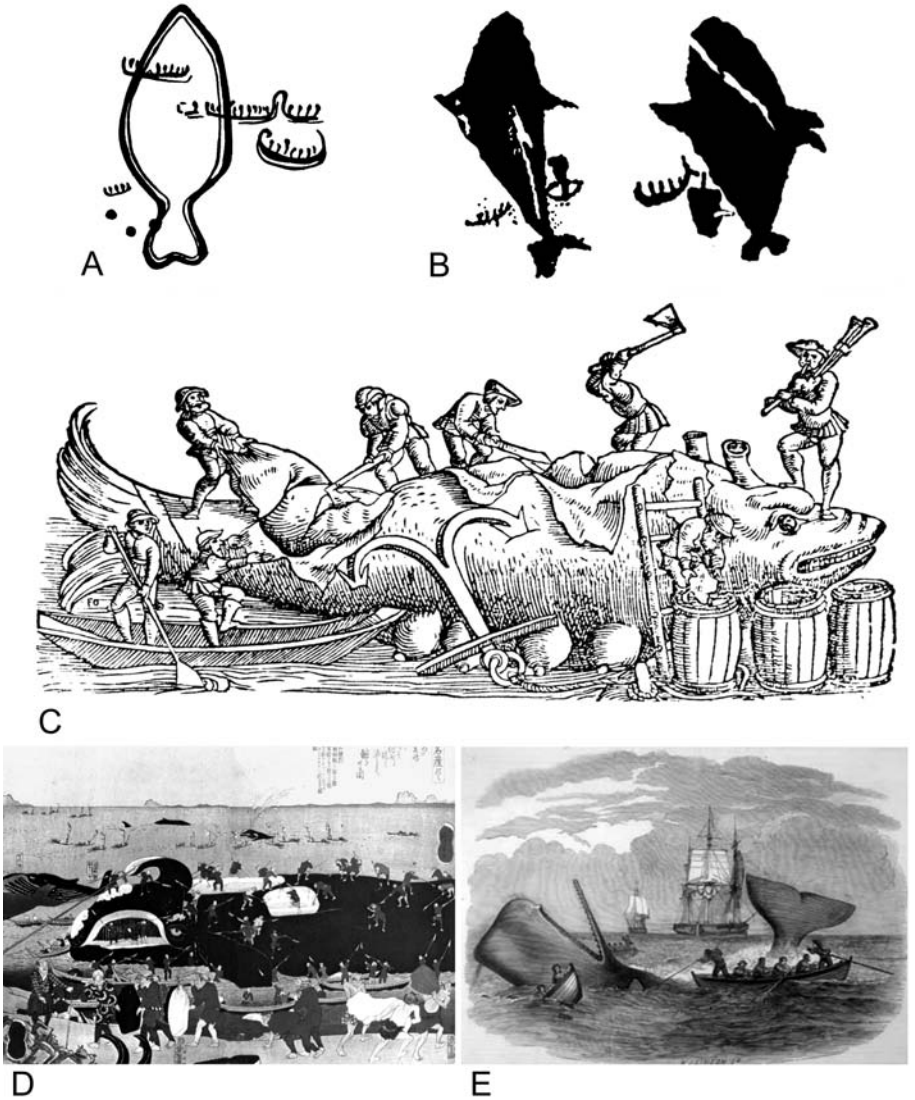

Fig. 1.8 Whaling illustration from the past. **A, B.** Prehistoric rock engravings in Meling-Rogaland, Norway (A) and Bangu-Dae, South Korea (B), showing the capture of large whales by manned boats; **C.** Whale dissection from Conrad Gessner's *Historia Animalium* (1551-1558). **D.** Whale dissection from a Japanese drawing of 1798. **E.** Whaling of a sperm whale (*Physeter macrocephalus*) in the South Seas from *Illustrated London New* (1847). A. From Shäfer 1972. Ecology and palaeontology of marine environments. The University of Chicago Press, Chicago, 568 pp., Fig. 13 (modified). B. From Lee and Robineau 2004. L'anthropologie 108: 137-151, Fig. 6 (modified).

archeological evidences found in Ra's al Hadd (Oman), strongly suggests that both *Tursiops* spp. and common dolphins (*Delphinus delphis*) were usually chased into a weir that was built across a lagoon by local communities. This whaling strategy also was used for large cetaceans. Around I century BC, Icelanders and Vikings in the North Atlantic and Japanese in the North Pacific Ocean used to trap their prey in fiords by closing the entry with fishing-nets. The cetaceans were killed with arrows. In the Faer Oer islands, between Scotland and Iceland, this strategy still survives as an old and traditional hunt involving many people. As soon as a pack of *Globicephala melas* (Pilot whale) reaches the coast, the sound of a horn calls the fishermen and the hunting starts. A small fishing fleet surrounds the pack, trapping it into a fiord. Then, armed with sticks and knives, the people throw themselves into the water from the shore or from their boats, and a massacre begins. Evidence suggests that an estimated 300 to 1700 animals have been killed in this manner each year since 1584.

1.3.3 A Shamanist Hunting: The Ocean Present

One of the strangest dolphin-hunting customs, also a magic-religious ritual, is practiced by Oceania tribes. In their tradition, the dolphin is a sacred animal and its killing is a sacrilege, except when the dolphin offers himself according to the gods' will. These tribes believe that they know the secret rhythm and gestures needed to lure the dolphins to the beach and this hunting is considered not a "killing" but a holy act in honor of the dolphins which sacrifice themselves to men.

In the Marquesas Islands, as soon as dolphin dorsal fins appear, some men make noise with rocks under water to confuse and scare the animals, which swim towards the shore where it is possible to catch them with no difficulty. Also in this case, the submissive behavior of the dolphin is considered a spontaneous sacrifice to the men, who receive the animals with songs and cries of joy. But as soon as the dolphins are carried onto the beach, this magic-religious representation ends and the animals are killed with knives, stones, and sticks.

The ritual used in the Gilbert Islands (Micronesia) was far more complicated. The approach of a dolphin pack was not considered a casual event, but was telepathically led by shamans. Native people thought their shamans had the power to communicate with these animals in their dreams. They also believed that their souls left their bodies to go looking for dolphins to invite them to a party in the village. Once the dolphins reached the coast, the ritual was similar to that described for the Marquesas Islands: first celebration and great welcome then slaughter.

Regardless of the details, all of these complex hunting ceremonials share a deep respect for the killed animals and celebrate whaling as an activity that gathers the whole tribe and makes all families work as a team.

1.3.4 Fasts, Penances, and Sexual Abstinences

The whale capture, so important to the survival of subsistence cultures, is a tragic event that breaks the harmony between the human and cetacean communities. Therefore, propitiatory rites and/or penances are required to expiate the sin of cetacean killing. This attitude is common to all subsistence cultures that recognize a Supreme Being as the creator of life and that follow rules and principles to make whaling favorable. James George Frazer (1854-1941), in his famous book, *The Golden Bough,* tells us that, before whaling, the North America natives of Nootka Sound fasted for a week and bathed many times a day, rubbing their faces with shells and spines in order to have a torn appearance. Similarly, whalers from Madagascar purified themselves for eight days. Fishermen confessed all their sins and, not to endanger the outcome of their whaling, they kept away those fishermen who had committed too many severe sins. Likewise, sexual abstinence was observed. In the Caroline Islands (Polynesia), the fisherman was considered taboo, such that during the fishing period, neither his wife nor other women were allowed to see him. Taboos and restrictive rules continued until the end of the fishing period, when fear and anxiety for the killing turned to joy for the good fishing.

1.3.5 The Power of Immortality

The whole social structure of the Makah tribe, living near the town of Neah Bay on the northwestern United States coast, is built around hunting. A purifying ritual dance precedes hunting and follows catching. Hunting success is celebrated by sharing the catch in a sophisticated, but at the same time informal way, with each family getting different parts of the animal.

In North Alaska, Inuit people, as in many other traditional hunting societies, use charms or amulets to ensure their luck and safety. They also give back dead whale skulls to the sea in order to assure the whale's immortality, reincarnation, and protection following whaling. Another custom is for the leader and a crew member to temporarily exchange wives in order to increase cooperation among fishermen.

Similar to the Inuits, other Arctic populations feel a strong sense of regret after whaling. To compensate, they give back some parts of the whale body to the sea, hoping that the animal will come back to life, or that the animal god will not be aware of the killing. The Eskimo of the Bering Straits consider the executor of whaling to be impure, and consequently he is not allowed to work amongst or touch anything in the tribe. All other members of the tribe behave as if the whale were still alive, speaking and offering food to it. Some populations of northeastern Siberia celebrate the same rite. They believe that the killed whale has come to their village of its own accord and they behave as if it were still alive. Moreover, some parts of the whale body, like fins or tails, are thrown into the sea or onto a tree to cover up the act of killing the whale, which is considered a depredation of the Supreme Being. In this way, they believe that they are giving the whale back to the animal's god. In Alaska,

according to Aleut traditions, the fisherman who kills the whale stays alone for four days in his hut, reproducing the whale's cry. At the end of this period, he takes a bath in the sea, shouting and hitting the water's surface with his hands. At last, part of whale body is thrown to the sea to hide the whale's agony from the animal's god.

1.3.6 The Commercial Whaling

The trend from subsistence hunting to more organized and efficient hunts started in the VII century on the Atlantic coast of France. In the XII century Basque populations give rise to systematic whaling in the Bay of Biscay. In fact, catching large cetaceans as a regular industry needed not only considerable skills, but also organization and equipment. The first commercial whaling ships were constructed around the XV-XVI centuries, and sighting towers were built on the coast to aid whalers in detecting the whale's presence.

Eubalaena glacialis were easily caught because of their slow movements and because they lived in groups. Furthermore, they floated after being killed, making it possible to drag them onto land with little difficulty. After two centuries, these whales disappeared from the Bay of Biscay, and the Basques were forced to begin pelagic hunting with sailing ships carrying the traditional whalers. This transition from coastal to pelagic hunting gave birth to a systematic and intensive exploitation of vast oceanic areas.

Since the XVII century, all the major sea-powers (Basques, Dutch, Englishmen, and Norwegians) continued whaling, first in the North Atlantic, especially in the waters between Greenland and Spitzbergen islands, and later in the North Pacific and Bering Sea. Whalers were mainly Basques, Danish, Dutch, English, French, German, Norwegian, and Portuguese, sometimes working together, but more often quarreling over whaling rights and prey sharing. The solution was dividing the coasts, giving each nation a whaling area. Similar to the cities of Amsterdam, Flushing, Middleburg and others, the Dutch founded a whaling town named Smeerenburg or "Blubber Town" in Spitzbergen islands. The Dutch obtained whaling supremacy, having the most ships (300) and the most men (18,000). The sea became a place of battles among European powers to obtain hunting rights. Eventually, France and England gained supremacy, leaving behind the Netherlands, whose activities in the North Atlantic ended in 1798.

North American colonists discovered the value of stranded whales around the first half of the XVII century, spawning a new interest in the whaling industry on Nantucket and Long Island using simple ships. Before the end of the XVII century, whaling was well organized and sighting towers had been built on the coasts. However, this changed in 1712 when *Physeter macrocephalus* was killed and taken to the harbor. *P. macrocephalus* were numerous in the Atlantic Ocean, but larger ships and well organized crews were required to hunt them.

The first factory ship was constructed between the XVII and the XVIII centuries, because hunting needed more efficient organization. The killed cetaceans were processed with rudimentary techniques alongside the ships. Around 1760, ovens were built on deck and were used to transform blubber into oil. These activities could be carried out in calm seas or near the coast where temporary bases were fitted out.

Ultimately, intensive hunting on *Eubalaena glacialis*, *Balaena mysticetus* (Bowhead whale), and *Physeter macrocephalus* decreased the number of these cetaceans in the Atlantic and Pacific northern waters. But a rising demand for raw materials, which supported a flourishing industry, was in conflict with the decline of these cetacean communities. The answer for the whaling industry was expansion of hunting territories towards the southern waters, and the addition of hunting of rorquals (Balaenopteridae), thanks to some technological innovations. Prior to the first half of the XIX century, these whales were not hunted mainly for two reasons: first, they were too fast and dangerous for whalers, and second their carcasses did not float, unlike other large cetaceans. So, since the XIX century, the South Seas, in large part unexplored until the XVIII century, were now exploited by American and European fishing-fleets.

The transition from commercial to industrialized whaling began in 1863, with the use of a cannon that fired a 100 lb explosive harpoon and was mounted on the bow of a 90 ft steamship. The harpoons had a long cable for holding the prey. Compressed air was blown into the whale's thorax and abdomen using air pumps so that the prey would float making it possible to tow them to the bases on land.

1.3.7 "The Big Whale War"

At the beginning of the XVIII century, exploratory routes started to cross the southern waters and, as for other great geographical explorations, the main motivation was the economics rather than the pleasure of geographic and scientific discovery. Whales themselves induced men to sail towards the Far South to discover the so called *Terra Australis Incognita.* In fact, the fierce hunting of American, English, Norwegian, French, Japanese, and Russian whalers made it harder to find prey and the previously unexplored southern waters became hunting waters. For a long time whalers did not reveal the secrets of these far oceans full of whales feeding on the abundant krill. The first whaler making an honest report was James Weddell who, in 1823, surveyed the Orkney Islands (discovered a year before by another whaler) and ventured to the South as far as latitude 74° 15' in the sea bearing his name.

The exploitation of southern baleen whales at the beginning of the XX century increased thanks to the creation of a base in the sub-Antarctic island called South Georgia and to factory-ships moored in seaports near the hunting areas. Even if the pelagic whaling industry did not entirely replace processing on the land bases, it was the main reason for the decline of

southern cetaceans. In 1911, an initial concern issued by the British Museum of Natural History stimulated scientific research on cetacean slaughter, specifically, slaughter of *Megaptera novaeangliae* (Humpback whale) in the Antarctic waters. By the beginning of the 1930s, the whaling industry had 41 factory-ships and more than 200 speedboats equipped with light guns with explosive harpoons.

The first international Convention for the Regulation of Whaling was formulated in Geneva in September 1931 to limit unrestrained hunting; however, neither this nor subsequent conventions gained force because not all countries signed the agreement. Between 1931 and 1945, the aims of the international conferences were: the defense of young whales, the preservation of *Eubalaena glacialis* (Right whale), *Eschrichtius robustus* (Gray whale) and *Megaptera novaeangliae* from extinction and, at last, to limit the activity of factory ships. Antarctic catches were regulated, establishing the "Blue whale unit" to limit the numbers of cetaceans which were allowed to be hunted: 1 *Balaenoptera musculus* (blue whale) corresponded to 2 *Balaenoptera physalus* (Fin whale), to 2.5 *M. novaeangliae*, or to 6 *Balaenoptera borealis* (Sei whale).

In 1946, the "International Whaling Commission" (IWC) was founded to decide the maximum sustainable use of whale stocks and to defend the future of the stocks. The IWC estimates statistical data yearly to set capture limits. Again, not all countries signed or observed the agreements. Fortunately, even with improved hunting techniques, the inability to process great numbers of whales in a small time frame serves as an internal limiting factor to whaling.

After 1945, British whaling decreased and by 1965, it disappeared completely. Similarly, Norwegian whaling decreased but remains active. Japanese and Russian whaling increased after the Second World War, and continued until 1960. Unfortunately, subsequent large whaling industries were established in Peru, Chile, Australia, and South Africa.

Since 1950, improved hunting techniques have allowed fishing fleets and factory ships to operate, as well as land bases, making whaler actions free and uncontrolled. Hunting has been very well organized: helicopters sight the prey and relay the location to the whalers who surround the whale with probes and frighten it with ultrasound. The prey was killed with explosives or electric harpoons. This efficient method drastically decreased the last Antarctic reserves.

In 1975, the IWC divided whales into stocks based on level of protection according to the "New Management Policy," whose goal was to define exploitation quotas so as to not exceed the maximum sustainable use of each stock. Subsequently, the IWC became more conservative and tried to stop all pelagic whaling activities until whale stock increases could be supported by scientific evidence. In 1979, moratorium measures against whaling and factory-ships were adopted and came into force in January 1986. Nevertheless, in 1987, Japan, Iceland, and South Korea were still catching whales for "scientific purposes."

In 1993, Norway raised objections against the moratorium, started whaling, and eight years later participated in the international trade of whale meat and blubber. In 2000, Japan extended "scientific" whaling to *Physeter macrocephalus*, *Balaenoptera edeni* (Bryde's whales) and *Balaenoptera acutorostrata* (Minke whale), and, in 2002, included *B. borealis*, which is already on the way to extinction. In the same year, Iceland became a member of IWC and, opposing a reserve against the moratorium that was valid until 2006, began "scientific" whaling of *B. acutorostrata* in 2003. Perhaps before deciding on monitoring systems for whale stocks, an international agreement should be reached regarding the preservation of cetacea, rather than considering them as a mere resource.

1.3.8 Can Chemistry Save Whales?

In the past, the majority of the profit obtained from whale trade was derived from oil and, to a lesser extent, meat and bones. Whale oil had many uses, including lighting and lubrication, as well as the production of soaps and industrial margarine. Baleen plates were processed to whalebone used in corsets, umbrellas, and shoehorns. Teeth and bones were carved and decorated as scrimshaws. Other remains were used as animal food and fertilizer. Sperm-oil was transformed into solid waxes and used to produce candles, while ambergris, a waxy substance originating in the intestines of *Physeter macrocephalus*, was used as a fixative in the cosmetic industry. Today, most of these products have been replaced by synthetic substances and, ironically, cetacean numbers may ultimately be protected in large part due to the efficacy of these chemical products compared to traditional ones.

1.4 THE NATURAL HISTORY

1.4.1 Early Writings

For centuries people from all over the world have celebrated and sung of whales and dolphins. The first who wrote about cetaceans was Aristotle. In the *History of Animals,* he described whales, dolphins, and porpoises as cetaceans and distinguished them from fishes for having a blowhole instead of gills, generating embryos, being viviparous, and producing milk. This detailed description means that Aristotle directly observed these animals and dissected some specimens. Moreover, he described one of the first non-lethal techniques used by fishermen for cetacean identification and age evaluation. According to the Greek philosopher, fishermen used to capture dolphins and nick their tails, before letting them go freely, so that later identification was possible.

About one century after Aristotle, the Baiji (*Lipotes vexillifer*) was described by scholars of the Han Dynasty in the first Chinese dictionary, *Er-Ya,* as an aquatic mammal found in freshwaters. A more detailed description of this river dolphin was given by Guo Pug (276-324 AD) in his *Annotations to Er-Ya*.

Surprisingly, this book reveals that the almost extinct baiji was very abundant in that period.

The Roman naturalist, Pliny the Elder, in *Naturalis Historia*, recorded some of the first descriptions of cetacean pulmonary respiration, even if the anatomic particulars were not exact. Moreover, Pliny wrote that whales utter sounds similar to human voices and love to be called Simon ("*simos*" in Greek means flat-nose). Pliny described porpoises as similar to dolphins but with a sad appearance and a slothful behavior: "They are not playful or jumping like dolphins, they are similar to snarling dogs." About large cetaceans, Pliny reports minimal original information instead getting most of his data from Aristotle. Pliny exaggerates their dimensions, reporting measures ten times larger than *Balaenoptera musculus*, the largest living cetacean. He also described a fantastic symbiosis between whales and the "marine mouse" or *Musculus marinus*, similar to that known for the *Naucrates doctor* (Pilot fish), which usually swim with large marine animals like sharks and cetaceans. According to Pliny, *M. marinus* acts as an organ of sight by swimming in front of the whale, whose eyes are obstructed by their eyelashes, to warn them about shallow waters. Finally, Pliny describes *Orcinus orca* as the terror of all marine animals because they persecute baleen whales, eating their tongues and flippers.

1.4.2 The Dark Years

After Pliny, cetacean studies were completely neglected for many centuries. The complex animal world of the medieval culture resulted from the confluence of two tendencies: scientific knowledge, begun with Aristotle, and mythical-magic beliefs inherited from Oriental cultures, and including Hellenic and Roman cultures. Accordingly, the cosmos was woven with hidden relationships linking stars and animals. On the base of these models, the Middle Ages' "Imaginary Zoology" developed from the great encyclopedic works born between the VII and the XII centuries and the pre-scientific attitude of the XIII century.

Only between the XII and the XIII centuries did cetacean researches re-establish contact with the science of Aristotle. The German philosopher Albertus Magnus (1193?-1280) in *De Animalibus* founded his classification of animals on the Aristotelian one, based on advancement and articulation of the organs. He considered whales and dolphins the most perfect marine animals, because they were *parentia* and *spirantia*, that is, mammals with lungs. In the XII century, *The Cambridge Bestiary* summarized the acquired knowledge on cetaceans by stating that dolphins are considered fish that respond to human voice or music, and assemble in groups. They are the quickest sea animals and encircle boats with great jumps. Common tradition believes that dolphins are storm messengers.

The first tribute to whales, including original naturalistic observations, was *Speculum regale* or *Kongespeil*, which was written in Iceland in 1240. This book is about North Sea cetaceans, but it also shows for the first time the differences

between *Eubalaena glacialis* and *Balaena mysticetus*. Nevertheless, in the following five centuries zoologists continued to confuse these two species that were correctly identified only by whalers.

1.4.3 The Rise of Science

During the Renaissance, the rapid increase of ocean explorations was followed by several scientific publications. Pierre Belon in his *Histoire naturelle des éstranges poissons marins avec la vrai paincture et description du dauphin et de plusieurs autres de son espèce* (1555) described cetacean anatomy in detail but still classified whales as fish. Guillaume Rondelet in *Universae aquatilium Historiae* (1555) affirmed the difference between cetaceans with lungs and fish with gills. After comparing cetacean anatomical structures with those of other mammals, such as pigs and men, he reach the conclusion that cetaceans are "not true fishes" but an "aquatic quadruped."

After Aristotle, the first important work on the animal kingdom was written by Conrad Gesner. In *Historia Animalium* (1551-1558), his chapter that deals with fishes also described cetaceans, and was taken from Belon and Rondelet. In this work, Gesner portrayed the absurdity of many mythical animals; however, according to Medieval and Renaissance culture, some parts of his writings are based on fact because the marine monsters he drew are based on Olaus Magnus illustrations (Fig. 1.6B).

In the XVII century, a large part of the world was still unknown and voyages to discover new lands were frequent. The voyage chronicles and the works about fishing give information on the different cetacean species, hunting techniques and processing methods, while no information is given about their anatomy and behavior. One of the best works is *Spitzbergische oder Groenlandische Reise-Beschreibung gethan im Jahr 1671,* published in 1675 by Friederick Martens, whose drawings engraved on copper have been reproduced for centuries in many publications.

In these years, stranded whales were the main source for material for anatomical studies. Thomas Bartholin in his *Historiarum anatomicarum rariorum centuria I et II* (1654-1661) described the dissection of a pregnancy porpoise, underlining the close analogies with human beings. Other exhaustive porpoise and dolphin dissections were published in this period by John Ray and Johan Major. Another significant contribution to the study of cetacean anatomy was given by Edward Tyson who discovered the *retia mirabilia* ("wonderful nets"). Caspar Bartholin Jr., reviewing the book by his father Thomas, *De Unicornu Observationes Novae* (1678), concluded that the mythic unicorn horn was actually a narwhal tooth.

In the last decades of the XVIII century, other original anatomical works were published. Among these, *The Structure and Physiology of Fishes*, published by Alexander Monro Secundus in 1785, illustrates the dissection of porpoise anatomical parts and organs with original engravings. A more important work was *Observations on the Structure and Economy of Whales* by John Hunter, published in 1787 in the Philosophical Transactions of the Royal Society of

London. Hunter dissected some specimens of *Tursiops* spp. and was the first to describe the anatomical structures and ontogenetic development of *Balaenoptera acutorostrata*.

Some significant contributions on cetacean research in the XVIII century were the result of explorations and whaling activity. A complete history about whaling in Northern seas, with the description of fleets and hunting data, was *Bloeyende opkomst der aloude en hedendaagsche Groenlandsche Visschery*, written by Cornelis Gisbert Zorgdrager in 1720. Later, the naturalist Georg Wilhelm Steller was among the first Europeans to explore Alaska and the Aleutian Islands. In his *The Beasts of the Sea* (1751) he reported scientific observations about several marine mammals.

1.4.4 The Prevalence of Anatomical Studies

In the XIX century, cetology became more and more important thanks to an increasing number of scientific papers mainly focused on systematics and anatomy. If contributions about cetacean biology were relatively scarce, a new interest grew about paleontological research. In that century a very high number of works were published, many in scientific journals, but here we will cite only the most significant.

In 1820, a posthumous edition of the original studies by Peter Camper, *Observations anatomiques sur la structure intérieure et le squelette de plusieurs espèces de cétacés,* contained new information about the skeleton and soft tissues of different species. The English explorer, William Scoresby published two works about the Arctic: *An Account of the Arctic Regions with History and Descriptions of the Northern Whale-Fishery* (1820) and *A Journal of a Voyage to the Northern Whale-Fishery* (1823). The first one is a basic work about the scientific studies on the oceans and their fauna, especially cetaceans. Scoresby, in fact, was the first man of science to approach large cetaceans in their natural environment. In twenty years of whaling and dissection, he learned much about *B. mysticetus'* anatomy, behavior, and feeding. The second work is an illustrated description of porpoise and *B. mysticetus* anatomy.

Other original and significant contributions to cetology, in relation to whaling and explorations, were given by Thomas Beale in *The Natural History of the Sperm Whale* (1835) and by Frederick Debell Bennett in *Narrative of a Whaling Voyage Round the Globe* (1840). *The History of the American Whale Fishery*, published in 1878 by Alexander Starbuck, is a treatise on American whaling from its origins until 1876. Down through the years, Starbuck reported a list of all whalers, their expeditions and the size of their captures.

New observations on living and fossil cetacean skeletons were made by two great naturalists, the French Georges Cuvier and the English Richard Owen. In particular, *Le Règne Animal* (1817) and *Recherches sur les ossements fossiles* (1823) by Cuvier contained comparative anatomy descriptions of several cetaceans and also new descriptions of both extant and fossil species.

The Englishmen William Henry Flower and John Edward Gray published, in 1866 and 1885, respectively, two important catalogues about the whale

specimens kept in the British Museum. Between 1868 and 1879, two great European scientists – the Belgian zoologist Pierre-Joseph Van Beneden and the French paleontologist Paul Gervais – published *Ostéographie des cétacés des mers d'Europe*, a monograph about skeletal anatomy of fossil and extant species (Fig. 1.9). Other significant European contributions on cetacean anatomy were given by the Danish naturalists Daniel Frederik Eschricht and Johan Theodore Reinhardt. Moreover, whales that stranded continued to be a precious source of anatomical studies that often were accompanied by beautiful illustrations (Fig. 1.10).

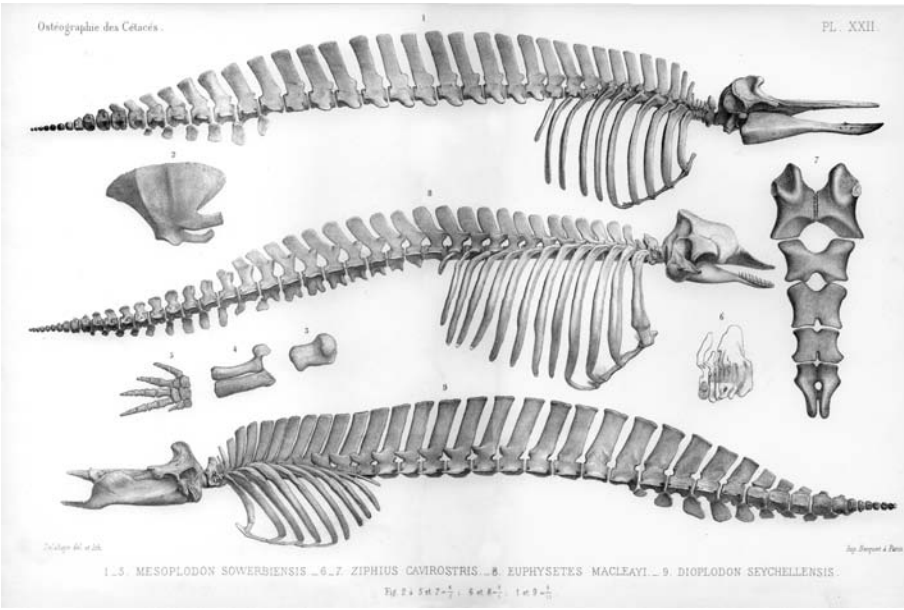

Fig. 1.9 Skeletons of beaked whales (Ziphiidae) and pygmy sperm whales (*Kogia breviceps*) from a plate of *Ostéographie des Cétacés vivants et fossiles* of P. J. Van Beneden and P. Gervais (1880).

In the United States of America, the studies by Edward Drinker Cope and William H. Dall are noteworthy. A significant contribution also was written in 1874 by captain Charles M. Scammon who described *Eschrichtius robustus* while analyzing its biology and its hunting techniques on the coast-lagoons of California in *The Marine Mammals of the North-Western Coast of North America* (1874).

Besides these zoological and anatomical works, a literature based on natural history treatises and on encyclopedia-like works was developed. We can include in this last category: the *Histoire Naturelle de Cétacés* (1804) by B. G. É. de la Ville Lacépède, the *Histoire naturelle générale et particulière des mammifères et des oiseaux vol. I* (1828) by René Primevère Lesson, *The ordinary Cetacea or whales* (1837) written by an anonymous author, and *The natural*

Fig. 1.10 Bowhead whale (*Balaena mysticetus*) caught near Taranto (Italy) in 1877 from Capellini, G. 1877. Memorie dell'Accademia delle Scienze dell'Istituto di Bologna, Serie 3, 7: 1-34, Pl. 1.

history of the order Cetacea and the inhabitants of the Arctic regions (1834) by Henry William Dewhurst. In the last work, the author also reported new observations taken during an expedition to Greenland in 1824.

1.4.5 Cetacean Conservation Awareness

During the first decades of the XX century, the works by Frederick William True and G.E.H. Barrett-Hamilton gave a great fillip to cetacean researches. In 1904, True published the original results of his research at Newfoundland station in *The Whalebone Whales of the Western North Atlantic*. In 1913, Barrett-Hamilton completed his study about baleen whales of southern seas, working at the coastal-stations of the island of South Georgia. The reports by Barrett-Hamilton, published in 1925 by M.A.C. Hinton, pointed out the excessive whaling in the Antarctic. Very interesting was the information about reproductive biology and hunting of *Megaptera novaeangliae*, which in 1913 was on the way to extinction. In the years following, research and data collecting sought to underline the increasingly uncertain condition of cetacean populations in fishing areas and to support the international committees in promulgating protectionist laws.

An investigative project, started in 1925, gave rise to 26 years of *Discovery* expeditions. The aim was to increase the knowledge on cetacean reproductive biology and Antarctic ecology for a more efficient management policy for sustainable exploitation. Despite the success of the research, the objectives of the project remain unrealized. By the time the Discovery investigations were completed, other scientific committees had provided the whale populations'

status (e.g. IWC, the U.S. Marine Mammal Commission since 1972 and the European Cetacean Society since 1987).

1.4.6 Modern Cetology

After World War II, a new phase began for cetology, thanks to new methodologies and innovations in oceanographic research and, mainly, to the re-discovery of these animals, no longer considered hunting resources. In this phase, one of the first great works on cetaceans was the book by A. G. Tomlin about Russia and adjacent countries. It was originally published in Russian in 1957 and later translated into English (1967). Subsequently, a branch of whale research developed that obtained useful information from captive cetaceans. Examples of these captive studies include dolphin echolocation (Au 1993) and cognitive capabilities (Tyack 1999). More recently, whale investigations are focused on studying these animals in their natural habitat. These free-ranging studies are possible thanks to advanced microelectronic technologies (e.g. satellite telemetry and time-depth recorders).

Some books (e.g., Norris 1966; Anderson 1969; Ridgway 1972; Matthews 1978; Slijper 1979; Gaskin 1982; Evans 1987) provide up-dated and exhaustive general information on whales. Recently, a general picture on cetacean studies was provided in the Handbook of Marine Mammals edited by Ridgway and Harrison (1985, 1989, 1993, 1998). The most comprehensive scientific accounts on cetacean research of the last years, however, are perhaps the works written by Rice (1998) and Berta and Sumich (1999), and the books edited by Reynolds and Rommel (1999), Hoelzel (2002), and Perrin *et al.* (2002). Other significant works focused on specific topics, such as those written by Fraser and Purves (1960) on cetacean hearing and those edited by Harrison (1972, 1974, 1977) on functional anatomy and by Thewissen (1998) on whale origins. Moreover, some books are dedicated only to a single cetacean, such as that edited by Leatherwood and Reeves (1990) on *Tursiops* spp. and that written by Whitehead (2003) on *Physeter macrocephalus*. However, as pointed out by Berta and Sumich (1999), it is hard to synthesize all the cetacean investigations of the last decades. In fact, research encompasses all sorts of biological-naturalistic studies, from systematics to ecology, from behavioral science to functional morphology, from evolutionary biology to paleontology. Unfortunately, it is impossible to give an exhaustive bibliography of all these studies, even in summary; however, in the following chapters, some of the most stimulating fields of cetacean research will be investigated with the help of useful references.

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CHAPTER 2

Fossil History

Giovanni Bianucci and Walter Landini

2.1 INTRODUCTION

In one of his essays published in 1994, Stephen Jay Gould described, in his exciting style, the contemporary discoveries of the oldest whales. He emphasized their importance in the demolition of one of the strong points of the creationists' theories against Darwinism: the presumed lack of fossil evidence in the transition between land mammals and whales. In fact, the discoveries of the last two decades, especially those made in Pakistan, India and Egypt and published by Philip D. Gingerich, J.G.M. Thewissen, Mark Uhen and others, have revealed the transitional phases that occurred over 15 million years, from terrestrial mammals to whales perfectly adapted to aquatic life. Therefore the origin of whales represents one of the best-documented examples of macroevolution.

These discoveries have drawn the attention of scientists of several branches of Natural Sciences to the fossil history of the cetaceans. Under this impulse much research has been done, and some results on phylogeny, functional morphology, embryogenesis, and other topics already have been published using a multidisciplinary approach to the paleontological data. The number of paleontologists interested in cetacean studies increases each day and the attention is focused not only on archaic whales but also on the more specialized toothed and baleen whales.

Fossil cetaceans have been known for more than four centuries. The first described and illustrated fossil was a mandible fragment with three teeth of a squalodontid from the Miocene of Malta. Curiously this fossil was described and figured (Fig. 2.1) in one of the first scientific contributions to paleontology, the famous "*La vana speculazione disingannata dal senso*" written by the Italian Agostino Scilla in 1670.

Nevertheless, serious studies on fossil cetaceans began only about 1800. One of the first detailed descriptions appeared in 1824 – the *Recherches sur les ossemens fossiles* by Georges Cuvier. Research considerably increased in the

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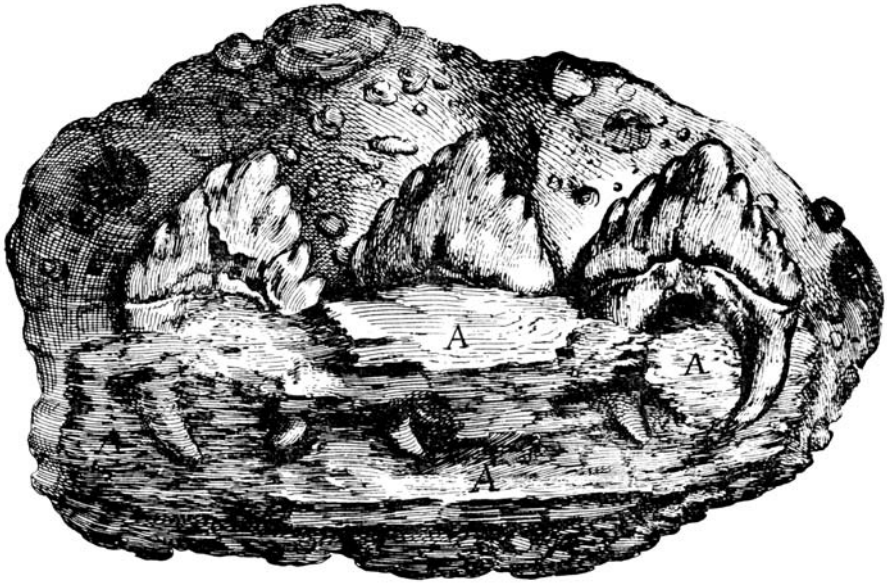

Fig. 2.1 Earliest illustration of fossil cetacean. Fragment with three teeth of a squalodontid mandible of the Miocene of Malta from Agostino Scilla's *La vana speculazione disingannata dal senso* (1670).

second half of 1800 and in the early 1900s, particularly in Europe, with the contributions by many authors, like Othenio Abel, Johann F. von Brandt, Giorgio Dal Piaz, Giovanni Capellini, Paul Gervais, Frederick W. True and Pierre-Joseph Van Beneden.

Later on and up until 1970, the number of scientists who published on fossil cetaceans was curiously very scarce. In the United States most of the contributions of that period were by Remington Kellogg, author of several publications and, among these, a monograph on archaeocetes (1936) and an important general review of fossil whales (1928). After Kellogg, the contributions of a relatively small number of paleontologists, including Karlheinz Rothausen, Lawrence Barnes, Ewan Fordyce, and Christian de Muizon, anticipated and supported the recently-expanding interest in fossil whales.

2.2 STRATIGRAPHICAL AND GEOGRAPHICAL DISTRIBUTION

The fossil record of cetaceans covers a time interval of about 50 million years. In the relative time scale, this interval represents most of the Cenozoic Era, corresponding to six epochs (from Eocene to Holocene). Fossil whales have been found on all continents, Antarctica included. The most significant fossil sites are located in some restricted areas of Europe (Belgium, Georgia, and Italy), America (USA, Peru, Chile and Argentina), Africa (Egypt), Asia (Japan, Pakistan, and India), and Oceania (Australia and New Zealand) (see

Fordyce and Muizon 2001 and Fordyce 2002d for a detailed description of the most significant localities).

The stratigraphical distribution is not homogeneous. In some intervals of time, the fauna is well known (e.g., Early-Middle Miocene) while in others the fossil record is very scarce (e.g., Early Oligocene). Eocene fossil whales have been found mainly in Pakistan, India, Egypt, and the United States. Fossils from Pakistan and India are the oldest and they principally were collected in the last twenty years from four sedimentary deposits: the Kuldana Formation (northern Pakistan), the Domanda Formation (central Pakistan), and the Subathu and the Hrudi formations (northwestern India). Eocene fossils from Egypt have been collected near Cairo in the nummulitic limestones outcropping at the Gebel Mokattam and southward at the Fayum in the Gehannan and Sahaga formations. All Eocene whales from the United States were collected on the eastern and Gulf coasts (South Carolina, Louisiana, Alabama, etc.).

Entering into the Early Oligocene stratum, fossil whales are very rare but by the Late Oligocene they are relatively common, although generally localized to a few areas. The poor and fragmentary Early Oligocene cetaceans were collected in New Zealand, France, Austria, and Washington State (USA). The most significant whale assemblages from the Late Oligocene are those of Waitaki Valley (New Zealand), Caucasus (Georgia), and some localities of the United States.

Next was the Miocene stratum. Fossil cetaceans from Miocene marine sediments are very abundant in several localities around the world. The most significant are those of Antwerp Basin (Belgium), Salento Peninsula and Belluno (Italy), Chesapeake Bay and Californian Sharktooth Hill (USA), Pisco-Sacaco areas (Peru), and Patagonia (Argentina).

Pliocene cetacean localities are less numerous than those of the Miocene. The most significant are in Tuscany and Po Valley (Italy), San Diego (California), and Pisco-Sacaco areas (Peru). Further, Pleistocene-Holocene fossil cetaceans are rather rare and generally consist of fragmentary remains. Specimens from Japan and California are probably the most significant.

In any case the analysis of the known fossil record allows one to reconstruct with some reliability the principal phases of the history of whales from the origin and first radiation of the basal archaeocetes to the emergence and establishment of the modern fauna.

2.3 THE ANCESTORS OF WHALES

Within the last decade, the unanimous opinion among paleontologists was that the ancestors of cetaceans were Mesonychia, an extinct group of hoofed mammals (ungulates) living in the Northern Hemisphere during the Paleocene-Early Oligocene [about 60-30 million years ago (Ma)] (Van Valen 1966; Szalay 1969; Gatesy and O'Leary 2001) (Fig. 2.2). Cetacean-mesonychian affinities have been deduced principally from details of dentition and the ear region.

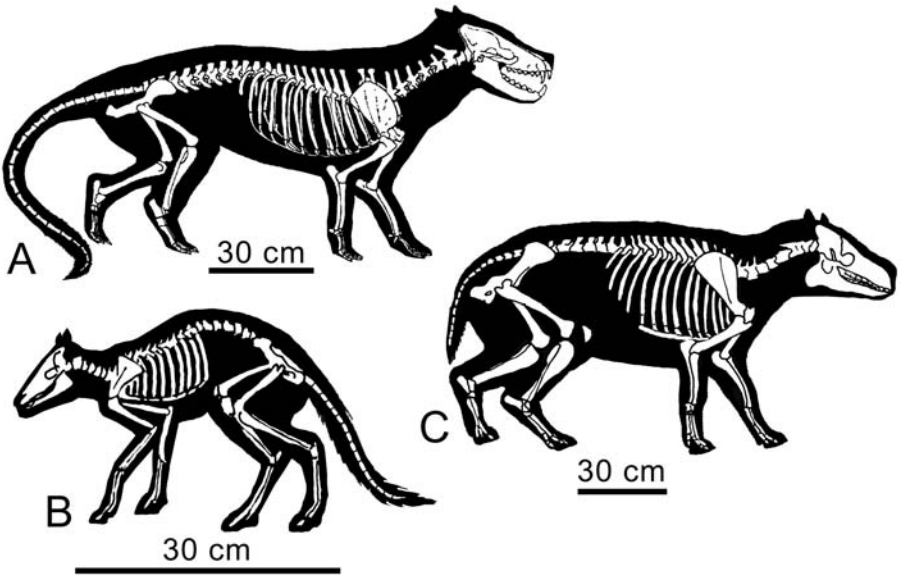

Fig. 2.2 Extinct hoofed mammals that may be closely related to whales. **A.** *Mesonyx* (mesonychian). **B.** *Diacodexis* (earliest perissodactyl). **C.** *Elomeryx* (possibly ancestor of hippopotamids). Skeletons of *Mesonyx* and *Elomeryx* are from Carroll 1988. Vertebrate paleontology and evolution. W.H. Freeman and Company, New York, 698 pp., Figs. 21.14, 21.28 (redrawn). Skeleton of *Diacodexis* from Rose 1982. Science 216: 621-623, Fig. 1 (redrawn).

Over the last 25 years, the discovery of several well-preserved skeletons of basal cetaceans and some cladistic analyses based on morphological and/or molecular data partially supported, but partially refuted, the mesonychian hypothesis. In particular, the discovery of an almost complete skeleton of the basal cetacean *Ambulocetus natans* revealed that cetaceans originally had paraxonic feet (Thewissen *et al.* 1996a). This discovery caused the rejection of the previous hypotheses of a close affinity between cetaceans and Perissodactyla (all with mesaxonic arrangement) (Prothero *et al.* 1988; Thewissen 1994), but it did help to understand that whales might be more closely related to Artiodactyla or mesonychians, both with the paraxonic arrangement of the feet (Fig. 2.3).

Concurrently, some cladistic analyses based on molecular data supported a close cetacean-artiodactyl affinity, interpreting cetaceans as the sister taxon of hippopotamuses (Gatesy *et al.* 1996, 1999; Montgelard *et al.* 1997; Shimamura *et al.* 1997; Gatesy 1998; Nikaido *et al.* 2001; Arnason *et al.* 2004). In this context the traditional artiodactyls were considered a paraphyletic group including the cetaceans, while the mesonychians were reinterpreted as archaic ungulates with some convergent features but not in close relation with the earliest cetaceans. The hypothesis of closer relationships between hippopotamuses and whales is also supported by a recent cladistic analysis

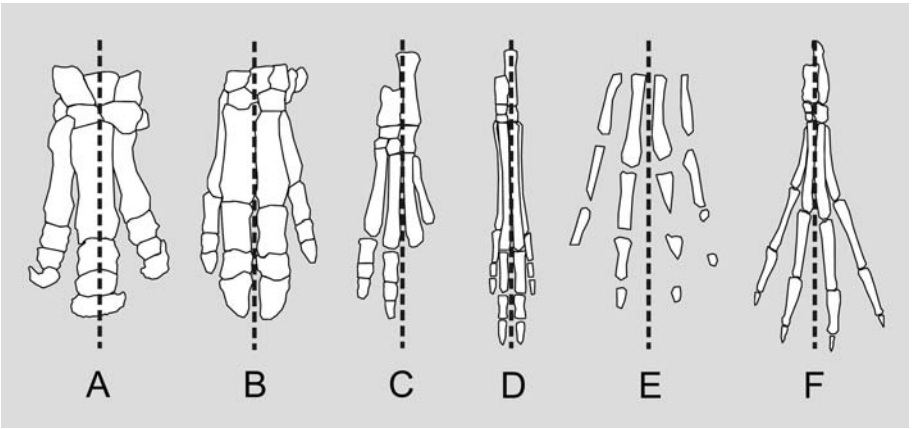

Fig. 2.3 Mesaxonic (**A**) and paraxonic (**B-F**) foot arrangement in ungulates (**A-D**) and archaic whales (**E-F**). **A**. *Rhinoceros* (rhino). **B**. *Sus* (pig). **C.** *Pachyena* (mesonychian). **D**. *Diacodexis* (earliest artiodactyl). **E**. *Ambulocetus* (archaic whale). **F**. *Rodhocetus* (archaic whale). C-E from O'Leary 2002. Pp. 735-738. In W. F. Perrin, B. Wursig and J. G. M. Thewissen (eds), Encyclopedia of Marine Mammals. Academic Press, San Diego, California, Fig. 3 (redrawn). F from Gingerich *et al.* 2001. Science 293: 2239-2242, Fig. 2C (redrawn).

based on morphological data (Geisler and Uhen 2003). The data of the cladogram presented by Geisler and Uhen is here utilized to draw a phylogenetic tree (Fig. 2.4) including the stratigraphic ranges of most of the considered taxa. Because monophyletic sister taxa have the same time of origin, this tree shows ghost lineages – inferred gaps in the fossil records. In particular if we admit a whales-hippos sister relationship, the ghost lineage of the hippopotamuses is longer than 40 million years (Theodor 2004).

Other recent analyses based on both molecular and morphological data of extant and fossil taxa reevaluate the mesonychians as a sister taxon to cetaceans (Geisler and Luo 1998; O'Leary and Geisler 1999; O'Leary and Uhen 1999; Luo 2000; Gatesy and O'Leary 2001). Moreover, in these studies the mesonychian-cetacean clade is interpreted as a sister group of the monophyletic artiodactyls. According to the authors of these studies, hippopotamuses are the mammals most closely related to cetaceans, not considering the extinct mesonychans. The phylogenetic tree supporting this hypothesis, presented by O'Leary and Uhen (1999) and here simplified (Fig. 2.5), indicates ghost lineages of about 10 million years for both cetaceans and the monophyletic artiodactyls.

Other recent fossil discoveries reveal close affinity among the basal cetaceans and archaic artiodactyls. In particular, some astragali of basal cetaceans include specialized artiodactyl features for running, for example, the typical double trochlea (Fig. 2.6) (Thewissen *et al.* 1996b, 2001; Thewissen and Madar 1999; Gingerich *et al.* 2001; Rose 2001). These characteristics are absent in the ankle bone of mesonychians, which is similar to that of other archaic

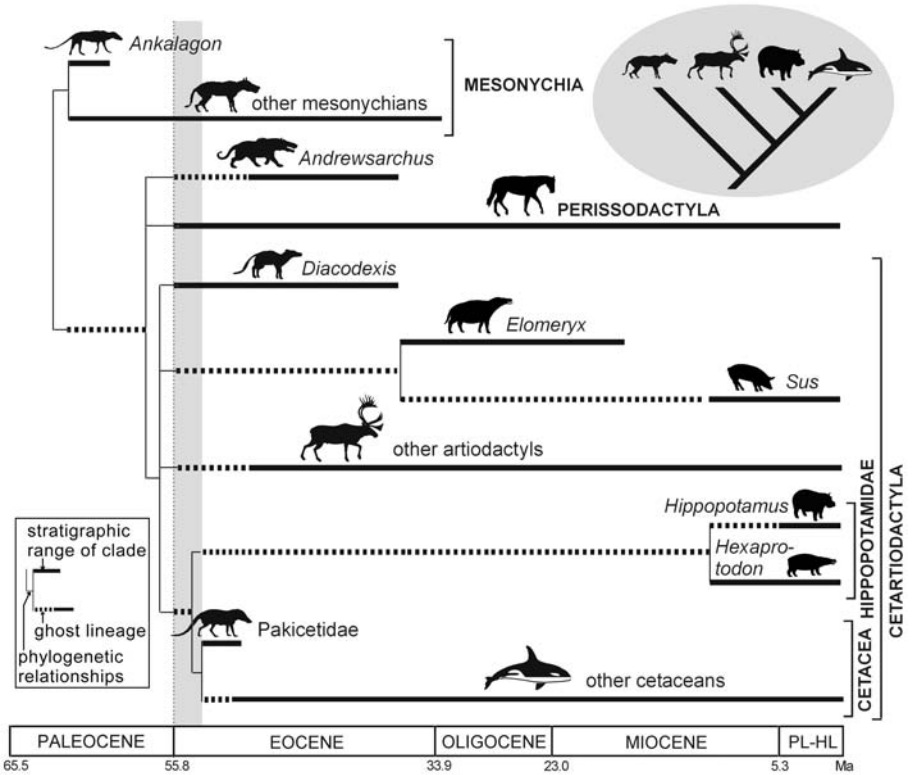

Fig. 2.4 Phylogenetic relationships of cetaceans according to the hypothesis considering whales as a sister group of hippopotamuses. Inferred relationships derived from Geisler and Uhen 2003. Journal of Vertebrate Paleontology 23(4): 991-996, Fig. 1. Stratigraphical ranges from O'Leary and Uhen 1999. Paleobiology 25(4): 534-556, Fig. 3. Gray band indicates a cetacean ghost lineage. Original.

ungulates. Moreover, the postcranial skeleton of basal whales has characteristics of archaic artiodactyls, such as the mesaxonic arrangement of the hand. Based on these discoveries, a cladogram with a cetacean-artiodactyl clade sister group of the mesonychians has been proposed (Thewissen *et al.* 2001).

Hippopotamuses may indeed be the closest *living* relatives of the whales considering that the primitive artiodactyl characteristics of the limbs of basal cetaceans also are observed in the ancestors of living hippopotamuses (the Eocene-Oligocene anthracotheres). In fact, a phylogenetic tree (Fig. 2.7) constructed using data presented by Thewissen *et al.* (2001) shows shorter ghost lineages in comparison with both the mesonychian and the hippopotamus hypotheses. Recently, new evidence supporting this cetacean-artiodactyl affinity emerged from the Late Eocene artiodactyls from France revealing a deciduous dentition similar to that of the archaeocetes (Foss and Theodor 2003).

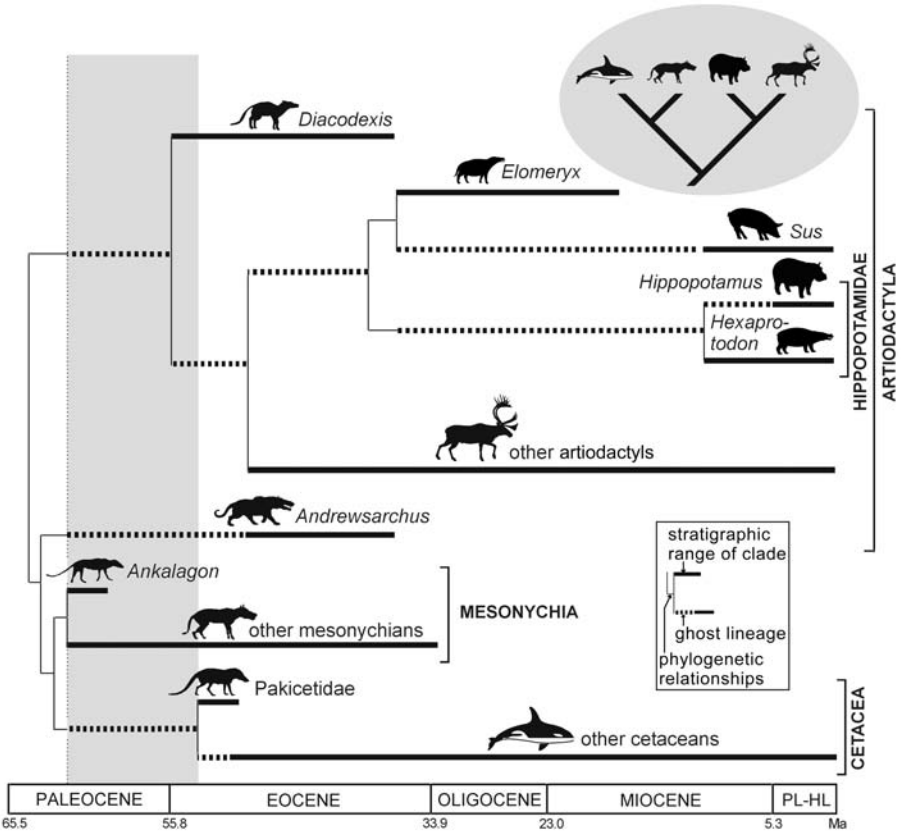

Fig. 2.5 Phylogenetic relationships of cetaceans according to the hypothesis considering whales as a sister group of mesonychians. Inferred relationships and stratigraphical ranges are derived from O'Leary and Uhen 1999. Paleobiology 25(4): 534-556, Fig. 3. Gray band indicates a cetacean ghost lineage. Original.

2.4 TIME OF ORIGIN

The oldest reported fossil cetacean is *Himalayacetus,* dated at about 53.5 Ma (Bajpai and Gingerich 1998). This age is contested by some authors (see below), who consider *Pakicetus*, at 48 Ma, as the oldest known fossil whale. Considering the rarity of findings, 53.5 or 48 Ma do not necessarily represent the time of origin of the whales, and a gap between the effective appearance of the first cetacean and the first fossil known is probable. Consequently, some indirect estimations of the time of origin for whales were proposed and were based on different approaches.

One of these methods is based on probability. It is calculated by considering the date of the oldest fossil and the distribution of known fossils in the stratigraphic record. An application of this method resulted in a

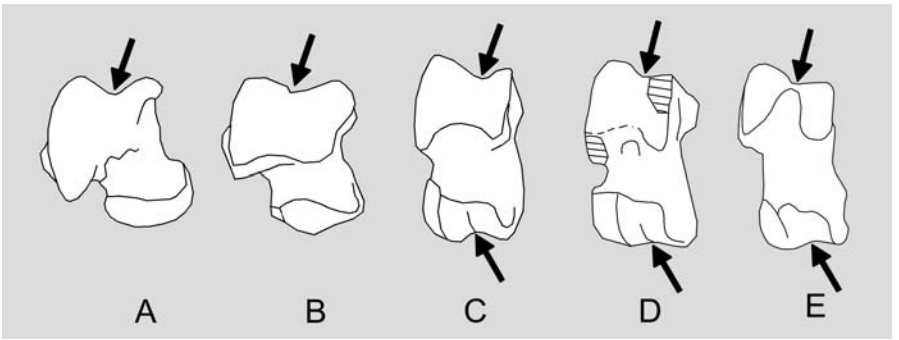

Fig. 2.6 Comparison of astragali of ungulates (A-C) and early whales (D-E). **A**. *Phenacodus* (early ungulate). **B**. *Pachyena* (mesonychian). **C**. *Bunophorus* (artiodactyl). **D**. *Pakicetus* (archaic whale). **E**. *Artiocetus* (archaic whale). A-C from O'Leary and Geisler 1999. Systematic Biology 48: 455-490, Fig. 1 (redrawn). D from Thewissen *et al.* 2001 Nature 413: 277-281, Fig. 1 (redrawn). E from Gingerich *et al.* 2001. Science 293: 2239-2242, Fig. 3 of the supplemental text online (drawn from photo). D-E are speculative drawings of left bones.

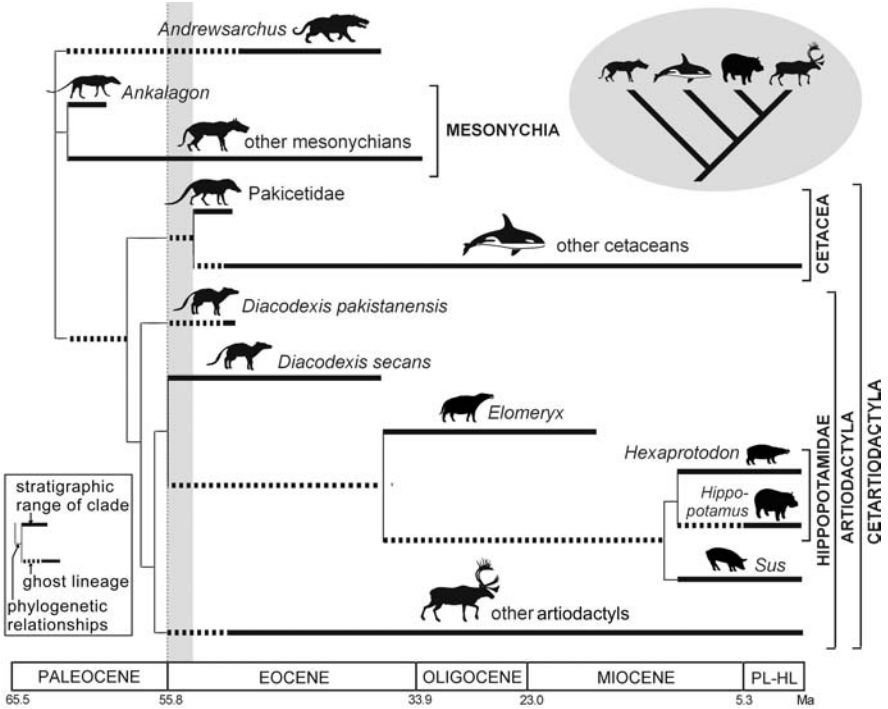

Fig. 2.7 Phylogenetic relationships of cetaceans according to the hypothesis considering whales as a sister group of artiodactyls. Inferred relationships derived from Thewissen *et al.* 2001. Nature 413: 277-281, Fig. 4. Stratigraphical ranges from O'Leary and Uhen 1999. Paleobiology 25(4): 534-556, Fig. 3, and from Gingerich 2003. Journal of Vertebrate Paleontology 23(3): 643-651. Gray band indicates a cetacean ghost lineage. Original.

predicted origin of whales of ca 54-55 Ma (Bajpai and Gingerich 1998; Gingerich and Uhen 1998).

Another approach to estimating the time of origin uses ghost lineages, where the oldest known record of a monophyletic sister taxa indicates the minimum age of divergence of the clade. For cetaceans, the time of origin based on this method varies considering different phylogenetic relationships among the taxa (Gatesy and O'Leary 2001). For example, considering a phylogenetic analysis where the cetaceans are a sister group to mesonychians, the ghost lineage of cetaceans is approximately 10 million years and its estimated origin dates back to the beginning of the Middle Paleocene (Fig. 2.5). In fact the oldest known cetacean is 53.5 Ma (considering *Himalayacetus* the first fossil) and the oldest mesonychians (*Ankalagon* and *Dissacus*) are from the North American Torrejonian Stage (62.5-60.5 Ma) (O'Leary and Uhen 1999). Considering the cetaceans as a sister group to artiodactyls, the ghost lineage is reduced to about 2 million years and the time of origin of cetaceans approximates the Paleocene-Eocene boundary (Gingerich 2002; Tuinen and Hadley 2004). In this case the time of origin was calibrated with the oldest known artiodactyl *Diacodexis* (Fig. 2.7).

Finally, recent data based on molecular clock estimates indicate that the divergence of whales from artiodactyls occurred 60 Ma (Árnason and Gullberg 1996; Theodor 2004).

2.5 CLASSIFICATION

Cetaceans are traditionally divided into three suborders: Archaeoceti, Mysticeti, and Odontoceti. The archaeocetes are paraphyletic because the other two groups originated from the specialized archaeocete subfamily of Dorudontinae from the Late Eocene (Uhen and Gingerich 2001) or from other archaeocetes that survived to the Oligocene (Fordyce 2004). The odontocetes plus the mysticetes form a clade of crown-group Cetacea, named Neoceti by Fordyce (2002e). This clade also has been indicated as "Autoceta" (McKenna and Bell 1998; Geisler and Sanders 2003), a name that firstly appeared in Ernst Haeckel's *Generelle Morphologie der Organismen* (1866). Nevertheless the name Neoceti must be preferred because it is the first formalized (Fordyce 2002e) for this taxon.

Odontocetes and mysticetes are generally considered as monophyletic groups. Milinkovitch and colleagues (1993, 1994) proposed close relationships between sperm whale odontocetes and rorqual mysticetes, evocative of odontocete paraphyly. This hypothesis was rejected by later molecular studies (Gatesy *et al*. 1999; Cassens *et al*. 2000; Nikaido *et al*. 2001). Surprisingly, in a recent molecular analysis (Verma *et al.* 2004), the mysticetes are considered a sister group of the platanistids, clearly inside the odontocete clade.

The cetaceans are distributed in 38 families of which only 13 (about one-third) are living. The relationships within the archaeocetes have been investigated in some recent cladistic studies emphasizing the paraphyletic

condition of some families (Uhen 1998; O'Leary and Uhen 1999; Uhen and Gingerich 2001; Thewissen and Williams 2002). The relationships among the 33 known families of neocetes have been intensely investigated in the past using morphological data only (Muizon 1987, 1988c, 1991; Heyning 1989, 1997; Barnes, 1990; Fordyce, 1994, 2002a; Kimura and Ozawa 2002; Geisler and Sanders 2003; Dooley *et al.* 2004; Deméré *et al.* 2005; Lambert 2005a), molecular data only (Cassens *et al*. 2000; Nikaido *et al.* 2001; Árnason *et al.* 2004; Verma *et al.* 2004; Rychel *et al.* 2004), and both morphological and molecular data (Messenger and McGuire 1998). Despite the many analyses, some questions are still not fully answered. Of note are the first phases of the neocete radiation (due to the scarcity of Early Oligocene fossil records), the relationships within the mysticetes (partly due to minimal interest afforded by paleontologists to these fossils), the location of the sperm whales within the odontocetes, and the debated monophyly or polyphyly of the river dolphins.

In their recent phylogenetic analysis based on 304 morphological characteristics of 54 fossil and extant cetaceans (including some unnamed basal Oligocene taxa), Geisler and Sanders (2003) tried to answer some of these questions. Their analysis partially contradicts some apparently firm results of previous studies. One of the most surprising results is that river dolphins are considered a monophyletic group together with the morphologically similar eurhinodelphinids. This datum, even if in accordance with some previous studies (Kasuya 1973; Barnes 1985b; Zhou 1982), contradicts many recent analyses which consider the river dolphins as polyphyletic (Heyning 1989; Muizon 1994, 2002; Messenger and McGuire 1998; Hamilton *et al*. 2001; Nikaido *et al.* 2001; Árnason *et al.* 2004; Lambert 2005a). Another result of the Geisler and Sanders paper is the relocation of the physeterids as a sister taxon to the ziphiids, contradicting some recent molecular (Cassens *et al*. 2000; Nikaido *et al.* 2001; Árnason *et al.* 2004) and morphological (Lambert 2005a) analyses that regard sperm whales as the most basal extant odontocetes.

Considering Geisler and Sanders admission that additional analyses (including both anatomical and molecular data) are needed, we propose a more conservative classification (Table 2.1) and phylogeny (Fig. 2.8) for fossil and extant cetaceans. In particular, the classification here proposed (Table 2.1) is after Fordyce and Muizon (2001) with the following modifications reflecting some recent papers:

- the following newly erected taxa have been added: Aulophyseterinae Kazár, 2002; Cetotheriopsidae Geisler and Sanders, 2003; Eomysticetidae Sanders and Barnes, 2002; Eomysticetoidea Sanders and Barnes, 2002; Simocetidae Fordyce, 2002, Xenorophiidae Luo and Gingerich, 1999 (emended and reproposed by Fordyce 2003a);
- the Balaenopteridae, Eschrichtiidae, and Neobalaenidae are included in the superfamily Balaenopteroidea according to the phylogenetic analysis by Dooley *et al.* 2004 and Rychel *et al.* 2004;

Table 2.1 Classification and stratigraphic ranges (in brackets) of fossil and extant Cetacea. E., Early. Eoc., Eocene. M., Middle. Mio., Miocene. L., Late. Olig., Oligocene. Pleis., Pleistocene. Plio., Pliocene. Rec., Recent. †. extinct taxa.

Archaeoceti (E. Eoc.-L. Olig.)
- †Family Pakicetidae (E.-M. Eoc.)
- †Family Ambulocetidae (M. Eoc.)
- †Family Remingtonocetidae (M. Eoc.)
- †Family Protocetidae (M. Eoc.)
  - †Subfamily Protocetinae (M. Eoc.)
  - †Subfamily Makaracetinae (M. Eoc.)
  - †Subfamily Georgiacetinae (M. Eoc.)
- †Family Basilosauridae (M.-L. Eoc.)
  - †Subfamily Dorudontinae (M.-L. Eoc.)
  - †Subfamily Basilosaurinae (M.-L. Eoc.)
- †Family Kekenodontidae (L. Olig.)

Neoceti (L. Eoc.-Rec.)

Mysticeti (L. Eoc.-Rec.)
- †Family Llanocetidae (L. Eoc.-L. Olig.)
- †Family Mammalodontidae (L. Olig.)
- †Superfamily Aetiocetoidea (E.-L. Olig.)
  - †Family Aetiocetidae (E.-L. Olig.)
- †Subfamily Aetiocetinae (L. Olig.)
- †Subfamily Chonecetinae (L. Olig.)
- †Subfamily Aetiocetinae (L. Olig.)
- †Subfamily Morawanocetinae (L. Olig.)
- †Superfamily Eomysticetoidea (L. Olig.)
  - †Family Eomysticetidae (L. Olig.)
  - †Family Cetotheriopsidae (L. Olig.)
  - †Family Cetotheriidae (L. Olig.-E. Plio.)
- Superfamily Balaenopteroidea (M. Mio.-Rec)
  - Family Balaenopteridae (L. Mio.-Rec)
  - Family Eschrichtiidae (Pleis.-Rec.)
  - Family Neobalaenidae (Rec.)
- Superfamily Balaenoidea (L. Olig.-Rec.)
  - Family Balaenidae (L. Olig.-Rec.)

Odontoceti (L. Eoc.-Rec.)
- † Family Agorophiidae (L. Eoc.-L. Olig.)
- † Family Xenorophiidae (L. Olig.)
- † Family Simocetidae (L. Olig.)
- Superfamily Platanistoidea (L. Olig.-Rec.)
  - † Family Squalodontidae (L. Olig.-M. Mio.)
  - † Family Prosqualodontidae (E. Mio.)
  - † Family Waipatiidae (L. Olig.)
  - † Family Squalodelphinidae (L. Olig.-E. Mio.)
  - † Family Dalpiazinidae (L. Olig.-E. Mio.)

Table 2.1 Contd. ...

Table 2.1 Contd. ...

- Family Platanistidae (E. Mio.-Rec.)
  - †Subfamily Pomatodelphininae (E.-L. Mio.)
  - Subfamily Platanistinae (Rec.)
- Superfamily Physeteroidea (L. Olig.-Rec.)
  - †Stem-group Physeterida (L. Olig.-E. Plio.)
    - †Subfamily Aulophyseterinae (M. Mio.)
    - †*Incertae sedis* (including *Diaphorocetus*, *Zygophyseter* and *Naganocetus*)
  - Family Physeteridae (E. Mio.-Rec.)
    - Subfamily Physeterinae (E. Mio.-Rec.)
  - Family Kogiidae (L. Mio.-Rec.)
    - Subfamily Kogiinae (L. Mio.-Rec.)
    - Subfamily Scaphokogiinae (L. Mio.)
- Superfamily Ziphioidea (E. Mio.-Rec.)
  - Family Ziphiidae (E. Mio.-Rec.)
    - Subfamily Ziphiinae (M. Mio.-Rec.)
    - Subfamily Hyperoodontinae (M. Mio.-Rec.)
    - †Subfamily Squaloziphiinae (E. Mio.)
- †Superfamily Eurhinodelphinoidea (L. Olig.-M. Mio.)
  - † Family Eurhinodelphinidae (L. Olig.-M. Mio.)
  - † Family Eoplatanistidae (E. Mio.)
- †Superfamily Delphinoidea (L. Olig.-Rec.)
  - † Family Kentriodontidae (L. Olig.-L. Mio.)
    - †Subfamily Kentriodontinae (E-M. Mio.)
    - †Subfamily Lophocetinae (E.-L. Mio.)
    - †Subfamily Pithanodelphinae (M.-L. Mio.)
  - Family Delphinidae (L. Mio.-Rec.)
    - Subfamily Delphininae (E. Plio.-Rec.)
    - Subfamily Lissodelphininae (?E. Plio.-Rec.)
    - Subfamily Stenoninae (E. Plio.-Rec.)
    - Subfamily Orcininae (E. Plio.-Rec.)
    - Subfamily Orcaellinae (Rec.)
  - Family Phocoenidae (L. Mio.-Rec.)
    - Subfamily Phocoeninae (L. Mio.-Rec.)
    - Subfamily Phocoenoidinae (L. Mio.-Rec.)
  - †Family Albireonidae (L. Mio.-E. Plio.)
  - Family Monodontidae (L. Mio.-Rec.)
    - Subfamily Delphinapterinae (L. Mio.-Rec.)
    - Subfamily Monodontinae (Pleis.-Rec.)
  - †Family Odobenocetopsidae (E. Plio.)
- Superfamily Inioidea (M. Mio.-Rec.)
  - Family Pontoporiidae (M. Mio.-Rec.)
    - Subfamily Pontoporiinae (L. Mio.-Rec.)
    - †Subfamily Brachydelphinae (M.-L. Mio.)
  - Family Iniidae (L. Mio-Rec.)
  - Family Lipotidae (E. Plio.-Rec.)

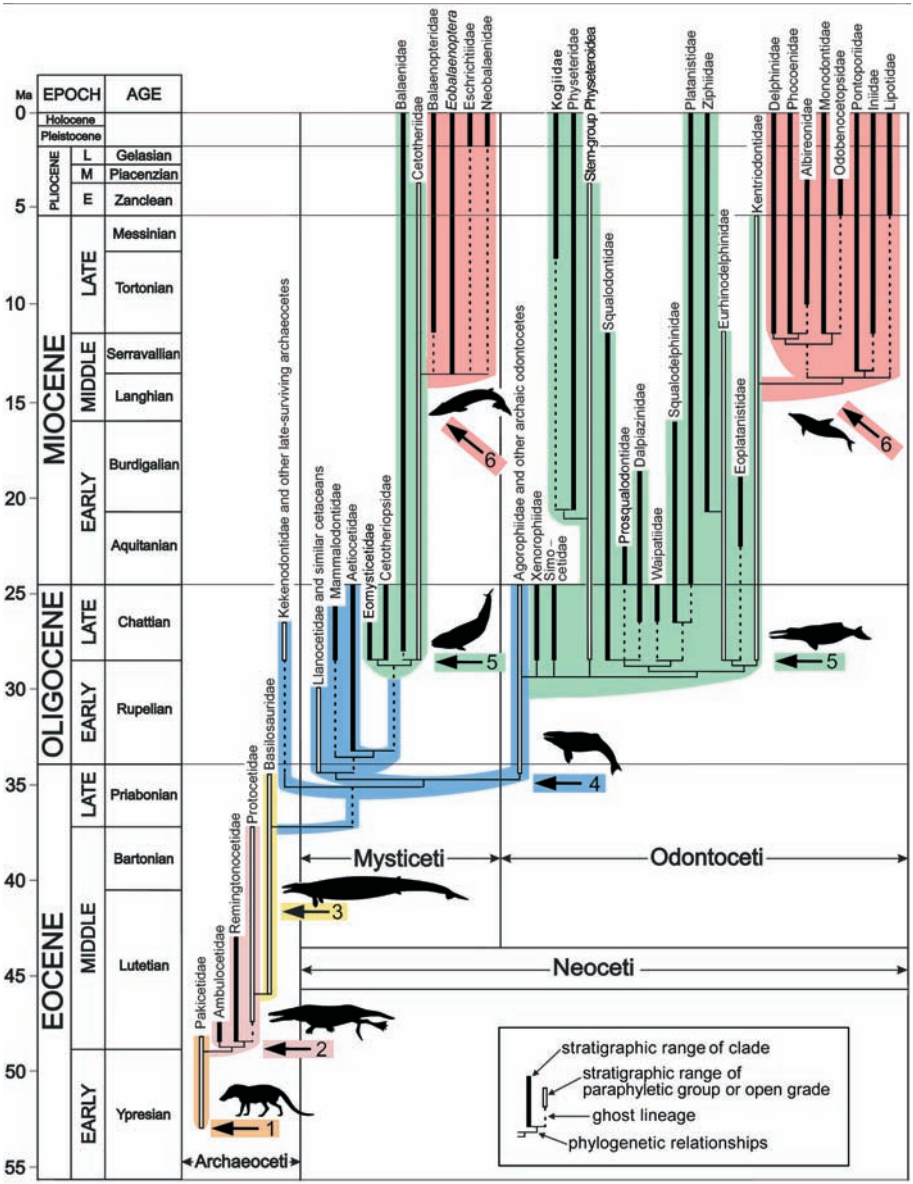

Fig. 2.8 Phylogenetic tree of cetaceans showing the six phases of radiation. Inferred relationships and stratigraphical ranges derived from Fordyce 2002c, Pp. 453-471. In W. F. Perrin, B. Wursig and J. G. M. Thewissen (eds), Encyclopedia of Marine Mammals. Academic Press, San Diego, California, Fig. 2, integrated with data from Fordyce (2002a, 2003a, 2004), Geisler and Sanders (2003), Dooley *et al.* (2004), Lambert (2005a) and Bianucci and Landini (2006). Original.

- the Physeteridae are restricted to Physeterinae subfamily and are considered the sister group of Kogiidae, while the Aulophyseterinae and more basal sperm whales form the stem-group Physeteroidea, according to Bianucci and Landini (2006);
- the delphinid subfamilies are partially rearranged according to Bianucci (2005).

2.6 DIVERSIFICATION IN THE PAST

2.6.1 Archeoceti

Archeocetes are archaic toothed cetaceans. They are known as fossils for almost 30 million years, from the latest Early Eocene (about 53.5 Ma) to near the end of Oligocene (about 26 Ma). They are a paraphyletic group formed by 31 known genera assembled in six families.

The skull of these stem-group Cetacea differs from that of the more specialized whales (mysticetes and odontocetes) in lacking the typical telescoping formed by the partial overlap of adjacent bones (Miller 1923; Romer *et al*. 2002) (Fig. 2.9). The upper jaw is relatively elongated, bearing 11-12 teeth on each row. All archaeocetes are heterodont and all (with the possible exception of the dorudontine *Chrysocetus*) were diphyodont, having two generations of teeth (Uhen 2000; Uhen and Gingerich 2001).

The general trends of the archaeocetes concern ongoing adaptation to the water. The most obvious transformations concern the postcranial skeleton. The passage from limb locomotion to locomotion based on dorso-ventral movement of the tail fluke (fluke-based locomotion) was brought about by reduction of the limbs and modification of the vertebral column (Gingerich *et al*. 1990; Buchholtz 1998; Thewissen and Williams 2002; Gingerich 2003a).

Modifications of the skull emerge and are related to adaptation for aquatic life. These modifications include posterior migration of the external nares (though they do not reach the neurocranium) and the adaptation of the ears to underwater sound perception and control of locomotion. The sound transmission elements of the outer and middle ear underwent evolutionary changes during the history of the archaeocetes, as shown in Fig. 2.10 (Nummela *et al*. 2004). The semicircular canal system, involved in neural control of locomotion, in the basal archaeocetes is similar to the unique and specialized canal system of the extant whales, denoting a quick and early swimming adaptation (Spoor *et al*. 2002).

Pakicetidae. The pakicetids are small terrestrial animals with limited aquatic adaptations. They are the oldest known cetaceans, with a fossil record ranging between 53.5 and 48 Ma (Early Eocene). All remains, referred to the genera *Pakicetus*, *Nalacetus* and *Ichthyolestes,* have been recovered in northern Pakistan and northwestern India.

The type-genus *Pakicetus* was originally described on the basis of a partial skull and mandible of *P. inachus* from Pakistan (Gingerich and Russell 1981;

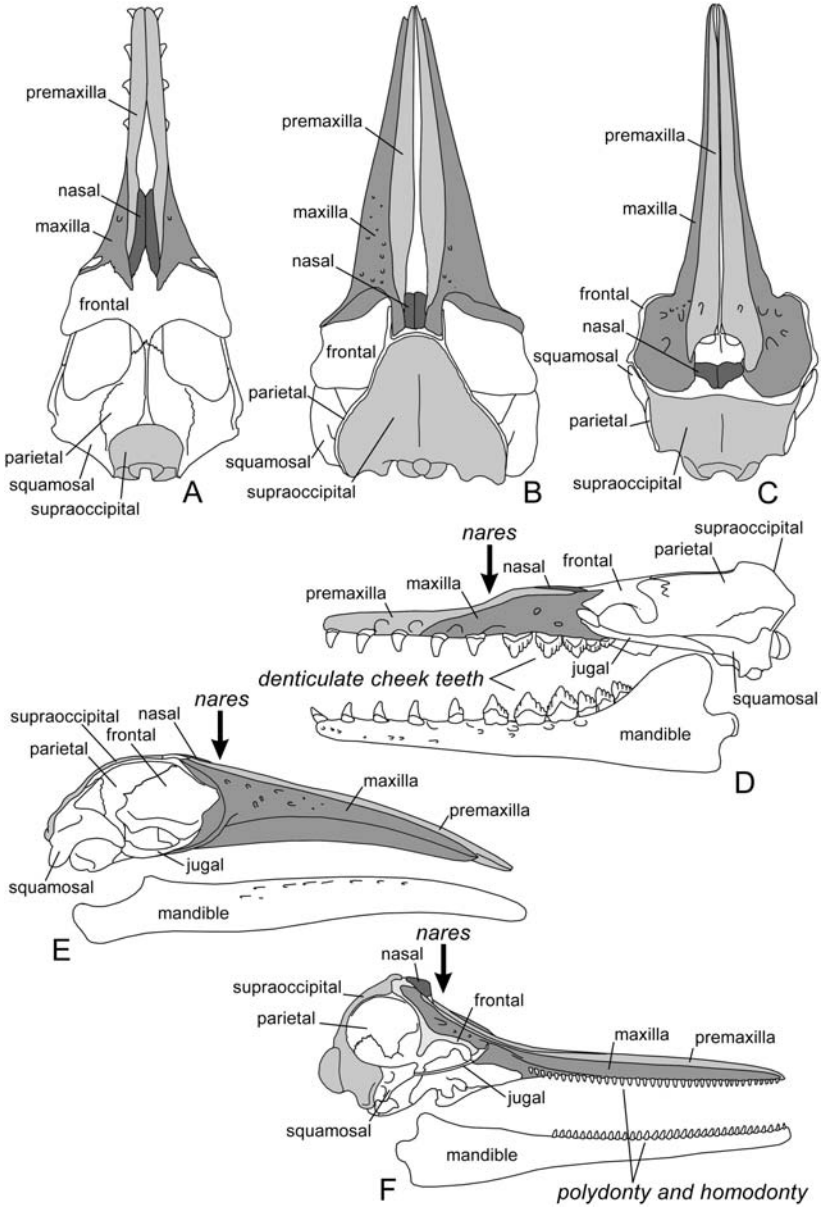

Fig. 2.9 Comparison of skull and mandible of representative derived genera of the three cetacean suborders. **A, D**. *Zygorhiza* (archaeocete). **B, E**. *Balaenoptera* (mysticete). **C, F**. *Sousa* (odontocete). A-C, dorsal view. D-E, lateral view. A, D from Kellogg. 1936. Carnegie Institute of Washington Publication 482: 1-366. Figs. 29, 31a (redrawn). B, C, E, F from Van Beneden and Gervais. 1880. Ostéographie des Cétacés vivants et fossiles. Atlas. Arthus Bertrand Éditeurs, Paris, Pl. 11, figs. 11-12, Pl. 37, figs. 1-3 (redrawn). Bones strongly modified for telescoping evidenced in different gray tones.

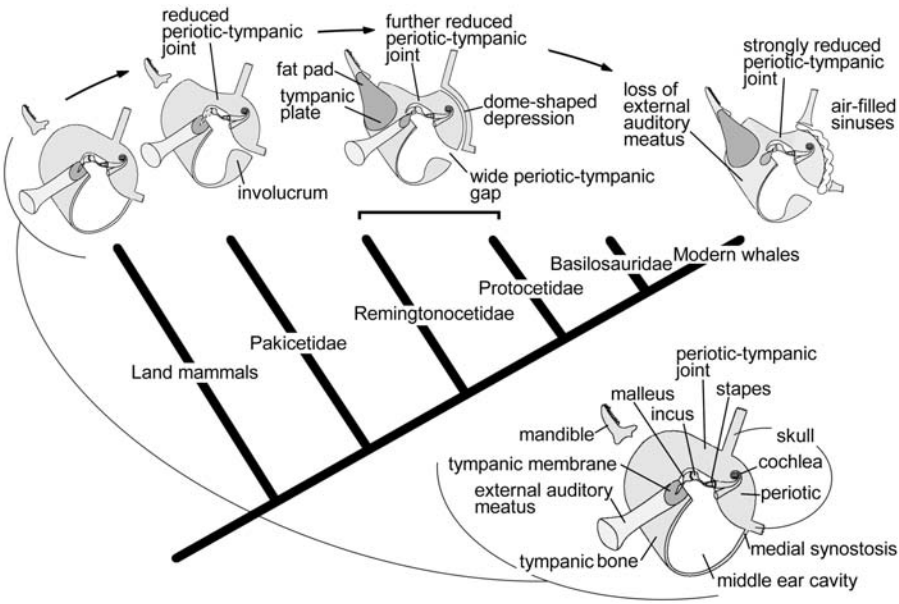

Fig. 2.10 Evolution of sound transmission mechanisms in whales. From Nummela *et al.* 2004. Nature 430: 776- 778, Fig. 2 (for the diagrams of the ear, modified) and Fig. 4 (for the phylogenetic relationships, redrawn).

Gingerich *et al*. 1983). Other remains from Pakistan and India have been referred to *Pakicetus* (Thewissen and Hussain 1993, 1998; Luo and Gingerich 1999; Thewissen *et al.* 2001). The skull of *Pakicetus* maintains features reminiscent of land mammals, such as the external nares being located at the tip of the snout and the orbits not being positioned at the most dorsal aspect of the head. The ear bones differ substantially from those of terrestrial mammals in the thickening of the tympanic bulla (involucrum) – a specialization interpreted by Thewissen *et al.* (2001) as related to the reception of sounds and vibrations from the ground through the contact of the head with the ground. Several isolated postcranial bones referred to *Pakicetus* (see Thewissen *et al.* 2001) revealed that this cetacean had a wolf-sized skeleton not obviously adapted to swimming, with elongated neck vertebrae, a vertebral column not capable of wide flexion, a distinct sacrum formed by the fusion of four vertebrae, and anterior and posterior limbs similar to those of running terrestrial mammals (Fig. 2.11).

The genus *Ichthyolestes* is another pakicetid originally known on the basis of a few teeth. *Ichthyolestes* is a fox-sized archaeocete. Several bones belonging to this genus were excavated from the same Pakistani site as the postcranial bones referred to *Pakicetus* (Thewissen *et al.* 2001).

The genus *Himalayacetus* was considered the oldest cetacean known and referred to pakicetids (Bajpai and Gingerich 1998). It is described on the basis of a mandible fragment with two teeth collected in northern India. There

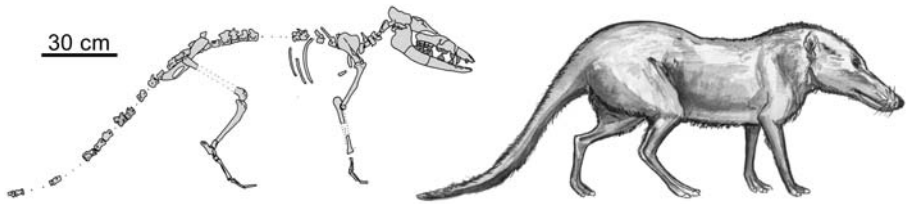

Fig. 2.11 Skeleton and body reconstruction of *Pakicetus* (Pakicetidae). Skeleton from Thewissen *et al.* 2001. Nature 413: 277-281, Fig. 2a (modified). Original drawing of body reconstruction based on Thewissen and Williams 2002. Annual Review of Ecology and Systematics 33: 73-90, Fig. 2.

remains disagreement regarding its age and placement in the pakicetid family (Thewissen *et al.* 2001; Thewissen and Williams 2002).

Pakicetus, *Nalacetus,* and *Ichthyolestes* remains were collected in fluvial deposits, while the presumed oldest *Himalayacetus* was found in marine strata associated with marine fauna, indicating colonization of marine waters early in cetacean history. The oxygen isotope composition of the tooth-enamel phosphate of this basal cetacean confirms that *Himalayacetus* probably spent some time in marine waters (Bajpai and Gingerich 1998).

Ambulocetidae. The ambulocetids are seal-like amphibious marine cetaceans known on the basis of some specimens collected in Middle Eocene strata of northern Pakistan and northwestern India. The knowledge of these archaeocetes is essentially based on an almost complete skeleton of *Ambulocetus natans* (Thewissen *et al.* 1994, 1996; Madar *et al.* 2002). *Ambulocetus* had a bizarre body shape, with long posterior limbs suited for both swimming (paddling with their large feet) and walking on land (Thewissen and Fish 1997) (Fig. 2.12). Other ambulocetids based on fragmentary remains are *Gandakasia* and, according to Thewissen and colleagues, also the presumed pakicetid *Himalayacetus*. Ambulocetids probably lived in estuaries or bays because they are always found in near-shore shallow marine deposits associated with fossils of littoral molluscs and abundant marine plants (Williams 1998; Thewissen and Williams 2002). Moreover, studies on stable oxygen isotopes indicated that ambulocetids were probably partly dependent on freshwater at some stages of their life (Roe *et al*. 1998).

Remingtonocetidae. The remingtonocetids are a specialized and diversified group of archaeocetes found in the Middle Eocene of Pakistan and India. Besides the type-genus *Remingtonocetu*s, this family is known on the basis of four other genera: *Andrewsiphius, Attockicetus*, *Dalanistes,* and *Kutchicetus* (see Williams 1998 and Thewissen and Williams 2002 for a detailed review and bibliography). A derived feature of remingtonocetids is the long, narrow snout denoting a specialized feeding adaptation. The hearing of remingtonocetids exhibits some adaptations to water sound transmission, such as the large mandibular foramen, indicating the presence of the mandibular fat pad, and the partial acoustic insulation periotic to the skull (Nummela *et al*. 2004) (Fig. 2.10). Postcranial bones of *Kutchicetus* suggest that these cetaceans swam

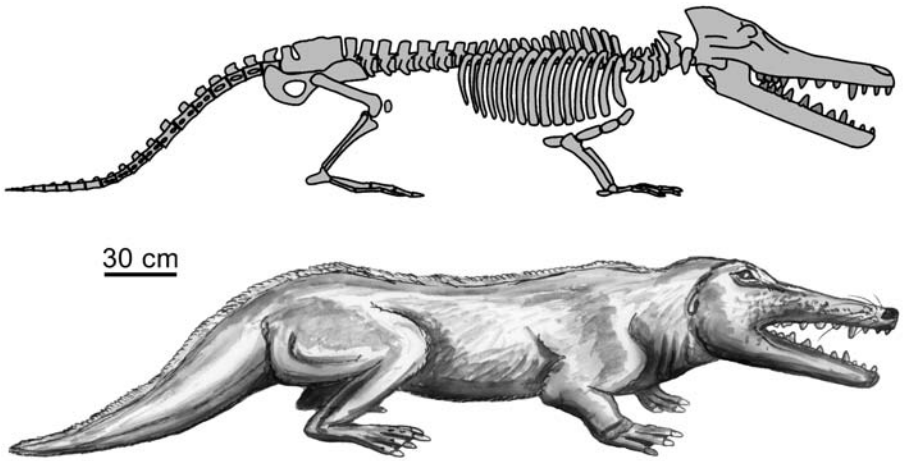

Fig. 2.12 Skeleton and body reconstruction of *Ambulocetus* (Ambulocetidae). Skeleton from Thewissen. 2002. Pp. 36-39. In W. F. Perrin, B. Wursig and J. G. M. Thewissen (eds), Encyclopedia of Marine Mammals. Academic Press, San Diego, California, Fig. 3 (modified). Original drawing of body reconstruction based on Thewissen and Williams. 2002. Annual Review of Ecology and Systematics 33: 73-90, Fig. 2.

by means of a long and flat tail similar to *Pteronura,* the living South American giant freshwater otter (Bajpai and Thewissen 2001). As for the ambulocetids, the remingtonocetids lived in coastal marine or lagoonal environments, but most of them were independent of freshwater (Williams 1998; Roe *et al*. 1998; Thewissen and Williams 2002).

Protocetidae. The protocetids were a diverse (and probably paraphyletic) group of Middle Eocene marine cetaceans that were more specialized for marine life than other basal archaeocetes. Protocetid remains were found in Egypt, Pakistan, India, and North America, indicating the first cetacean dispersion outside the southwestern Tethys. Some protocetids lived in coastal and lagoon environments, others in the open sea (see Williams 1998 and Thewissen and Williams 2002 for a detailed review and bibliography).

The type-genus, *Protocetus,* known on the basis of a skull and some postcranial bones from Egypt, has been the best known basal archeocete for about 80 years. Up to now, 14 protocetid genera have been described, of which the best-known is *Rodhocetus*. Generally the skull of protocetids is characterized by a long snout and a large and flat supraorbital shield denoting lateral placement of large eyes. The nasal opening is more posteriorly placed with respect to other basal archaeocetes. The ear bones are specialized similar to that of remingtonocetids (Nummela *et al*. 2004) (Fig. 2.10). A well-preserved specimen of *Rodhocetus* provides the best information on the axial skeleton of protocetids: the neck vertebrae are short and four lumbar vertebrae have fused transverse processes forming a rigid

sacrum (Gingerich *et al.* 1994; Gingerich *et al.* 2001; Gingerich 2003a); the tail fluke is absent (Buchholtz 1998; Gingerich *et al.* 2001); and the limbs are short but hands and feet have long fingers probably connected by a membrane (Fig. 2.13).

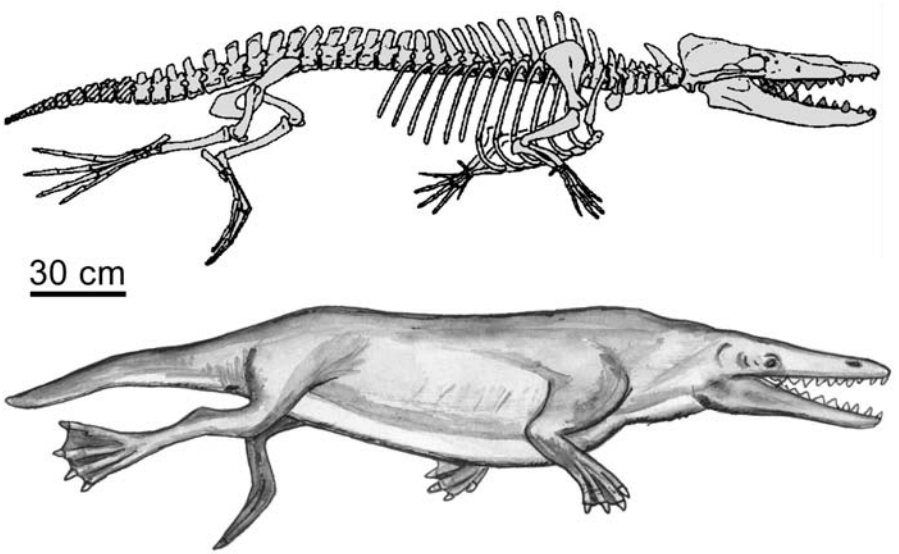

Fig. 2.13 Skeleton and body reconstruction of *Rodhocetus* (Protocetidae). Skeleton from Gingerich *et al.* 2001. Science 293: 2239-2242, Fig. 3 (modified). Original drawing of body reconstruction based on Gingerich 2002. LSA Magazine, University of Michigan College of Literature, Science, and the Arts (LSA) Magazine, University of Michigan, 2002: 25-27, unnumbered Fig. at bottom of p. 27.

Protocetids have been recently divided in three subfamilies: Protocetinae, Georgiacetinae and Makaracetinae. The Protocetinae are the more generalized feeders and swimmers, the Georgiacetinae are the more specialized for marine life and the monogeneric Makaracetinae are a highly specialized feeder with a primitive postcranial skeleton (Gingerich *et al.* 2005).

Basilosauridae. The basilosaurids are a paraphyletic group of fully aquatic marine archaeocetes known from the Middle-Late Eocene on all continents (see Uhen 1998, 2002 for reviews). The skull of basilosaurids still lacks the derived features of odontocetes and mysticetes except for the expanded basicranial air sinuses. The teeth are specialized in that the cheek-teeth have complex denticles. The axial skeleton shows a marked adaptation to water: the forelimbs have broad, fan-shaped scapula, with the humerus, radius and ulna flattened into a single plane; the hindlimbs are markedly reduced and not connected to the vertebral column; and the sacral vertebrae are missing (Gingerich *et al.* 1990; Buchholtz 1998; Uhen 2002).

Basilosaurids are divided in two subfamilies (Basilosaurinae and Dorudontinae) (Miller 1923; Uhen 1998), sometimes elevated to familial rank

(Thewissen and Williams 2002). Basilosaurinae have a large (about 16 m long) and bizarrely snake-like body. They are known on the basis of several skeletons from Egypt, North America, and Pakistan. All remains are referred to the genus *Basilosaurus* except for one single caudal vertebra and one single cervical vertebra described, respectively, as the holotypes of species of *Basiloterus* and *Pontogenus*. The axial skeleton of *Basilosaurus* is characterized by extremely elongated vertebral bodies amongst the posterior thoracic, lumbar, and anterior caudal vertebrae (Fig. 2.14). A tail fluke was probably present even if it was not the propulsive organ: swimming was probably realized by means of sequential dorsoventral undulatory waves of uniform amplitude passing posteriorly along the body (Buchholtz 1998).

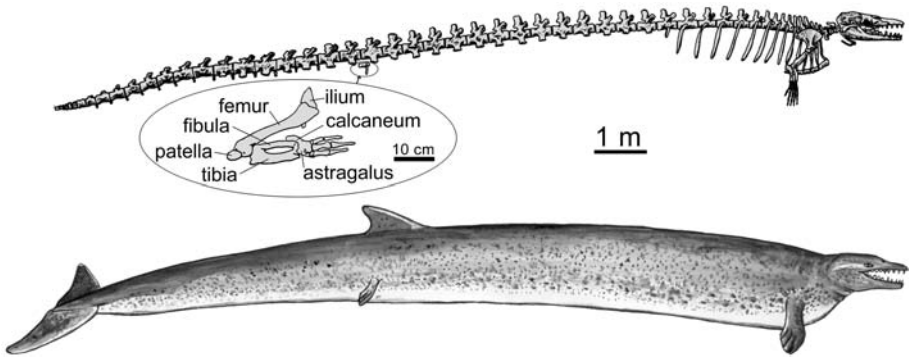

Fig. 2.14 Skeleton and body reconstruction of *Basilosaurus* (Basilosauridae). Skeleton from Kellogg 1936. Carnegie Institute of Washington Publication 482: 1-366. Pl. 1A (redrawn). Detail of limb from Gingerich *et al.* 1990. Science 249: 154-157, Fig. 1 (redrawn). Drawing of body reconstruction original.

Unlike the Basilosaurinae, the subfamily, Dorudontinae, had a body shape similar to that of modern dolphins, including a fluke and they probably swam as do living cetaceans (Buchholtz 1998; Uhen 1998, 2004). Dorudontines are known on the basis of six genera, of which *Dorudon* and *Zygorhiza* are the best known. A subadult dorudontine specimen described as a new genus, *Chrysocetus,* lacks deciduous teeth, indicating either possible monophyodonty, as in the neocetes, or acceleration in adult dental eruption (Uhen and Gingerich 2001).

Kekenodontidae and other Oligocene archaeocetes. Despite the most recent literature that considered the archaeocetes as becoming extinct at the end of the Eocene, Fordyce (2004) reported solid evidence of archaeocete-grade Cetacea into the Oligocene. This assumption is mainly based on two new unfigured fossils from the Late Oligocene of New Zealand. One of these consists of a skull, with associated teeth and periotics, showing intermediate features between the dorudontine archaeocetes and the archaic neocetes. Of note, unlike the situation in other archaeocetes, the mastoid or posterior

process of the periotic is not exposed laterally on the skull wall. According to Fordyce (2004), this fossil belongs to an unnamed sister-species of the Neoceti. The same author also referred to archaeocetes the previously described *Kekenodon onamata* from the Late Oligocene of New Zealand and "*Squalodon*" *gambierensis* from about the Early-Late Oligocene boundary of Australia. The genus *Kekenodon*, based only on teeth, ear bones, and other fragments, was referred to a new subfamily Kekenodontinae; it had already been placed in the archaeocetes (basilosaurids) by Mitchell (1989). Fordyce (1992) put *Kekenodon* in the mysticetes and elevated the kekenodontines to familial level. In conclusion, the recent paper by Fordyce (2004) re-evaluated the original interpretation by Mitchell (1989), considering *Kekenodon* as an archaeocete. In all cases, a future detailed description of the new Oligocene fossils is necessary to better clarify their relationships with kekenodontids and other archaeocetes.

2.6.2 Neoceti

The evolution of Neoceti from dorudontine archaeocetes is supported by several common characteristics of skull, teeth, mandible and postcranial skeletons (Uhen and Gingerich 2001; Fordyce 2002e). Among these common characteristics, the most evident are the dentition and the advanced hind limb reduction for complete water adaptation. The common dentition is heterodont with multiple accessory denticles on the cheek teeth, which are lacking in the more-specialized neocetes. The more-specialized neocetes (relative to the archaeocetes) possess skull characteristics such as an open mesorostral groove of the rostrum and a relatively delicate jugal and robust zygomatic process of the squamosal (Fordyce 2002e, 2003a). Characteristics intermediate between those of the dorudontine archaeocetes and the most archaic neocetes were recently reported in new Oligocene archaeocetes (Fordyce 2004). Specifically, the periotic amastoid with the posterior process laterally unexposed on the skull wall was previously considered neocete synapomorphies (Fordyce 2002e) but is observed in the later archaeocetes. Further, all neocetes have only one tooth generation (monophyodont), but this apomorphy, as already stated, may also be present in the specialized dorudontine, *Chrysocetus* (Uhen and Gingerich 2001). Other characteristics, such as the increase in telescoping of the skull and the polydont and homodont dentition, are evolutionary trends of the neocetes but are absent in some basal taxa.

2.6.3 Mysticeti

All living mysticetes, except the medium size *Caperea*, are large or very large whales, while fossil mysticetes have more heterogeneous sizes. In fact, some archaic genera, such as *Aetiocetus,* are relatively small cetaceans. Fossil mysticetes date back to 34 Ma (Eocene-Oligocene boundary).

Evolutionary trends characterizing the mysticete history include increasing body size, loss of erupted teeth, progressive telescoping of the skull, bowing of

the mandibles, and shortening of the neck. In general, filter feeding characterizes all living mysticetes and this trophic strategy possibly was used since the first phases of mysticete radiation (Fordyce 1980).

Llanocetidae. The llanocetids are the oldest, and probably most basal, mysticete family. Llanocetids, along with the aetiocetids and mammalodontids, are archaic mysticetes that maintain erupted teeth and other archeocete skull features. They are characterized by a relatively broad rostrum and probably used their teeth in filter feeding, perhaps along with the baleen (Fordyce 2002c). The llanocetids are relatively large whales (estimated length more than 9 m) intermediate between basilosaurid archaeocetes and more specialized mysticetes (Fordyce 2003b).

The original description of the llanocetids was based on a mandible fragment from a brain cast found on Seymour Island (Antarctic) and determined to be from the latest Eocene (Mitchell 1989). This find was referred to as genus *Llanocetus* (Mitchell 1989). The skull and some postcranial bones of the same species were briefly described by Fordyce (2003b). This author also reported a skull and teeth, possibly belonging to the same genus, from the basal Oligocene of New Zealand.

Aetiocetidae. Originally referred to as archaeocetes (Emlong 1966), the aetiocetids are the most diversified toothed mysticetes (Fig. 2.15). The type-genus *Aetiocetus* and other cetaceans, such as *Chonecetus*, *Ashorocetus* and *Morawanocetus,* are referred to this family (Barnes *et al.* 1995). Most described aetiocetids are restricted in geographical (western and eastern coasts of North Pacific Ocean) and temporal (Late Oligocene) ranges and were interpreted as relict basal mysticetes (Barnes *et al.* 1995). Recently, records of aetiocetids have been reported from Early Oligocene in the eastern North Pacific Ocean

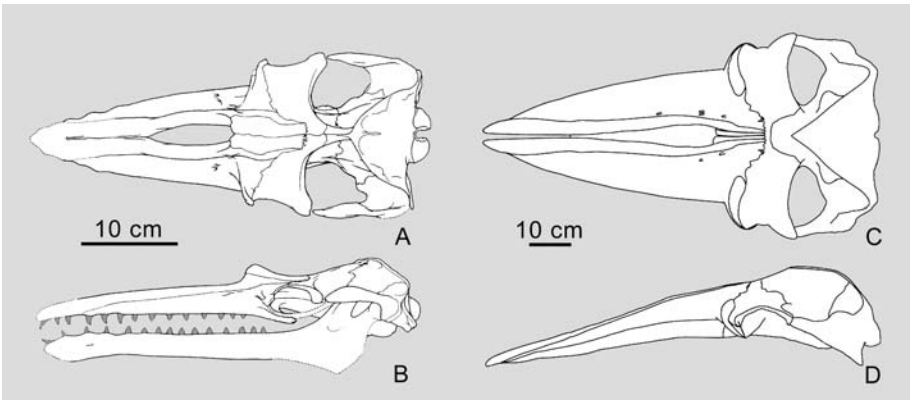

Fig. 2.15 **A, B**. Skull in dorsal view and skull and mandible in lateral views of *Aetiocetus* (Aetiocetidae). **C, D**. Skull in dorsal and lateral views of *Isanacetus* (Cetotheriidae). A, B from Barnes *et al.* 1995. The Island Arc 3: 392-431, Fig. 20 (modified). C, D from Kimura and Ozawa 2002. Journal of Vertebrate Paleontology 22: 684-702, Fig. 2 (modified).

(Goedert *et al*. 2001) and in South Indian Ocean (Pledge 2005), indicating that they are contemporary to the stem mysticete llanocetids.

Mammalodontidae. The only genus of this family, *Mammalodon* from the Late Oligocene-Early Miocene of Australia, is an archaeocete-like small mysticete characterized by a very short rostrum and heterodont dentition (Fig. 2.16) (Mitchell 1989; Fordyce and Muizon 2001).

Eomysticetidae. Based on two species of the genus *Eomysticetus* from the Late Oligocene of South Carolina (USA), the eomysticetids are relatively large toothless mysticetes which presumably had baleen for filter feeding (Sanders and Barnes 2002b). *Eomysticetus* has an elongated, broad and flat rostrum with nutrient foramina for baleen but it also maintains some archaeocete features of the neurocranium, such as the narrow and elongated intertemporal region and the elongated zygomatic processes of squamosals (Fig. 2.16). Because of its relatively low degree of cranial telescoping (in comparison with the contemporaneous aetiocetids and cetotheres), *Eomysticetus* was considered by Sanders and Barnes (2002b) as a relict.

Cetotheriopsidae. The cetotheriopsids are a Late Oligocene mysticete group (Sanders and Barnes 2002a) recently removed from Cetotheriidae and elevated to familial level (Geisler and Sanders 2003). Cetotheriopsids are the sister

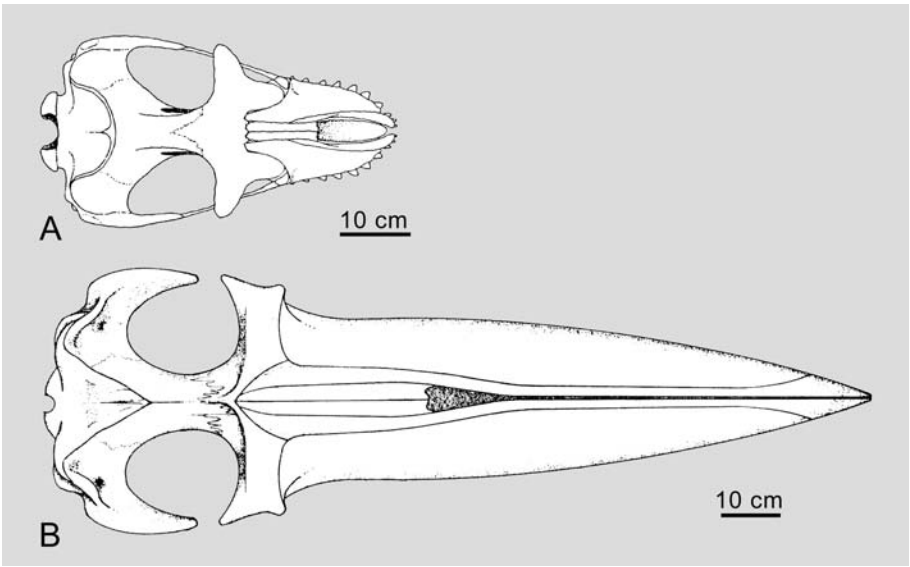

Fig. 2.16 **A**. Skull in dorsal view of *Mammalodon* (Mammalodontidae). **B**. Skull in dorsal view of *Eomysticetus* (Eomysticetidae). A from Fordyce and Muizon 2001. Pp. 169-233. In J. M. Mazin and V. Buffréenil (eds), Secondary adaptations of tetrapods to life in water. Verlag Dr. Friedrich Pfeil, München, Germany, Fig. 10A (modified). B from Sanders and Barnes 2002b. Smithsonian Contributions to Paleontology 93: 313-356, Fig. 3B (modified).

taxon of the eomysticetids from which they differ essentially by having a shorter intertemporal region and zygomatic process of the squamosal. *Cetotheriopsis* from Austria and *Micromysticetus* from Germany and North Carolina (USA) are the only two genera referred to this family.

Cetotheriidae. As generally portrayed in the literature, the cetotheres are a polyphyletic and paraphyletic wide group of cosmopolitan, extinct, toothless, baleen-bearing mysticetes ranging from the Late Oligocene to the Early Pliocene (Kimura and Ozaka 2002). Cetotheres, in the conventional sense, are defined on the basis of some plesiomorphic characteristics such as moderate telescoping of the skull, gradual sloping of the frontal bone, and slightly concave glenoid fossa of the squamosal (Fig. 2.15). Some late cetotheres are thought to be similar and possibly related to the balaenopterids.

Balaenopteridae. Fossil balaenopterids are reported since the Middle Miocene (Fordyce and Barnes 1994) but well-described remains, referred to as rorquals, are from the Late Miocene, in particular the primitive genera *Plesiocetus*, *Parabalaenoptera,* and possibly the *Megaptera miocaena* (Humpback whale) (see Zeigler *et al*. 1997). *Plesiocetus* extends to the Pliocene with several nominal species and a wide geographical distribution. It is a basal balaenopterid sometimes placed within the cetotheres. *Parabalaenoptera,* from California, was assigned to the monogeneric subfamily Parabalaenopterinae by Zeigler *et al*. (1997). It differs from extant Balaenopterinae and Megapterinae in characteristics such as the very elongated and narrow nasal passages. The same authors considered *Megaptera miocaena*, from the Late Miocene of California, more closely related to balaenopterines than to the extant Humpback whale (*Megaptera novaeangliae*). Another presumed humpback whale fossil is the relatively well-preserved skeleton from the Pliocene of Chile described by Dathe (1983) as *Megaptera hubachi*. In a recent review Deméré *et al*. (2005) considered *Parabalaenoptera* closely related to other balaenopterids and they retained that both fossil species of humpback whales (*Megaptera novaeangliae* and *M. hubachi*) should be reassigned to two new genera.

The extant genus *Balaenoptera* is known as a fossil from the Pliocene and most of the member species are from Belgium and Italy (Fig. 2.17) (Deméré 1986; Deméré *et al.* 2005). Recently, Dooley *et al.* (2004) believed the extant balaenopterids, *Balaenoptera* spp., and *Megaptera novaeangliae,* to be sister taxa belonging to a wide and unresolved clade with *Eschrichtius*, *Parabalaenoptera*, *Megaptera miocaena,* and a new genus from the Middle Miocene of the eastern United States, named *Eobalaenoptera*.

Eschrichtiidae. The oldest described record of eschrichtiids is a fragmentary skeleton from the Late Pliocene of Japan (Hichishima *et al.* 2006). The only reported fossil record of *Eschrichtius robustus* (Gray whale) is a skull and partial skeleton from the Pleistocene of California described as *Eschrichtius* cf. *E. robustus* by Barnes and McLeod (1984). An ancient presence of *E. robustus* in the North Atlantic is supported by subfossil remains (Bryan 1995). The partial skull that Dooley *et al.* (2004) described and named *Eobalaenoptera*, exhibits

Fig. 2.17 Skeleton of *Balaenoptera cortesii* collected from the Pliocene of Italy in 1806. This drawing first appeared in Cortesi's *Saggi geologici degli strati di Parma e Piacenza* (1819) and was reproposed in Cuvier's *Recerche sur les ossements fossiles* (1824).

intermediate features between the balaenopterids and the eschrichtiids. According to Dooley *et al.* (2004), eschrichtiids and balaenopterids split by at least 14 Ma. The hypothesis of a monophyly of the clade formed by eschrichtiids and balaenopterids is also supported by Deméré and Berta (2003) who reported a partial undescribed skeleton from the Pliocene of California similarly presenting intermediate balaenopterid-eschrichtiid features. The recent phylogenetic analysis made by Deméré *et al.* (2005) confirmed close relatinships beween eschrichtiids and balaenopterids.

Neobalaenidae. A presumed fossil *Caperea marginata* (Pygmy right whale) from Chile, referred to the extant genus *Caperea* by Donoso-Barros (1976), is too incomplete to warrant attribution to this family (Fordyce 1984; McLeod *et al*. 1993). According to recent phylogenetic studies based on both molecular (Rychel *et al.* 2004) and morphological (Geisler and Sanders 2003) data, neobalaenids are more strictly related to eschrichtiids and balaenopterids than to the balaenids. If this interpretation is correct, neobalenids might have originated together with the eschrichtiids and balaenopterids at the end of the Middle Miocene (Fig. 2.8).

Balaenidae. The balaenids have a wide stratigraphic range even if significant fossil records are not common (McLeod *et al.* 1993; Bisconti 2003, 2005). The oldest right whale is traditionally considered *Morenocetus*, a genus based on a partial skull (Fig. 2.18) from the Early Miocene of Patagonia (Cabrera 1926). Recently, a partial skull and some associated postcranial bones of a putative balaenid were reported from the Late Oligocene (about 28 Ma) of New Zealand, extending the range of this family by 5+ Ma (Fordyce 2002f). Other Miocene balaenid records are fragmentary. One of the most significant is perhaps an undescribed partial skull from the Late Miocene of southern Italy (Bianucci *et al.* 2000).

Pliocene records are relatively more common, especially from the North Atlantic and Mediterranean, with significant remains mainly referred to the fossil genera *Balaenella*, *Balaenula,* and *Balaenotus* and the extant genera *Balaena* and *Eubalaena*. *Balaenella* and *Balaenula* (Fig. 2.18) are small whales, with the skull length of the first being about 1 m and the body length of the

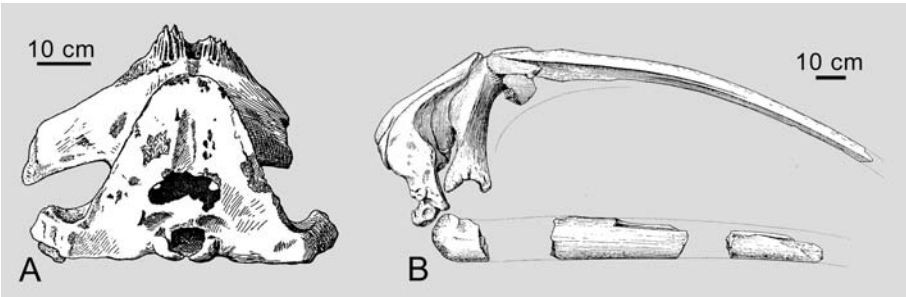

Fig. 2.18 **A**. Partial skull in dorsal view of *Morenocetus* (Balaenidae). **B**. Skull and mandible in lateral view of *Balaenula* (Balaenidae). A from Cabrera 1926. Revista Museo de La Plata 29: 363-411, Fig. 1 (modified). B from Trevisan 1941. Palaeontographia Italica 40: 1-13, Fig. 11 (modified).

second about 5 m. A recent phylogenetic analysis maintains small body size to be a common condition in the two different balaenid clades represented by *Balaenella* + *Balaena* and by *Balaenula* + *Eubalaena* (Bisconti 2005). *Balaenotus* is a primitive balaenid lacking the anterior torsion of the mandible and the fusion of the atlas to the other cervical vertebrae. The most significant described remains belonging to the genus *Balaena* are the holotypes of the species *B. montalionis* and *B. ricei* from Italy and Virginia (USA), respectively (Bisconti 2000; Westgate and Whitmore 2002). Both fossil species have a body size slightly smaller than extant *B. mysticetus*. The oldest *Eubalaena* record is a partial skull from the Middle Pliocene of Italy referred to *Eubalaena* sp. (Bisconti 2002).

2.6.4 Odontoceti

Odontocetes are toothed whales which use high frequency sound to echolocate. The fossil record extends from the Eocene-Oligocene boundary. The oldest well-described odontocetes are from Late Oligocene.

Evolutionary trends characterizing odontocete history include an increase in telescoping of the skull (involving posterior movement of the supraorbital parts of the tooth-bearing maxilla) and an evolution of the dentition from heterodont to homodont and polydont. Moreover, the efficiency of echolocation seems to increase throughout odontocete evolution, possibly due to some skull modifications, such as increased facial asymmetry and development of complex basicranial sinuses. Body size increase and/or reduction in teeth number characterize the evolution of some odontocete clades (e.g., physeterids and ziphiids).

Recent studies using computer tomography evidenced that both fossil and living odontocetes are highly encephalized and that the large brain evolved in two phases of whale history: the first with the origin of odontocetes from the archaeocetes near the Eocene-Oligocene boundary and the second with the origin of the modern Delphinoidea (Delphinidae, Phocoenidae and Monodontidae) about 15 Ma (Marino *et al.* 2004) (Fig. 2.19). The increase in

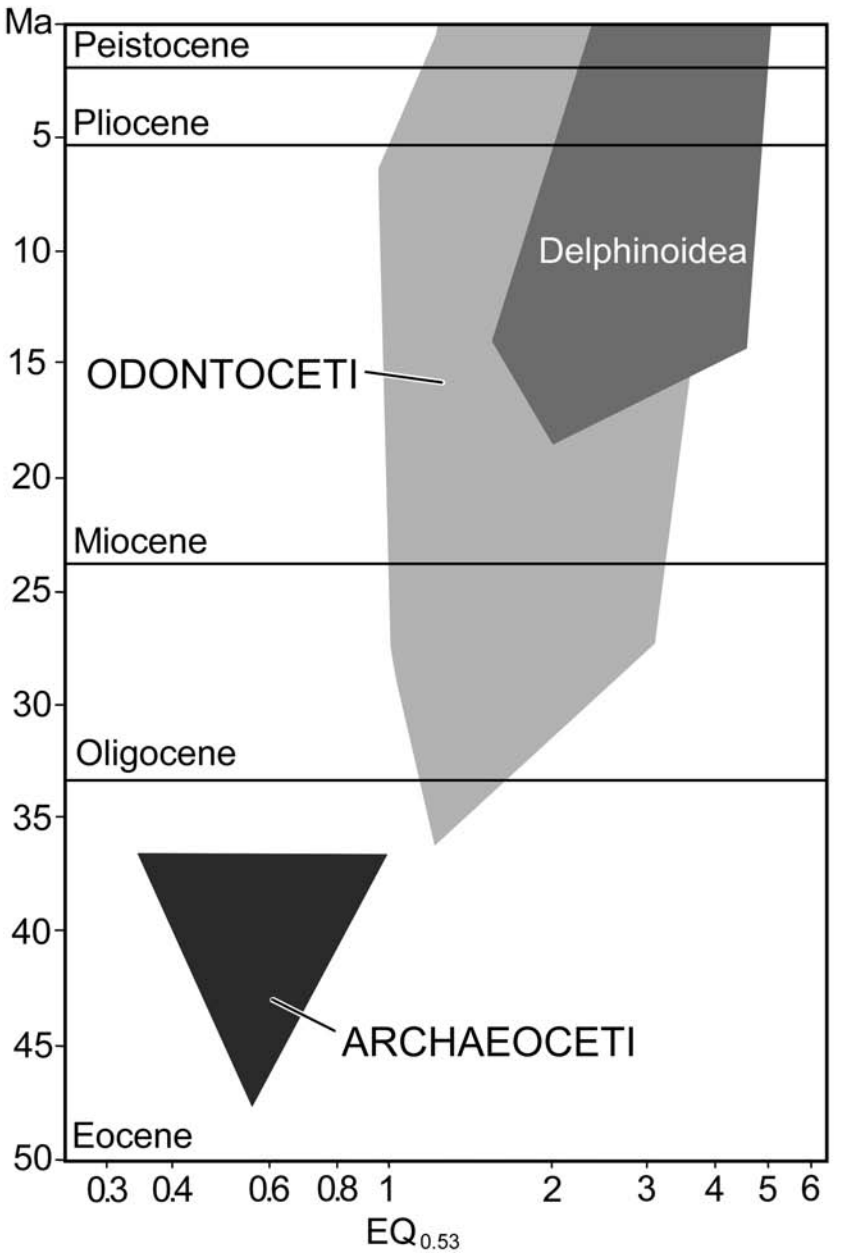

Fig. 2.19 Change over time in brain size relative to body size of archaeocetes and odontocetes (with the delphinoids evidenced). The brain size is expressed in mean log encephalization quotients: $EQ_{0.53}$ = brain weight (g)/[1.6 × body weight (g)]$^{0.53}$. From Marino *et al.* 2004. The Anatomical Record part A, 281(2): 1247-1255, Fig. 3 (redrawn and modified).

brain size among the odontocetes is probably related to the echolocation and in particular to the capability of perceiving and elaborating high-frequency sounds (Marino *et al.* 2004). Another factor that may have contributed to the high encephalization of the odontocetes is their social evolution (Connor *et al.* 1998).

Agorophiidae, Xenorophiidae and other basal odontocetes. The agorophiids were traditionally considered as primitive odontocetes intermediate between the archaeocetes and the odontocete squalodontids (Rothausen 1968; Whitmore and Sanders 1977). Fordyce (1981) redefined the agorophiids and restricted them to the single genus, *Agorophius*, from the Late Oligocene of South Carolina (USA). The only described skull (now lost) of *Agorophius* exhibits some plesiomorphic characteristics (e.g. the parietals dorsally exposed between the frontals and the supraoccipital and the heterodont dentition) but, according to Fordyce (1981), this is not sufficient to diagnose the family in terms of derived characteristics (Fig. 2.20). A second but undescribed skull belonging to *Agorophius* was reported by Geisler and Sanders (2003).

Other basal odontocetes, sometimes placed in the agorophiids, are *Archaeodelphis* and *Xenorophus* (Fordyce and Muizon 2001). *Archaeodelphis*, which is of uncertain origin and possibly from the Oligocene age, has long

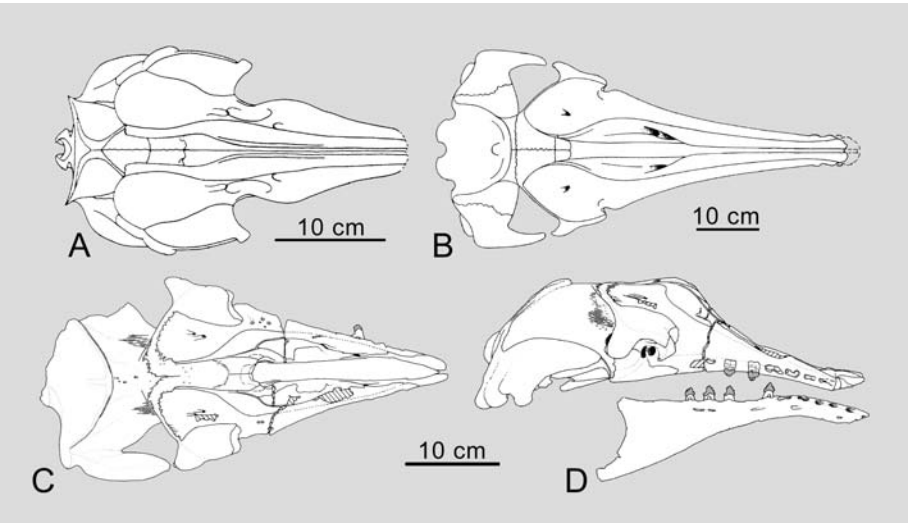

Fig. 2.20 **A**. Skull in dorsal view of *Xenorophus* (Xenorophiidae) (Mammalodontidae). **B**. Skull in dorsal view of *Patriocetus* (Squalodontidae). **C, D**. Skull in dorsal view and skull and mandible in lateral view of *Simocetus* (Simocetidae). A from Whitmore and Sanders 1977. Systematic Zoology 25(4): 304-320, Fig. 1a (modified). B from Dubrovo and Sanders 2000. Journal of Vertebrate Paleontolology 20: 577-590, Fig. 5 (modified). C, D, from Fordyce 2002a. Smithsonian Contributions to Paleobiology 93: 185-222, Figs. 3, 9, 10 (modified).

been considered the most archaic odontocete, even if it is probably not ancestral to the other known odontocetes because it has some specialized characteristics such as a large lacrimal bone. *Xenorophus*, from the Late Oligocene of South Carolina (USA), combines some archaic characteristics observed in *Agorophius* with some clearly derived features (e.g. a very large lacrimal bone, a large supraorbital process of the maxilla projecting far posteriorly, and a polydont dentition). *Xenorophus* was included in the family Xenorophidae (*sic*) by Luo and Gingerich (1999, Fig. 29) emended as Xenorophiidae by Fordyce (2003a, Fig. 9.1). Another *Xenorophus*-like odontocete with evidence of a derived premaxillary asymmetry was recently reported from the Late Oligocene of South Carolina (Mannering and Geisler 2003). According to Gaisler and Sanders (2003), *Archaeodelphis*, *Agorophius,* and *Xenorophus* belong to the stem-group Odontoceti, and in particular *Archaeodelphis* and *Xenorophus* belong to a clade with a lower grade respective to *Agorophius*.

Simocetidae. The simocetids include a single named species, *Simocetus emlongi*, from the Late Oligocene from Oregon (USA) (Fordyce 2002a). *Simocetus* is an archaic, small odontocete that combines some primitive characteristics (e.g., nares anterior to the orbit, narrow supraorbital processes of the premaxillae, and nonpolydont dentition) with some specialized features (e.g., toothless premaxillae anterior of the rostrum, and downturned mandible) (Fig. 2.20). *Simocetus* is considered a possible bottom-feeder that preyed on soft-bodied invertebrates by means of suction feeding.

Squalodontidae. The squalodontids are archaic odontocetes characterized by a narrow and rather elongated rostrum and heterodont dentition (Kellogg 1923; Rothausen 1968; Muizon 1991) (Fig. 2.21). Most of the features designed to distinguish these odontocetes are actually plesiomorphies (Cozzuol 1996; Muizon 2002) and Geisler and Sandres (2003) removed *Patriocetus* (Fig. 2.20) from squalodontids and assigned it to a lower grade in our cladogram. The squalodontids are relatively common in the Late Oligocene-Middle Miocene fossiliferous sediments and they disappear by the Late Miocene. The type-genus, *Squalodon* is from the Early-Middle Miocene of the North Atlantic and the Mediterranean. In the past, isolated, non-diagnostic squalodontid-like teeth were frequently referred to this family.

Prosqualodontidae. The family Prosqualodontidae is only based on the broad- and short-beaked *Prosqualodon* from the latest Oligocene-Early Miocene of the Southern Hemisphere (Patagonia, Tasmania and New Zealand) (Fordyce 1984; Cozzuol 1996; Muizon 2002). *Prosqualodon* differs from the apparently similar squalodontids in its shorter rostrum and its lack of certain derived characteristics of the ear bones.

Waipatiidae. The waipatiids are extinct archaic dolphins with a slightly asymmetrical skull, heterodont and polydont teeth (Fordyce 1994). The type-genus, *Waipatia,* is from the Late Oligocene of New Zealand. *Microcetus* from

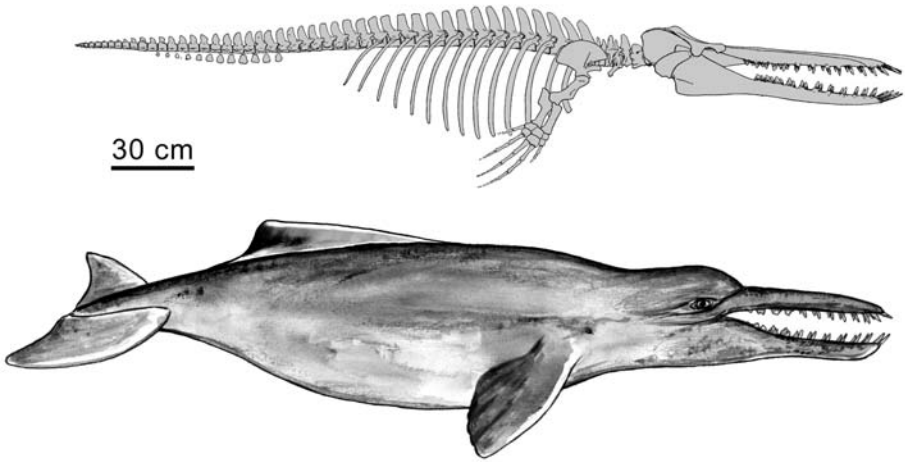

Fig. 2.21 Skeleton and body reconstruction of *Squalodon* (Squalodontidae). Skeleton from Dal Piaz 1916. Memorie dell'Istituto Geologico della Reale Università di Padova 4: 1-94, Fig. 10 (modified). Drawing of body reconstruction original.

the Late Oligocene of Germany, *Sachalinocetus* (Fig. 2.30B) from the Early or Middle Miocene of Sakhalin (Russia), and *Sulakocetus* from the Late Oligocene of Caucasus probably also are waipatiids (Fordyce and Muizon 2001; Muizon 2002).

Squalodelphinidae. The squalodelphinids are Early-Middle Miocene odontocetes characterized by an asymmetrical skull, narrow and rather elongate rostrum and slightly heterodont but single-rooted dentition (Muizon 1987, 2002). The type-genus, *Squalodelphis,* is from the Early Miocene Belluno sandstones (Italy). Other referred genera are: *Notocetus* from the Early Miocene of Argentina, New Zealand, and The United States; *Medocina* from the Early Miocene of France; and *Phocagenus* from the Early-Middle Miocene of the USA. Wrongly considered related to ziphiids in the past (Simpson 1945), the squalodelphinids are very similar to squalodontids and platanistids (Muizon 1991, 1994; Fordyce 1994).

Dalpiazinidae. The dalpiazinids are longirostral odontocetes with homodont and polydont dentition, perhaps related to the squalodontids (Muizon, 1988b). They are represented by the genus, *Dalpiazina,* from the Early Miocene of Italy (Muizon 1988b, 2002), and by undescribed remains from the Late Oligocene of New Zealand (Fordyce and Samson 1992).

Platanistidae. The platanistids, today represented only by the river dolphin genus, *Platanista*, are known as fossils from the Early-Late Miocene of the North Atlantic and Mediterranean (Muizon 1987; Barnes 2002; Bianucci and Landini 2002b) and the Early Miocene of the California coast (Barnes *et al.* 2003). The four known fossil genera, all marine, are *Prepomatodelphis*, *Pomatodelphis, Zarhachis,* and *Allodelphis*. These genera have, as do the extant

Platanista, a narrowed, elongated rostrum and homodont and polydont teeth, but they lack the complete bony skull crests of *Platanista*. The fossil genera have been included in the subfamily Pomatodelphininae and the extant *Platanista* in the monogeneric subfamily Platanistinae (Barnes 2002). *Zarhachis* was considered closely related to eurhinodelphinids by Geisler and Sandres (2003).

Physeteridae. Sperm whales (superfamily Physeteroidea) are known as fossils since about 25 Ma and they are particularly common during the Miocene (Kazár 2002; Bianucci and Landini 2006). The oldest record of this superfamily, named *Ferocetotherium*, was collected from late Oligocene sediments of the Caucasus (Mchedlidze 1970, 1976; Barnes 1985c). Many fossil physeteroids have teeth retaining the enamel crown and are traditionally referred to the polyphyletic subfamily "Hoplocetinae" (Fig. 2.22). Together with the Aulophyseterinae they form the stem-group Physeteroidea (Bianucci and Landini 2006). Among these, some have a large body size and strong teeth in both the upper and lower jaws. Examples include *Naganocetus shigensis* from Japan (= *Scaldicetus shigensis* of Hirota and Barnes 1995) and a well preserved specimen from Italy, recently described with the name of *Zygophyseter vaolai* (Bianucci and Landini 2006). They probably were active predators adapted to large-prey feeding, similar to the extant killer whale (*Orcinus orca*) (Bianucci and Landini 2006).

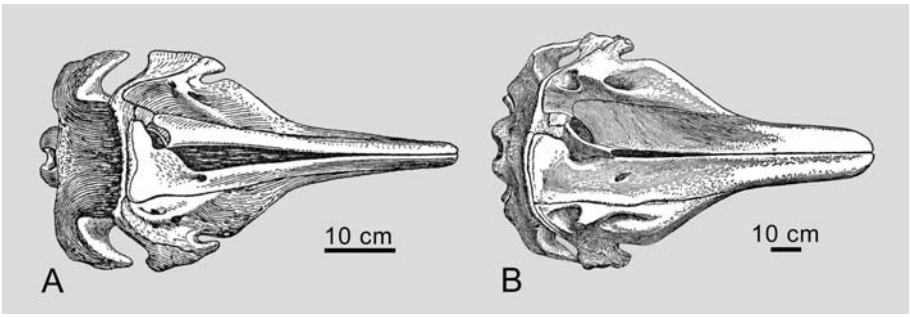

Fig. 2.22 **A**. Skull in dorsal view of *Diaphorocetus* (Physeteridae). **B**. Skull in dorsal view of *Aulophyseter* (Physeteridae). From Kellogg 1928. The Quarterly Review of Biology 3: 174-208, Fig. 14 (modified).

The family Physeteridae, here restricted to the subfamily Physeterinae, including the extant large-sized *Physeter*, is known as a fossil as far back as the Early Miocene. Physeterines are represented by medium-sized odontocetes, such as *Orycterocetus* and *Placoziphius*. The last two genera are characterized by a cranial fossa that does not extend onto the rostrum and by a relatively narrow distal portion of rostrum. *Orycterocetus* is known from the Miocene sediments of both the coast of the North Atlantic and from the Mediterranean (Bianucci *et al*. 2004). *Placoziphius* is reported from the Miocene sediments of Belgium and Austria (Kazár 2002). The extant *Physeter* is represented as fossil mainly by fragmentary remains (mostly teeth) and the most significant

specimen referred to this genus is an almost complete postcranial skeleton from the Pliocene of North Italy (Parona 1930).

Kogiidae. Our recent cladistic analysis (Bianucci and Landini, 2006) evidences that *Kogia* and related fossil genera are a separate family, as already affirmed by some authors in the past (Miller 1923; Barnes 1973; Kasuya 1973; Muizon 1991; Bianucci and Landini 1999). Kogiidae are small physeteroids lacking both nasals in the skull. They are known as fossils since the Late Miocene with the extinct genera *Scaphokogia* and *Praekogia* respectively from Peru (Muizon 1988a) and California (Barnes 1973). The extant genus *Kogia* is reported in the Pliocene from Italy with *K. pusilla*. This fossil species principally differs from extant *K. breviceps* and *K. sima* by the more elongated rostrum and the smaller antorbital process (Bianucci and Landini 1999).

Ziphiidae. The oldest reputed ziphiid is the eurhinodelphinid-like *Squaloziphius* from the Early Miocene of Washington State (USA) (Muizon 1991). This genus was originally considered the sister taxon of all other ziphiids by Muizon (1991), an interpretation recently confirmed by the phylogenetic analysis of Lambert (2005c). Nevertheless, Muizon (1991) considered *Squaloziphius* as still belonging to the beaked whales, while Lambert (2005c) retained it as probably outside this family. This genus also has been considered as a possible eurhinodelphinid (Fordyce and Barnes 1994) or as sister taxon of a large clade formed by physeterids, ziphiids, delphinoids, and river and/or long-beaked dolphins (Geisler and Sanders 2003). Three partial skulls from the Middle Miocene of Belgium, unequivocally belonging to beaked whales, were recently described by Lambert and Louwye (2006) as *Archaeoziphius microglenoideus*. Excluding *Squaloziphius*, *Archaeoziphius* may be considered the oldest reported ziphiid known by significant cranial material.

Most fossilized ziphiid remains consist of isolated portions of pachyostosed rostra and are referred to the genus *Mesoplodon*. Further, many of these remains are referred to the fossil species *M. longirostris* (see Whitmore *et al.* 1986; Bianucci 1997 for revision). Some well known extinct genera, such as *Choneziphius*, *Ziphirostrum, Beneziphius, Aporotus,* and *Messapicetus* (Figs. 2.23, 2.30F), are characterized by a strong rostrum with premaxillae medially connected and, consequently, a closed mesorostral groove (Bianucci *et al.* 1992, 1994; Lambert, 2005c). *Choneziphius* and *Ziphirostrum* are relatively common in the Middle and Late Miocene of Belgium while the long-beaked *Messapicetus* is from the Late Miocene of Italy (Fig. 2.23). The Late Miocene *Caviziphius* from Belgium (Bianucci and Post 2005) and the Early Pliocene *Tusciziphius* from Italy (Bianucci 1997) could represent intermediate forms between all previously cited extinct genera (*Messapicetus*, *Ziphirostrum*, *Beneziphius, Aporotus* and *Choneziphius*) and extant *Ziphius*. The Early Pliocene *Ninoziphius* from Peru (Muizon 1984) is a long-beaked ziphiid closely related to extant *Berardius* and *Tasmacetus*. The reduction in number of teeth, observed in all living ziphiids other than *Tasmacetus*, seems to be a less common

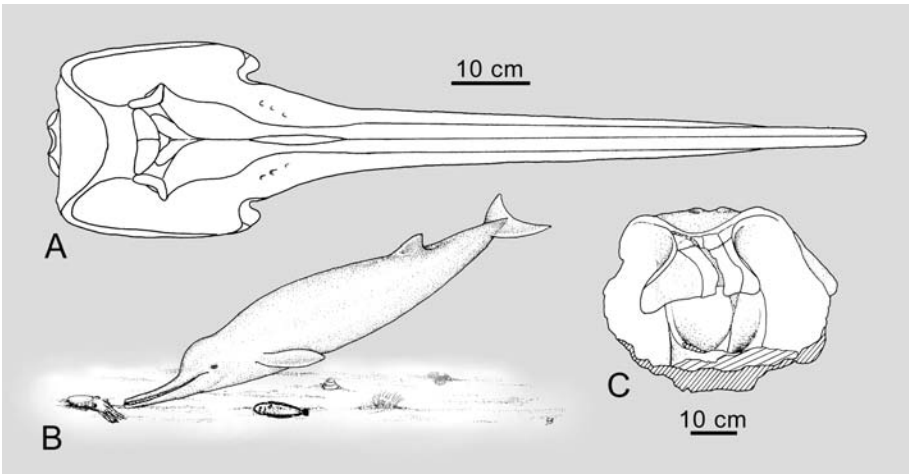

Fig. 2.23. A, B. Skull in dorsal view and body recostruction of *Messapicetus* (Ziphiidae). **C**. partial skull in dorsal view of *Tusciziphius* (Ziphiidae). A, B from Bianucci *et al.* 1994. Bollettino della Società Paleontologica Italiana 33: 231-242, Figs. 1, 9 (modified). C from Bianucci 1997. Palaeontographia Italica 84: 163-192, Fig. 2a (modified).

character in the fossil genera. In fact, *Ziphirostrum*, *Messapicetus*, and *Ninoziphius* exhibit functional maxillary teeth.

Eurhinodelphinidae. The eurhinodelphinids are extinct bizarre long-beaked dolphins characterized by an edentulous premaxillary anterior part of the rostrum that is longer than the mandible (Kellogg 1925; Myrick 1979, Muizon 1988b; Lambert 2004, 2005a,b) (Fig. 2.24). These swordfish-like dolphins originated in the Late Oligocene and became very common during the Early and Middle Miocene, but disappeared during the Late Miocene. The eurhinodelphinids are considered paraphyletic in a recent publication that put the origins of ziphiids inside this clade (Lambert 2005a). Remains of

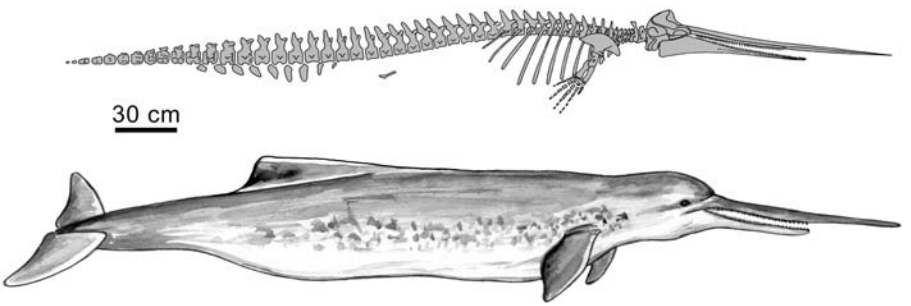

Fig. 2.24 Skeleton and body reconstruction of *Eurhinodelphis* (Eurhinodelphidae). Skeleton from Abel 1931. Mémoires du Musée Royal d'Histoire Naturelle de Belgique, 48: 191-334, Pl. 29 (redrawn). Drawing of body reconstruction original.

eurhinodelphinids are particularly common in the fossiliferous sediments of the United States, Italy, and Belgium. *Vanbrenia trigonia* from the Middle Miocene of the Netherlands could represent a case of shortening of the rostrum in the eurhinodelphinids, probably related to a specialized adaptation to bottom suction feeding (Bianucci and Landini 2002a).

Eoplatanistidae. The eoplatanistids are extinct odontocetes closely related to the eurhinodelphinids (Muizon 1988b). They show a very elongate and slender gavial-like rostrum with many teeth. The eoplatanistids are only represented by the genus *Eoplatanista* from the Early Miocene of Italy.

Kentriodontidae. The kentriodontids are probably a polyphyletic group of archaic extinct delphinoids (Kellogg 1927; Barnes 1978; Muizon 1988c; Dawson 2002). Their fossil record shows a cosmopolitan distribution from the Late Oligocene to the end of the Miocene (Ichishima *et al.* 1995; Bianucci 2001; Lambert *et al.* 2005). Most genera (e.g., *Liolitax* and *Rudicetus*) are small generalized odontocetes with a medium-elongated rostrum (Fig. 2.25A, B). Cases of shortening (e.g., *Leptodelphis,* Fig. 2.30C) and extreme elongation (e.g., *Belonodelphis*) of the rostrum presumably represent specialized distinct trophic adaptations. Some genera (e.g., *Kampholophos* and *Hadrodelphis*) still have heterodont dentition even if all teeth have a single root. The skull of the kentriodontids generally shows less asymmetry in comparison to the modern delphinids.

Delphinidae. The delphinids are known as fossils since the Late Miocene of California (Barnes 1977). Older supposed delphinid finds probably represent other odontocete families or are too incomplete to confirm relationships. The delphinids became very diversified in the Pliocene and are particularly common in fossiliferous sediments of Italy (Bianucci 1996, 2005). The recorded Italian genera are the fossil genera, *Arimidelphis*, *Astadelphis*, and *Hemisyntrachelus,* and the living *Orcinus*, *Tursiops*, *Stenella*, and possibly *Globicephala*. An even more diversified delphinid fauna is actually indicated by numerous isolated periotics. The extinct genus *Arimidelphis* is a small, killer whale-like animal characterized by a strong mandible but relatively small and numerous teeth. *Astadelphis*, with a relatively narrow rostrum and elongate mandibular symphysis, could be closely related to the extant Stenoninae (Fig. 2.25E, F). *Hemisyntrachelus* is a generalized delphinid differing from the relatively similar extant *Tursiops* principally by having a larger body size and a smaller number of teeth. The living genus *Orcinus* is represented by the fossil species *Orcinus citoniensis* (Fig. 2.30J) which differs from the extant killer whale principally in its smaller body size (about 4.5 m long) and larger number of teeth (14 in each tooth row). The fossil species *Tursiops osennae* may not belong to the same genus of the extant Bottlenose dolphin. The most significant specimens referred to *Stenella* are those described as *Stenella giulii,* a fossil species differing from the extant ones principally because of its larger size. *Globicephala* is dubiously reported from the Italian

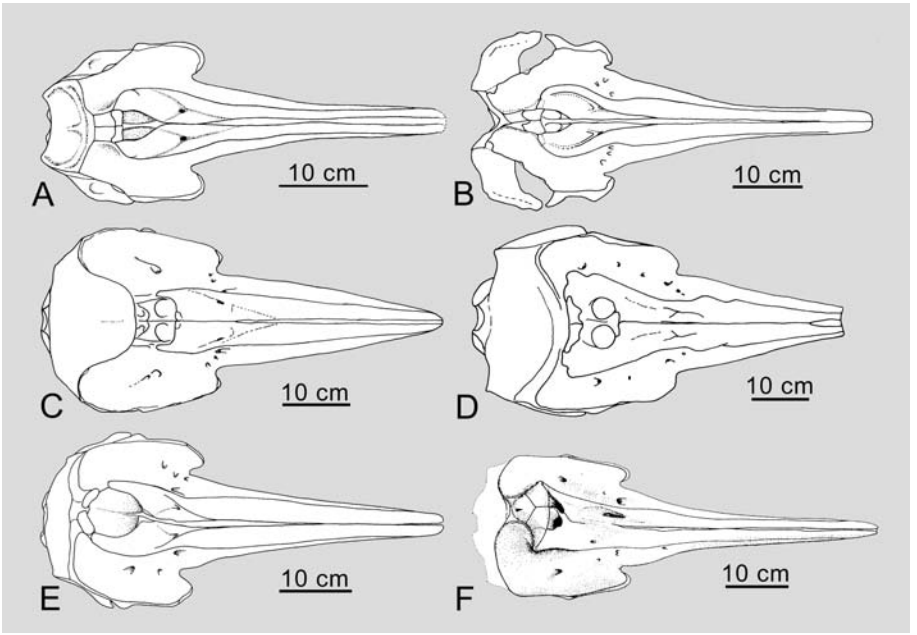

Fig. 2.25 Skulls in dorsal view of some fossil delphinoids. **A**. *Rudicetus* (Kentriodontidae). **B**. *Liolithax* (Kentriodontidae). **C**. *Piscolithax* (Phocoenidae). **D**. *Albireo* (Albireonidae). **E**. *Astadelphis* (Delphinidae). **F**. *Australodelphis* (Delphinidae). A from Bianucci 2001. Journal of Vertebrate Paleontology 21(3): 573-577, Fig. 3C (modified). B from Barnes 1978. Natural History Museum of Los Angeles County Science Bulletin 28: 1-35, Fig. 14b (modified). C from Muizon 1984. Travaux de l'Institut Français d'Études Andines 27: 1–188, Fig. 36a (redrawn). D from Barnes 1984. Paleobios 42: 1-46, Fig. 11a (redrawn). E from Bianucci 1996. Palaeontographia Italica 83: 73-167, Fig. 41 (modified). F from Fordyce *et al.* 2002. Antarctic Science 14(1): 37-54, Fig. 3b (modified).

Pliocene with an incomplete mandible described as *Globicephala etruriae*. Elsewhere, the most significant delphinid assemblage is that of the Pliocene Yorktown Formation (USA) (Whitmore 1994). Although not described in detail, the extant genera *Stenella*, *Tursiops*, *Globicephala,* and *Pseudorca* are reported and *Delphinus* and *Lagenorhynchus* are uncertainly signaled from this formation. Finally, from the Pliocene sediments of Eastern Antarctica, a bizarre delphinid genus, named *Australodelphis*, has recently been described (Fordyce *et al.* 2002). *Australodelphis* is a toothless ziphiid-like dolphin and perhaps it was a suction-feeding squid-eater.

Phocoenidae. The porpoises are extant delphinoids with a significant fossil record since the Late Miocene (Barnes 1985a; Muizon 1984, 1988a). *Salumiphocaena* from the Late Miocene (about 10-11 Ma) of California is the oldest reported porpoise (Barnes 1985a). *Australithax*, *Piscolithax* (Fig. 2.25C) and *Lomacetus* are known from the Late Miocene-Early Pliocene of Peru

(Muizon 1984, 1988a), while *Numataphocoena* and *Haborophocoena* have recently been described from the Early Pliocene of Japan (Ichishima and Kimura 2000, 2005).

All the fossil porpoises mentioned above share with the extant genera of this family some derived characteristics such as the premaxillary eminences and spatulate teeth. The rostrum of these fossil genera, when preserved, is more elongated in comparison with that of extant porpoises. The supposed short-beaked phocoenid, *Microphocoena,* from the Late Miocene of the Paratethys was referred to kentriodontids by Barnes (1978) and reconsidered as a porpoise by Muizon (1988c). Undescribed porpoise ear bones also are from the Pliocene of Italy.

Albireonidae. The albireonids are a monogeneric extinct family based upon *Albireo* from the Late Miocene-Early Pliocene of Baja California, Mexico (Barnes 1984), and Japan (Barnes and Furusawa 2001). *Albireo* is a porpoise-like odontocete (Fig. 2.25D) considered to have arisen from kentriodontids (Barnes 1984). Alternatively, it could be a sister taxon of phocoenids (Muizon 1988c).

Monodontidae. *Denebola* from the latest Miocene of Isla Cedros (Mexico) is the only reported fossil genus of the monodontids (Barnes 1984). This extinct delphinoid of the warm equatorial waters of the eastern Pacific was characterized by a short rostrum and a wide cranium.

The extant genus *Monodon* (Narwhal) also is found as a fossil from the Pleistocene in the North Atlantic (Fordyce and Muizon 2001) and the extant *Delphinapterus* (Beluga) is found as fossils from the Pliocene in North Carolina (Whitmore 1994). Indeterminate monodontids also are described from the Pliocene in Peru (Muizon 1988a). Supposed monodontids from the Early Miocene in Baltringen (Germany) actually belong to platanistids (Bianucci and Landini 2002b).

Odobenocetopsidae. *Odobenocetops*, the only known genus of odobenocetopsids, is a bizarre walrus-like cetacean from the Early Pliocene of Peru that is characterized by two large tusks similar to those of the living Walrus, *Odobenus* (Muizon 1993; Muizon *et al.* 1999, 2002; Muizon and Domning 2002) (Fig. 2.26). In particular, the right tusk of the male is very elongated and can reach one meter or more in length. The skull of *Odobenocetops* also differs from other cetaceans in that the very short and rounded rostrum is almost exclusively formed by the premaxillae and the bony nares are displaced far anteriorly. Their extremely salient occipital condyles indicate great mobility of the neck, probably related to bottom-feeding.

Pontoporiidae. The pontoporiids – represented today only by the long-beaked Franciscana, *Pontoporia blainvillei* – are known since the Middle-Late Miocene. The oldest fossil is the very short-beaked *Brachydelphis* from the marine sediments of Peru (Muizon 1984). Surprisingly, this genus was recently

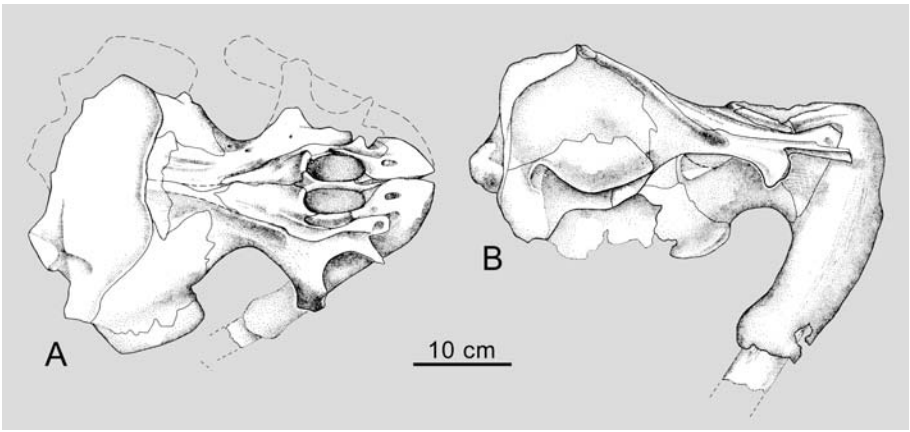

Fig. 2.26 Skull of *Odobenocetops* (Odobenocetopsidae). **A.** Dorsal view. **B.** Lateral view. From Muizon 1993. Nature 365: 745–748, Fig. 1 (modified).

identified as the sister taxon of all river dolphins and eurhinodelphinids (Geisler and Sanders 2003).

Other fossil marine pontoporiids include *Pliopontos*, reported from the Late Miocene in Paranà (Argentina) and from the Pliocene in Peru (Muizon 1984; Cozzuol 1996). Undescribed significant pontoporiid remains were recently found in the Miocene of Chile (see Fig. 2.30G). A partial skull similar to *Pliopontos* also was reported from the Late Miocene in Maryland (USA) (Godfrey 2001) and indeterminate pontoporiids are described from the Early Pliocene in Florida (USA) (Morgan 1994), extending their geographical range along the North Atlantic. The large geographical fossil distribution of the pontoporiids was confirmed by the recent assignation to this family of *Protophocaena*, a small toothed whale from the Miocene of North Sea (Lambert and Post 2005).

The longirostral *Parapontoporia*, reported from the latest Miocene in Isla Cedros (Mexico), was referred to pontoporiids by Barnes (1985b) but to lipotids by Muizon (1988c).

Iniidae. The only surely known fossil belonging to iniids is *Ischyrorhynchus* from the Late Miocene freshwater sediments in Argentina (Cozzuol 1996). This genus differs from extant *Inia* essentially in its larger size and relatively longer rostrum (Muizon 2002). Other fragmentary remains previously referred to iniids actually belong to other families or are based on nondiagnostic material (e.g. *Goniodelphis*, *Hesperoinia*, and *Saurocetes*).

Lipotidae. *Prolipotes* from the freshwater deposits of China, of uncertain stratigraphic position, was considered closely related to extant *Lipotes* (Zhou *et al.* 1984). This fossil genus is actually based only on a fragment of mandible and should be referred to Odontoceti *incertae sedis* (Fordyce and Muizon 2001). The already cited latest Miocene *Parapontoporia* may represent a marine lipotid from the subtropical north-east Pacific coast (Muizon 1988c).

2.7 EVOLUTION AND ENVIRONMENTAL CHANGES

2.7.1 Eocene (56-34 Ma): From the Land to the Sea

The oldest archaeocete remains, referred to pakicetids, ambulocetids, remingtonocetids, and protocetids, all are from Pakistan and northern India. During the Eocene, this geographical area represented the eastern portion of Tethys, an epicontinental warm sea separating the Eurasian continent from the African and Indian blocks. The geographical location and the climatic optimum (Fig. 2.27) characterizing the Early Eocene support a warm water stenotherm affinity for these first cetaceans. The high food availability in this warm epicontinental sea could have favored the initial radiation of the whales (Lipps and Mitchell 1976; Gingerich *et al.* 1983).

Pakicetids were terrestrial and/or semiaquatic mammals that lived on the coastal floodplains during the late Ypresian-early Lutetian. The ambulocetids were semiaquatic animals inhabiting the bays and estuaries in the early Lutetian. All pakicetid and ambulocetid remains only have been found in a small area between northwestern Pakistan and northern India indicating a very limited geographical distribution of the first whales (Fig. 2.28).

The remingtonocetids were the first whales totally independent of freshwater. They inhabited coastal and lagoon environments during the early-middle Lutetian together with the first protocetids. All remingtonocetids and three archaic protocetids (*Artiocetus*, *Rhodocetus*, and *Takracetus*) were found in central Pakistan and western India. The more specialized protocetids, although retaining hind limbs, could swim in open seas. They radiated into the western Tethys (Egypt) and along the Atlantic African coasts (Nigeria) during the middle-late Lutetian and were present in the western Atlantic waters (eastern coast of northern America) during the Bartonian (Williams 1998). The decline of the basal archaeocetes after the end of the Lutetian may be partly due to the gradual closure of the Tethys and the related cooling. In fact, during this later Eocene phase all animals and plants of warm climates and tropical forests were deeply decimated (Berggren and Prothero 1992).

The fully-marine basilosaurids have a cosmopolitan distribution with fossils found in the latest Middle Eocene and Upper Eocene sediments of all continents (Uhen 1998). Basilosaurids are known since the Lutetian, on the basis of a single specimen from Austria (Uhen and Tichy 2000), although their radiation is in the late Middle Eocene. In fact, basilosaurids appear almost contemporaneously in the Bartonian sediments of the Tethys and the northern Atlantic (Pakistan, Jordan, Egypt, Senegal, Europe, and the eastern United States), as well as in New Zealand (Fig. 2.29). The geographical distribution of the Middle Eocene basilosaurids suggests that these whales were predominantly stenotherm, preferring rather warm waters. During the Priabonian (Late Eocene), the basilosaurids radiated to the high latitudes of both hemispheres (Canada, Peru, and Antarctica), indicating a general adaptation of the whales to cold waters.

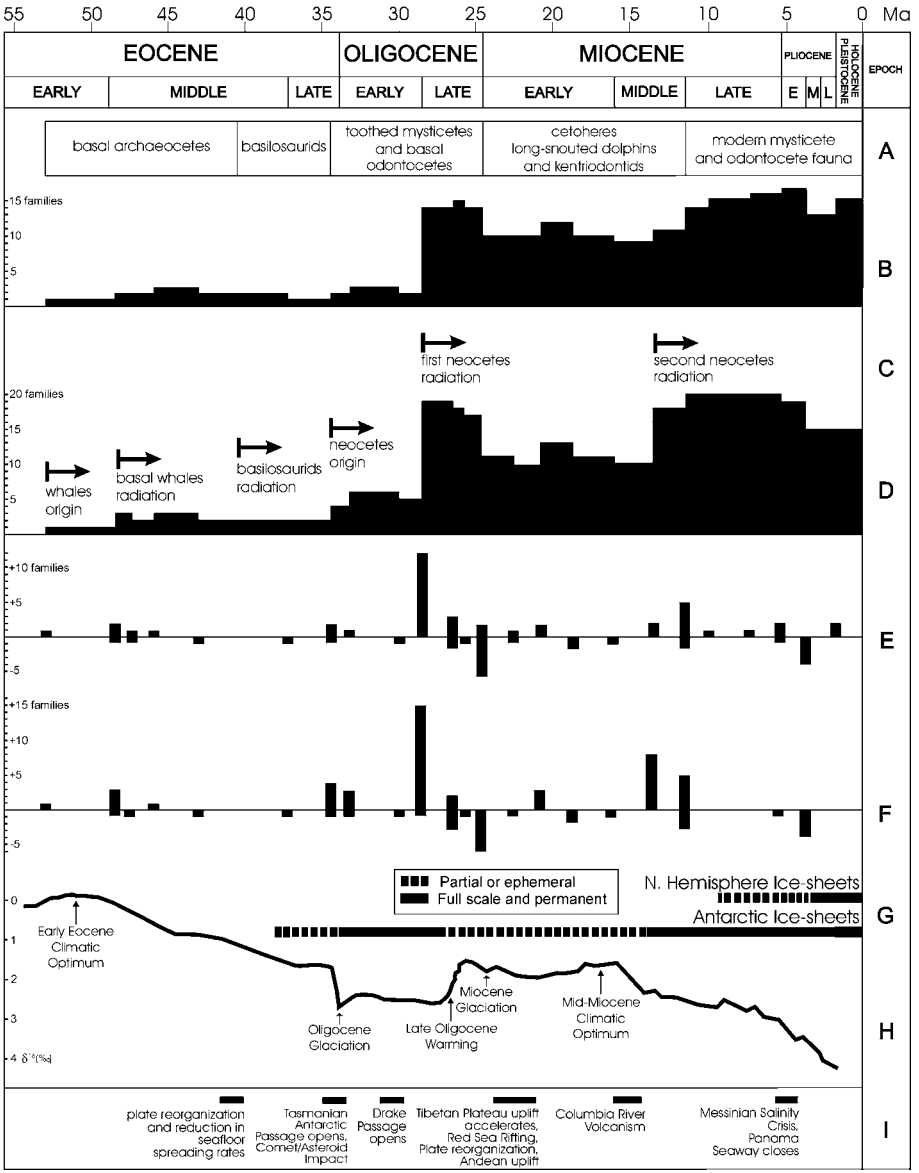

Fig. 2.27 Change in cetacean diversity at family level related to main physical events. **A**. Dominant cetacean fauna. **B**. Number of families based on fossil record. **C**. Origin and/or radiation events in the cetacean history. **D**. Number of families based on fossil record and ghost lineages. **E**. Number of families appearing (+) or disappearing (–) based on fossil record. **F**. The same as E considering the ghost lineages. **G**. Ice volume in each hemisphere. **H**. Global deep-sea oxygen isotope variations related to changes in temperatures. **I**. Main tectonic events. Data on cetaceans are extrapolated from Fig. 2.8. Data on physical events are derived mainly from Zachos *et al.* 2001. Science 292: 686-693, Fig. 2. Original.

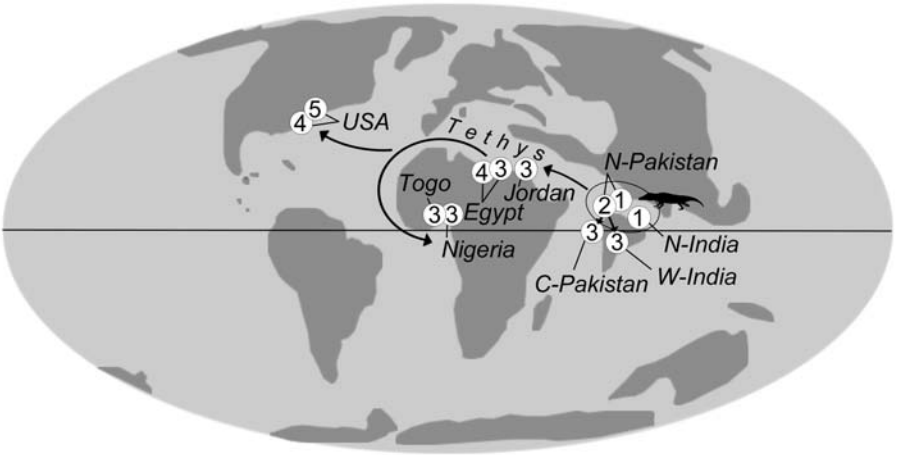

Fig. 2.28 Geographical distribution and radiation of early archaeocetes (pakicetids, ambulocetids, remingtonocetids, and protocetids). **1**. Late Ypresian-early Lutetian. **2**. Earliest middle Lutetian. **3**. Middle-late Lutetian: **4**. Bartonian. **5**. Early Priabonian. Data from Williams (1998), Gingerich and Uhen (1998), Fordyce (2003a), Zalmout *et al.* (2003). Original.

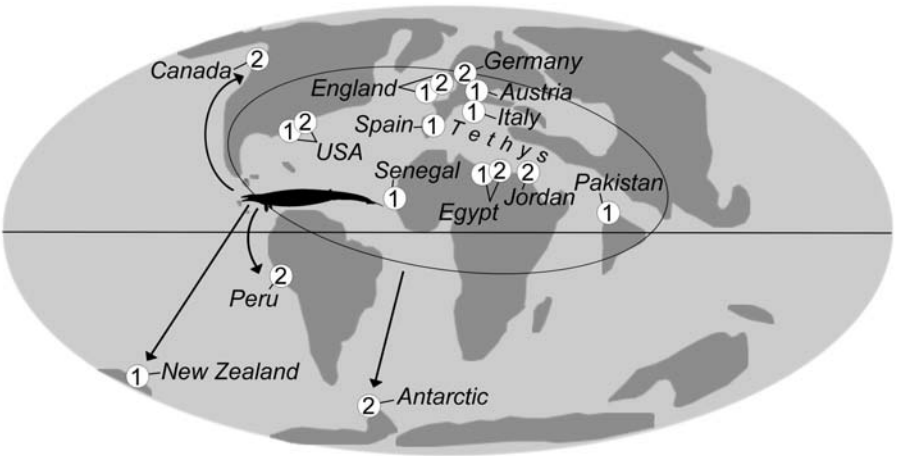

Fig. 2.29 Geographical distribution and radiation of basilosaurids. **1**. Lutetian. **2**. Bartonian. **3.** Priabonian. Data from Uhen (1998), Gingerich and Uhen (1998), Uhen and Tichy (2000), Zalmout *et al.* (2000), Fordyce (2003a). Original.

Basilosaurids are not known from rocks younger than Eocene and the possible cause of their decline and extinction may be the increase in cooling near the Eocene-Oligocene boundary related to the extension of the Antarctic ice sheet (Zachos *et al.* 2001) and to an asteroid or comet impact (Vonhof *et al.* 2000; Tagle and Claeys 2004). The global cooling and the progressive constriction of the Tethys may have caused the extinction in tropical and subtropical water of the possible stenotherm basilosaurids [e.g. *Saghacetus*

from lagoonal deposits of the Priabonian of Egypt (Gingerich 1992)]. More indirectly cooling and the related modifications of water circulation and food resources might have influenced cetacean ecology and diversity (Fordyce 2003a). Any basilosaurids that were adapted to the cold waters of high latitudes may have become extinct through competition with the first mysticetes and odontocetes, which likely were favored because of more advanced feeding strategies. Competition may have occurred with the more specialized archaeocetes recently reported from the Late Oligocene (Fordyce 2004) [the latter possibly originated before the end of Eocene (Fig. 2.8)]. Nevertheless the hypothesis of archaeocete-neocete competition is rather speculative, considering the scarcity of whale fossils at the Eocene-Oligocene boundary.

2.7.2 Oligocene (34-23 Ma): An Experimental Phase

Fossil cetaceans in the Early Oligocene are rather rare and mostly are represented by fragmentary remains or significant but undescribed specimens. In particular, fossil archaeocetes are reported in Early Oligocene strata on the basis of some teeth or poor diagnostic postcranial elements, such as the isolated vertebrae of *Platyosphys* from Ukraine and a tooth of *Phococetus* from France (Kellogg 1936). Actually, these remains are of uncertain Early Oligocene age and recently have been placed in the Cetacea *incertae sedis* (Fordyce, 1992, 2003a). In any case, the recently reported fossil archaeocetes from the Late Oligocene (Fordyce 2004) represent indirect evidence of the presence of this suborder in the Early Oligocene.

The Early Oligocene remains referred to mysticetes are represented by the latest Eocene-earliest Oligocene *Llanocetus* from Seymour Island (Antarctic) (Mitchell 1989; Fordyce 2003b), by aetiocetids from Washington State (Goedert *et al*. 1995, 2001) and South Australia (Pledge 2005) and by some fragmentary remains uncertainly referred to this suborder along with an incomplete *Llanocetus*-like skull from New Zealand (Fordyce 2002d, 2003b). The oldest reported fossil odontocete is an undescribed skull with agorophiid affinities collected in sediments of about the Eocene-Oligocene boundary from Washington State (Goedert and Barnes 1996; Barnes 2000). The suborder and/or stratigraphic collocation of other supposed Early Oligocene odontocete remains is uncertain (Fordyce 2003a). This apparent scarcity of cetaceans in the Early Oligocene may be due to a general drop in sea level and subsequent erosion of most of the fossiliferous sediments deposited during this interval of time (Fordyce 1992, 2003a; Fordyce and Muizon 2001).

The Late Oligocene cetacean fauna is characterized by an explosive radiation. In fact, during this age 17 families belonging to archaeocetes (1), mysticetes (6) and odontocetes (10) are reported. Considering the ghost lineages, the number may be raised to 21 with an addition of four other odontocete families (Figs. 2.8, 2.27). Fordyce (2003a) reported 60-65 species during this age interval. Among the Late Oligocene cetaceans that survived into the following epochs are the delphinoid kentriodontids, the physeterids,

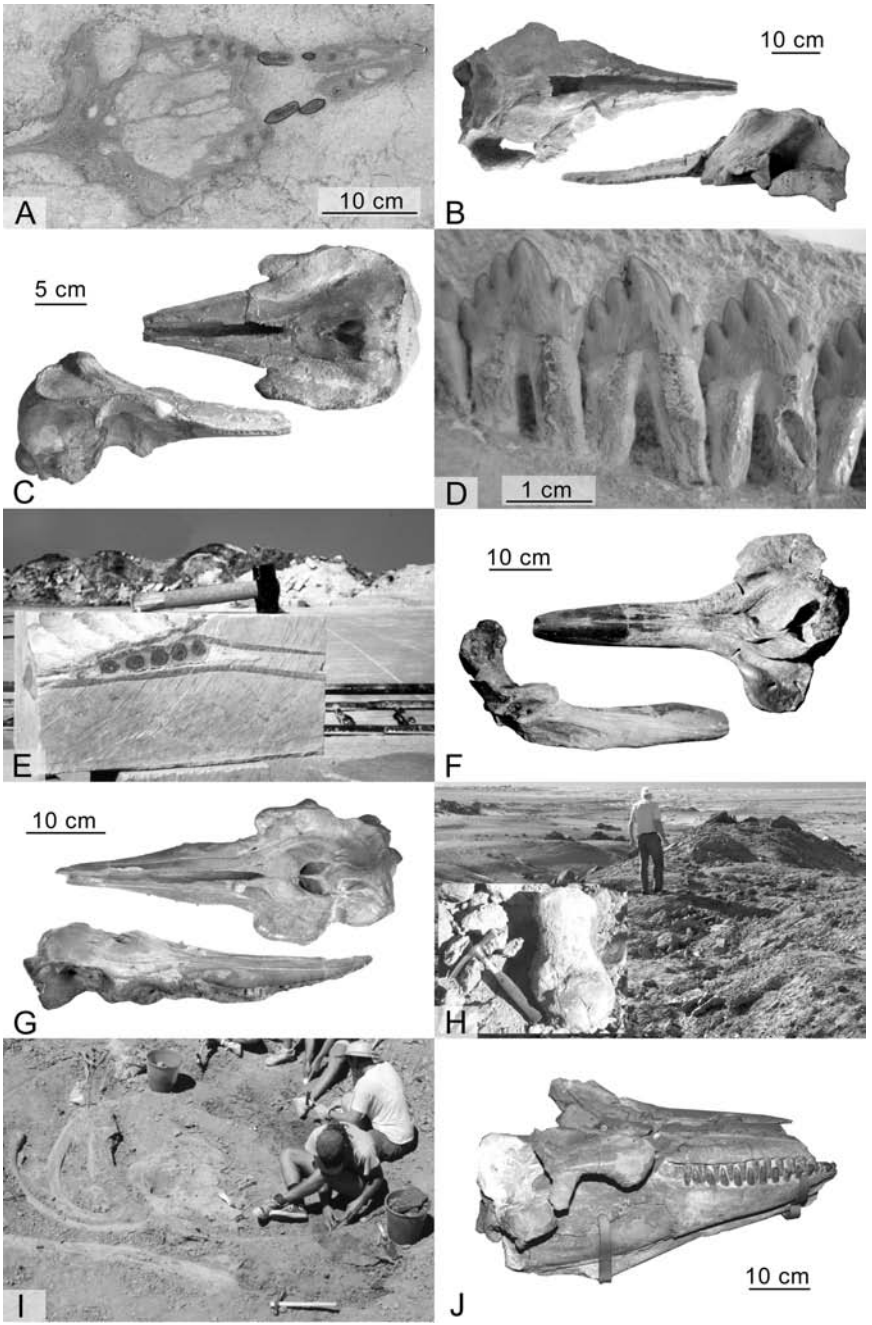

Fig. 2.30 **A**. Section of the skull of an undescribed protocedid-like archaeocete in a block of nummulitic limestone of the Middle Eocene of Egypt. **B**. Skull in dorsal

Fig. 2.30 Contd. ...

the long-beaked eurhinodelphinids, the shark-toothed squalodontids, and the baleen whales (cetotheres and balaenids).

The origin of mysticetes and odontocetes and their subsequent explosive radiation was correlated to some concurrent global physical events: the development of the Circum-Antarctic Current due to the final breakup of the austral continent Gondwanaland, the general cooling, the increase in temperature latitudinal gradients, and the changes in productivity of ocean ecosystems (Fordyce 1977, 1980, 2002b, 2003a). The new feeding strategies of mysticetes and odontocetes (filter feeding and echolocation) favored the rapid diversification and colonization of these more heterogeneous oceans. Speculations on the possible ecological interactions between the Oligocene archaeocete and the neocetes are premature because little is published on the latest archaeocetes.

The Late Oligocene may also be interpreted as an experimental phase of cetacean evolution. Several different morphologies (probably reflecting different ecological adaptations) evolved contemporarily and were deeply sifted by selection (Fordyce 1992). Among the exclusive Late Oligocene cetaceans are some bizarre forms such as the presumed suction feeder, *Simocetus*, the short-jawed gulp feeder, *Mammalodon,* and the presumed raptorial-bottom feeder, *Kelloggia* (a squalodontid characterized by a rostrum with unusual enlargement of the anterior portion).

During and at the end of the Late Oligocene, nine cetacean families disappear (one archaeocete, four mysticetes, and four odontocetes). These extinctions were partially compensated by the appearance of two odontocete families (even if, considering the ghost lineage, these two families may have originated in the Oligocene). A superficial analysis of this apparently critical phase of cetacean history denotes that the extinctions are selective. In fact, all extinct groups are characterized by heterodont dentition, while only one family (Squalodontidae) of the seven families which crossed the Oligocene-Miocene boundary is typically heterodont. Moreover, all the toothed mysticetes disappear about the end of the Oligocene. Among the odontocetes

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Fig. 2.30 Contd. ...

and lateral views of *Sachalinocetus cholmicus*, a possible waipatiid from the Early-Middle Miocene of Sakhalin (Russia). **C**. Skull in dorsal and lateral views of *Leptodelphis stavropolitanus*, a kentriodontid from the Late Miocene of Stavropol (Russia). **D**. Cheek-teeth of *Neosqualodon assenzae*, an Early Miocene squalodontid from Sicily (Italy). **E**. Portion of mandible of *Zygophyseter varolai* (physeteroid) in a block of "Pietra leccese" stone of the Late Miocene of Salento Peninsula (Italy). **F**. Skull in dorsal and lateral views of *Chonezipius planirostris* a Late Miocene ziphiid from the North Sea (Netherlands). **G**. Skull in dorsal and lateral views of an undescribed pontoporiid from the Late Miocene sediments near Caldera (Chile). **H**. Bones of a large mysticete outcropping in the Late Miocene sediments near Caldera (Chile). **I**. Field excavation of a mysticete skeleton from the Pliocene of Tuscany (Italy). **J**. Skull in lateral view of *Orcinus citoniensis* from the Pliocene of Tuscany (Italy). Photos by G. Bianucci and W. Landini. Original.

crossing the Oligocene-Miocene boundary, some squalodelphinids and kentriodontids show some degree of tooth differentiation, but less than other typical heterodont families because they (squalodelphinids and kentriodontids) tend to have single-rooted teeth. Among the odontocetes crossing the Oligocene-Miocene boundary, some squalodelphinids and kentriodontids show some degree of tooth differentiation, but all their teeth are single rooted. All cetacean families which appear after the Oligocene are homodont. Trophic competition may have been a cause of the changing patterns of feeding apparatus. Detailed studies on this topic seem worthwhile.

2.7.3 Miocene (23-5.3 Ma): The Time of the Long-beaked Dolphins

Nineteen cetacean families are known in the Miocene, although no more than 13 families are contemporary and only four are known for the full length of this epoch (Fig. 2.8), due to the progressive disappearance of some fossil families and the appearance of extant families (mostly since the Late Miocene).

In particular, during the Early-Middle Miocene the cetacean fauna is characterized by a wide radiation of platanistoids (squalodontids, squalodelphinids, platanistids, and dalpiazinids), eurhinodelphinoids (eurhinodelphinids and eoplatanistids), physeterids, and kentriodontids among the odontocetes and of cetotheres among the mysticetes. Many odontocetes of this age interval have a very long rostrum, a feature only observed in river and coastal equatorial and tropical dolphins among the extant fauna. Warm environments (Fig. 2.27) may have favored the radiation of these morphotypes, particularly in the northern Atlantic and Mediterranean waters where long-beaked odontocetes were very common (Bianucci and Landini 2002b). It is significant that the ice sheet was absent in the northern hemisphere until the end of the Miocene (Zachos *et al*. 2001) and consequently the climatic conditions of the North Atlantic and Mediterranean were even more favorable in the globally warm phase of the Middle Miocene. Most of the fossiliferous deposits rich in long-beaked dolphins are from deltaic (e.g., Molassa Bellunese of Italy) or coastal environments (e.g., Calvert Formation of the USA and Antwerp sandstones of Belgium), supporting their hypothesized habitat. Moreover eurhinodelphinid remains also are reported from freshwater sediments (Fordyce 1983). An origin of extant river dolphins from Middle Miocene odontocetes living in shallow epicontintental seas also has been hypothesized by Hamilton *et al*. (2001).

Around the Middle-Late Miocene boundary most of the platanistoids and all the eurhinodelphinoids disappeared while the kogiids and the modern delphinoid families of phocoenids, delphinids, and monodontids emerged along the eastern Pacific coasts. For the baleen whales, this time period marked the occurrence of the balaenopterids and, if considering the ghost lineages, also of the eschrichtiids and the neobalaenids.

The Middle-Late Miocene turnover may be partly due to the global deep-cooling that could have favored the disappearance of warm stenotherm dolphins and the radiation of open sea whales. Consistent with this hypothesis is the continued record of physeterids, ziphiids, and cetotheres, probably all of pelagic habitat, during the Middle-Late Miocene. Indirect interaction between the turnover in the cetacean fauna and cooling, such as changes in circulation and changes in vertical and horizontal temperature gradients, may have modified food distribution in the oceans (Fordyce and Barnes 1994; Whitmore 1994; Bianucci and Landini 2002b; Fordyce 2002b). Middle-Late Miocene extinctions of archaic families also may reflect competition with modern whales. In particular, the radiation of modern delphinoids also may have been favored by their greater encephalization relative to other odontocetes (Marino *et al.* 2004) (Fig. 2.19). Moreover, during the Late Miocene, the oldest representatives of most of the extant river dolphins, which are known as fossils (pontoporiids) or inferred to be present on the basis of the ghost lineages (iniids and lipotiids), may have competed with other long-beaked Miocene dolphins. Contrary to the hypothesis of competition, some families apparently disappeared before this boundary (squalodelphinids, dalpiazinids, and eoplatanistids) and others (eurhinodelphinids and squalodontids) were already in decline at this time. The latest Miocene extinction of kentriodontids and the mid-Pliocene extinction of cetotheres may have been due to their progressive substitution, respectively, by modern delphinoids and balaenopterids which may have occupied, at least in part, the same niches.

2.7.4 Pliocene (5.3-1.8 Ma): The Establishment of the Modern Fauna

The Pliocene fauna at familiar level is very similar to the extant fauna, except for the presence of the seal-like odobenocetopsids and some basal physeteroids among the odontocetes, the apparent lack of eschrichtiids and neobalaenids, and the survival of the fossil cetotheres among the mysticetes. From a quantitative point of view, the generic composition of the Pliocene odontocete families is relatively similar to the extant one. In fact, the delphinids are widely diversified (even if most fossil records are localized in a small number of areas). Some genera of phocoenids and ziphiids are recorded, while the physeterids are drastically reduced in both diversity and number of finds in comparison to the Miocene. Further, sperm whale declines may have been due to competition with delphinids, considering that some Miocene physeteroids (probably not adapted to deep-diving as is the extant *Physeter*) may have shared the same niches with delphinids, feeding on squids and/or large prey.

Despite a similar quantitative composition, not all Pliocene odontocete genera are presently extant: for example, among the delphinids, fossil genera such as *Astadelphis*, *Australodelphis* and *Hemisyntrachelus* are contemporaneous with the extant genera *Stenella*, *Tursiops* and *Orcinus* (see Bianucci 1996). There is

no clear evidence of extant odontocete species in the Pliocene, suggesting that many modern species have arisen since the Pliocene (within perhaps the last 2 M years). Further, even if Pliocene mysticete diversity in the northwestern Pacific was considered similar to the extant one on the basis of isolated tympanic bullae (Oishi and Hasegawa 1995a), in some other areas the baleen whales now show a greater diversity. For example, in the Mediterranean there is a consistent Pliocene record of a diversity of balaenids, cetotheres, and balaenopterids but low diversity among extant mysticetes. Indirect evidences based on fossil whale barnacles suggest that, during the Pliocene, the Mediterranean could have been a breeding area for some baleen whales, migrating into this basin from northern latitudes of the Atlantic Ocean (Bianucci *et al.* 2006).

During the Pliocene, extant genera such as *Balaenoptera*, *Eubalaena,* and *Balaena* shared the same geographical areas with several extinct mysticetes belonging to both cetotheres and extant families. Extinct genera such as *Balaenula* and *Idiocetus* were small, similar in size to the extant Pygmy right whale (*Caperea*). Although some extant baleen whale species have been reported in the Pliocene (e.g., *Balaenoptera acutorostrata*), the mysticete composition at specific levels seems substantially different from today.

Some fossil evidence suggests a shark-cetacean trophic interaction in the Pliocene. Cetacean bones marked by shark bites, as well as shark teeth found in close proximity to cetacean skeletal remains, reveal shark predation and scavenging on both mysticetes and odontocetes (Deméré and Cerutti 1982; Cigala-Fulgosi 1990). In the Mediterranean during the Pliocene, large sharks (*Carcharodon carcharias* and *Isurus hastalis*) predated not only odontocetes but also small-sized mysticetes such as balaenids and cetotheres (Bianucci *et al.* 2002).

2.7.5 Pleistocene-Holocene (1.8-0 Ma): The Appearance of the Neospecies

Based on a published list of fossil cetaceans from Japan (Oishi and Hasegawa 1995b), the Pleistocene and Holocene faunas are similar to the modern. In fact all the genera are still alive and just some species are extinct. Nevertheless, the specimens found consist primarily of fragmentary remains (mainly isolated vertebrae and teeth) and consequently their systematic interpretation is tentative. Fortunately, a few well-preserved specimens from widely-separated localities indicate the probable presence of neospecies since the Late Pleistocene. For example, a skull from the Late Pleistocene in California is morphologically inside the range of the extant Gray whale (*Eschrichtius robustus*) and was classified as *Eschrichtius* cf. *E. robustus* by Barnes and McLeod (1984).

Pleistocene cooling and glaciations are possible causes for the restriction in the geographical range of tropical forms of cetaceans (e.g., *Platanista*). It is surmised that the equatorial warm water may have represented a barrier to dispersal during glacial-interglacial oscillations, thus favoring the vicariant

antitropical speciation of the extant cetaceans (Davies 1963). Contrastingly, molecular studies (Cipriano 1997) of the delphinid genus *Lagenorhynchus* reveal a more ancient origin of its antitropical speciations.

2.8 CONCLUSION

At the moment, the history of cetaceans may be viewed as involving six short time intervals in which the appearences and/or radiations of all higher groups are concentrated (Fig. 2.8):

1. Early Eocene (Ypresian) – origin of cetaceans, probably from archaic artiodactyles during the warmest climatic Cenozoic phase in a small area around the Tethyan coasts (now between the northwestern Pakistan and the northern India).
2. Early Middle Eocene (Lutetian) – basal cetaceans radiated with ongoing adaptation to water, well documented by fossil records. Rich food resources of the warm epicontinental waters of Tethys could have favored this initial radiation.
3. Late Middle Eocene (Bartonian) – basilosaurids, fully adapted to the open sea, radiated and during the Late Eocene colonized all the oceans.
4. Late Eocene (Priabonian) – neocetes (odontocetes and mysticetes) originated during a general cooling phase and related changes in ocean ecology.
5. Late Oligocene (Chattian) – first explosive radiation of neocetes. The filter feeding mysticetes and the echolocating and encephalized odontocetes presumably had a competitive edge over surviving archaeocetes and colonized many new niches of the heterogeneous oceans.
6. Middle-Late Miocene boundary (Serravallian-early Tortonian) – second neocete radiation with the origin of most of the living families possibly related to middle Miocene general cooling and consequent ocean restructuring. Delphinoid radiation may have been favored by higher encephalization of their skull.

These radiation phases are intercalated with some extinction events. In particular a general great crisis is clearly evident around the Oligocene-Miocene boundary where the surviving archaeocetes and four families of both mysticetes and odontocetes disappear. These extinctions might have been due to competition among the highly diversified Oligocene fauna rather than to physical environmental changes.

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CHAPTER 3

Classification and Molecular Phylogeny

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3.1 INTRODUCTION

Cetacea includes 41 genera and more than 80 species (Table 3.1), representing 4% of the mammalian (placental) diversity. All Cetacea are adapted to a permanent aquatic life. From an evolutionary point of view, this means that they exhibit morphological traits that have evolved since their common ancestor that was a terrestrial placental mammal. Specialization to life in water has led to major modifications in all body parts and functions, such as loss of external hind limbs, a hydrodynamic torpedo-like body shape, disappearance of the external ear, the quasi disappearance of hair, and great modifications of the skull and head. Cetacea is thus a highly derived group, meaning that their members possess many synapomorphies (shared derived characters) that evolved after separation from their most recent common ancestor. Consequently they have probably lost most of the characters that allow us to trace their relationships with other mammalian orders. The difficulty experienced by morphologists in attempting to identify the cetacean sister taxa was well expressed by Simpson (1945, p. 213): "Their place in the sequence of cohorts and orders is open to question and is indeed quite impossible to determine in any purely objective way. There is no proper place for them in a *scala naturae* or in the necessarily one-dimensional sequence of a written classification."

This chapter will address classification and molecular phylogeny of Cetacea, using comparisons of DNA sequences and incorporating information from morphological and paleontological data. The scope of this review is to

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Table 3.1 Systematic of Cetacea including English names (Gunther, 2002; Wilson and Reeder, 2005). Number in parentheses gives the number of taxa in the corresponding category. GenBank accession numbers are given for complete mitochondrial genome (mtc), and the NADH Dehydrogenase 4 (ND4 in bold), cytochrome b (cytb), control region (CR), sex determining region of the Y chromosome (SRY), lactalbumin (lact) and, Interphotoreceptor retinoid binding protein (IRBP) genes

SUBORDER (N) *FAMILY (N)* *Genus species*	*English common name*	*Genebank accession number*					
		*Mtc/***ND4**	*cytb*	*CR*	*SRY*	*lact*	*IRBP*
MYSTICETI (14)							
BALAENIDAE (3)	RIGHT WHALES						
Balaena mysticetus	Bowhead Whale	AJ554051				AJ007809	AF304087
Eubalaena australis	Southern Right Whale	**AY398627**	X75587	AF395044	AB108514	AY398660	
Eubalaena glacialis	Northern Right Whale	**AY398626**	AY398662	X72199		AY398647	
BALAENOPTERIDAE (9)	RORQUALS						
Balaenoptera acutorostrata	Minke Whale	AJ554054			AB108509	AY398649	
Balaenoptera bonaerensis	Southern Minke Whale	**AY398633**	X75581	M60408	AB108510	AY398645	
Balaenoptera borealis	Sei Whale	**AY398632**	X75582	X72195		AY398658	
Balaenoptera brydei	Bryde's Whale						
Balaenoptera edeni	Eden's Whale	**AY398631**	X75583	X72196		AY398659	
Balaenoptera musculus	Blue Whale	X72204			AB108511	AY398655	
Balaenoptera omurai	Omura's Whale						
Balaenoptera physalus	Fin Whale	X61145			AB108512	AY398652	
Megaptera novaeangliae	Humpback Whale	**AY398624**	X75584	X72202	AB108513	AJ007810	AF304086
ESCHRICHTIIDAE (1)	GRAY WHALES						
Eschrichtius robustus	Gray Whale	AJ554053				AF304099	U50649
NEOBALAENIDAE (1)	PYGMY RIGHT WHALES						
Caperea marginata	Pygmy Right Whale	AJ554052				AY398657	

Table 3.1 Contd. ...

Table 3.1 Contd. ...

ODONTOCETI (73)							
KOGIIDAE (2)	SMALL SPERM WHALES						
Kogia breviceps	Pygmy Sperm Whale	AJ554055				AF304096	U50819
Kogia simus	Dwarf Sperm Whale		AF304072		AB108516	AF304097	AF304084
PHYSETERIDAE (1)	SPERM WHALES						
Physeter catodon	Sperm Whale	AJ277029			AB108515	AF304098	U50818
MONODONTIDAE (2)	WHITE WHALES						
Delphinapterus leucas	Beluga	**AY398628**	X92531	U18117	AB108518	AF228409	AF231341
Monodon monoceros	Narwhal	AJ554062					
INIIDAE (1)	AMAZON RIVER DOLPHINS						
Inia geoffrensis	Pink River Dolphin	AJ554059				AF304092	AF304080
PLATANISTIDAE (2)	INDIAN RIVER DOLPHINS						
Platanista gangetica	Ganges River Dolphin		AF304070			AF304094	AF304082
Platanista minor	Indus River Dolphin	AJ554058					
PONTOPORIIDAE (1)	LA PLATA RIVER DOLPHINS						
Pontoporia blainvillei	La Plata Dolphin	AJ554060				AF304093	AF304081
LIPOTIDAE (1)	CHINESE RIVER DOLPHINS						
Lipotes vexillifer	Yang Tze River Dolphin		AF158375	AF324747		AF304095	AF304083
ZIPHIIDAE (21)	BEAKED WHALES						
Berardius arnuxii	Arnoux's Beaked Whale						
Berardius bairdii	Baird's Beaked Whale	AJ554057					
Hyperoodon ampullatus	Northern Bottlenose Whale	AJ554056					
Hyperoodon planifrons	Southern Bottlenose Whale						
Indopacetus pacificus	Longman's Beaked Whale		AY162442	AY162436			
Mesoplodon bidens	Sowerby's Beaked Whale		X92538	U70456			
Mesoplodon bowdoini	Andrew's Beaked Whale						
Mesoplodon carlhubbsi	Hubb's Beaked Whale						

Table 3.1 Contd. ...

Table 3.1 Contd. ...

Mesoplodon densirostris	Blainville's Beaked Whale		X92536	U70464			
Mesoplodon europaeus	Gervais' Beaked Whale		X92537	U70460			
Mesoplodon ginkgodens	Ginkgo-toothed Beaked Whale						
Mesoplodon grayi	Gray's Beaked Whale						
Mesoplodon hectori	Hector's Beaked Whale		AY228110	AY228108			
Mesoplodon layardii	Strap-toothed Whale						
Mesoplodon mirus	True's Beaked Whale						
Mesoplodon perrini	Perrin's Beaked Whale		AF441259	AF441258			
Mesoplodon peruvianus	Pygmy Beaked Whale		AF304074	AF492413		AF228411	AF304085
Mesoplodon stejnegeri	Stejneger's Beaked Whale				AB108517		
Mesoplodon traversii	Spade-toothed Whale						
Tasmacetus shepherdii	Shepherd's Beaked Whale		AF334484	AF036226			
Ziphius cavirostris	Cuvier's Beaked Whale		X92540	AF516679		AF228412	AF231343
PHOCOENIDAE (6)	PORPOISES						
Australophocaena dioptrica	Spectacled Porpoise		U09681	U09695			
Neophocaena phocaenoides	Finless Porpoise		AF334489	U09696	AB108520		
Phocoena phocoena	Harbour Porpoise	AJ554063			AB108519	AJ007811	AF231340
Phocoena sinus	Gulf Porpoise		AF084051	U09703			
Phocoena spinipinnis	Burmeister's Porpoise		U09676	U09704			
Phocoenoides dalli	Dall's Porpoise		U09678	U09702			
DELPHINIDAE (36)	MARINE DOLPHINS						
Cephalorhynchus commersonii	Commerson's Dolphin		AF084073	AF39356			
Cephalorhynchus eutropia	Black Dolphin		AF084072	AF393544		AF304088	AF304076
Cephalorhynchus heavisidii	Heaviside's Dolphin		AF084070	AF393556			
Cephalorhynchus hectori	Hector's Dolphin		AF084071	AF057989			
Delphinus delphis	Short-beaked Common Dolphin		AF084084	AF242199	AB108522	AF304089	AF304077
Delphinus capensis	Long-beaked Common Dolphin		AF084086	AY185137			
Delphinus tropicalis	Arabian Common Dolphin		AF084088				

Table 3.1 Contd. ...

Table 3.1 Contd. ...

Feresa attenuata	Pygmy Killer Whale		AF084052				
Globicephala macrorhynchus	Short-finned Pilot Whale		AF084054	AY168599	AB108527	AF304090	U50821
Globicephala melas	Long-finned Pilot Whale		X92529				
Grampus griseus	Risso's Dolphin		AF084058	AB018584	AB108524		
Lagenodelphis hosei	Fraser's Dolphin		AF084098				
Lagenorhynchus acutus	Atlantic White-sided Dolphin		AF084075	AF113486			
Lagenorhynchus albirostris	White-beaked Dolphin	AJ554061					
Lagenorhynchus australis	Peale's Dolphin		AF084069	AF393532			
Lagenorhynchus cruciger	Hourglass Dolphin		AF084068	AF393533			
Lagenorhynchus obliquidens	Pacific White-sided Dolphin		AF084067	AF113490	AB108523		
Lagenorhynchus obsucurus	Dusky Dolphin		AF084066	AF113492		AF228410	AF304078
Lissodelphis borealis	Northern Right Whale Dolphin		AF084064				
Lissodelphis peronii	Southern Right Whale Dolphin		AF084065	AF393535			
Orcaella brevirostris	Irrawaddy Dolphin		X92527		AB108528		
Orcinus orca	Killer Whale		X92528	M60409	AB108526		
Peponocephala electra	Melon-headed Whale		AF084053	AY046904	AB108525		
Pseudorca crassidens	False Killer Whale		AF084057				
Sotalia fluviatilis	Tucuxi Dolphin		AF084078			AF304091	AF304079
Sousa chinensis	Indo-pacific Humpbacked Dolphin		AF084079				
Sousa plumbea	Indian Ocean Humpbacked Dolphin						
Sousa teuszii	Atlantic Humpbacked Dolphin						
Stenella attenuata	Pantropical Spotted Dolphin		X56294				
Stenella clymene	Clymene Dolphin		AF084083				
Stenella coeruleoalba	Stripped Dolphin		AF084081	AY046546			
Stenella frontalis	Atlantic Spotted Dolphin		AF084089				
Stenella longirostris	Spinner Dolphin		X56292	AY046903			
Steno bredanensis	Rough-toothed Dolphin		AF084076				
Tursiops aduncus	Indian Ocean Bottle-nosed Dolphin		AF084091	AF459518			
Tursiops truncatus	Bottle-nosed Dolphin		X92526	AF268357	AB108521		

combine as many genes as possible for the maximum number of taxa to obtain a general overview of cetacean phylogenetic relationships from the ordinal to the species level. Cetacean phylogeny will be treated at different taxonomic levels, ranging over the search for the sister taxa to Cetacea among eutherian mammalian orders, to the identification of the major lineages (suprafamilial relationships) among Cetacea, and finishing with phylogenetic relationships among the two sub-orders traditionally recognized in Cetacea, Mysticeti and Odontoceti.

3.2 SYSTEMATIC POSITION OF CETACEA AMONG MAMMALS

3.2.1 Relationships Based on Morphological Characters

The monophyly of cetaceans is now accepted even though a few authors have considered Mysticeti (baleen whales) and Odontoceti (toothed whales) as separate orders (see Fordyce and Barnes 1994). Morphological evidence for the monophyly of extant cetaceans is based on numerous characters of the skeleton:

- The skull of the cetaceans is telescoped, with external nares in a posterior position (nostrils in living animals open on the top of the head).
- The mandible is without an ascending branch (or coronoid process), and the condylar process is small and directed backwards. The mandible has an extremely wide aperture of the dental foramen and infundibulum.
- The tympanic bone (that surrounds the middle ear cavity) is a hollow, bullate, thick bone, associated with the very dense periotic bone (that includes the inner ear).
- Cervical vertebrae are compressed and form a stiff axis in line with the skull.
- There is no distinction between lumbar and sacral vertebrae, and there are no fused vertebrae that form a sacrum.
- Caudal vertebrae have isolated chevron bones, however the posterior ones have a simplified shape. Such a shape is correlated with the flukes of the caudal fin, which are supported by fibrous rods.
- The pectoral girdle is composed of one flat bone (the scapula), of which the flat spine is parallel to the bone and forwardly directed.
- Long bones of the forearm are shortened and flattened, and the joint of the elbow is not rotational.
- Carpals and metacarpals are flat and small. Fingers are elongated, and the second and third digits possess a high number of phalanges. Such a transformation is linked to the differentiation of forelimbs as flippers.
- The posterior girdle is very reduced, always isolated from the spinal column, and includes not more than a single rod-like bone, as the remnant of the basin. A reduced femur may be present.

Cetaceans consequently look very different from all other mammals and represent one of the few lineages that strongly diverge among the mammalian

radiation (Novacek 1992). The huge morphological hiatus that separates them from other placental mammals raises interesting problems. No characteristic connected to the adaptation to their peculiar way of life (cetaceans are all obligate swimmers) can be used to settle their relationships with any other order of placental mammals. On the contrary, the characteristics listed above unambiguously define the cetaceans. In classical textbooks of the past century, as Grassé's treatise of zoology (Bourdelle and Grassé 1955), only two orders of placental mammals have been considered as the possible sister group of the cetaceans: the Carnivora and the Ungulata. No characteristics of the skeleton were indicated as supporting these hypotheses, but some characteristics from the soft anatomy were suggested. The ungulate hypothesis relied at that time upon cerebral blood circulation that indicated a possible phylogenetic link with Artiodactyla. This question proved to be one of the most studied relative to cetacean origin (Messenger and McGuire 1998; Gatesy and O'Leary 2001).

To stress the major change introduced by this interpretation it must be recalled that the origin of cetaceans was first sought among carnivore-like terrestrial mammals called Mesonychidae (Van Valen 1966, and more recently in Luo 2000). This interpretation was based on similarities between teeth of mesonychids and archaeocete whales. It is necessary to point out that during early Cenozoic placental evolution, teeth, skulls and limbs were evolving independently in many morphological directions, resulting in the appearance of placental groups exhibiting combinations of characteristics, some of which are unknown among modern orders, such as mammals with hooves and carnivore-like teeth. As mesonychids were living at the right time interval, they have been proposed as the sister group of the cetaceans. These questions are still addressed in many papers (O'Leary and Geisler 1999; Luo 2000; Madar *et al.* 2002) and are now at least much better settled and defined.

3.2.2 Molecular Data

The first molecular work mentioning a possible relationship between Cetacea and Artiodactyla was based on immunological reactions (Boyden and Gemeroy 1950). Since that time, much literature has been devoted to the systematic position of Cetacea, this question being often included in the more general problem of ordinal relationships between extant eutherian mammals. An exhaustive review of the different molecular papers published until 1997 can be found in Gatesy (1998).

Here, we will begin this review with the paper of Irwin and Arnason (1994) because it was the first molecular study to propose a sister group relationship between Cetacea and Hippopotamidae. This study was based on cytochrome *b* sequences and was of crucial importance because cetacean and *Hippopotamus* DNA sequences were simultaneously included. This relationship was unexpected and raised a general outcry because, as both Artiodactyla and Suiformes were rendered paraphyletic, it was seriously in conflict with traditional systematics. Different arguments (Irwin and Arnason 1994; Philippe and Douzery 1994; Gatesy *et al.* 1996; Allard *et al.* 1996; Luckett

and Hong 1998) were put forward in support of the view that the hippo-cetacean relationship was artifactual and resulted from: (i) a nuclear copy obtained for the hippo cytochrome *b*; (ii) convergent evolution because of similar way of life in water; (iii) homoplasies in the cytochrome *b*; (iv) lack of representation of all mammal orders. Numerous papers subsequently published try to confirm or invalidate the Hippo-Cetacea association by including more mammalian diversity and more mitochondrial (Milinkovitch *et al.* 1994; Montgelard *et al.* 1997; Ursing and Arnason 1998) and/or nuclear markers (pancreatic ribonucleases: Philippe and Douzery 1994; caseins: Gatesy *et al.* 1996; interphotoreceptor retinoid binding protein [IRBP]: Stanhope *et al.* 1996; von Willebrand factor [vWF]: Porter *et al.* 1996; fibrinogen: Gatesy 1997; lactalbumin: Milinkovitch *et al.* 1998; nuclear introns: Matthee *et al.* 2001; alpha 2B adrenergic receptor [A2AB]: Madsen *et al.* 2002; apolipoprotein B [APOB]: Amrine-Madsen *et al.* 2003) used alone or in combination. All these studies, however, suffered from a lack of taxonomic representativeness and/or informative characters (number and choice of genes) to improve accuracy in the inferred molecular relationships. It is nevertheless interesting to note the congruence between individual datasets that support or do not resolve the hippo-cetacean association, but that no gene suggests a robust alternative to the hippo-cetacean sister group relationships (see gene review in Gatesy 1998; Waddell *et al.* 1999). We can say that the papers published in 2001 by Madsen *et al.* and Murphy *et al.* finally closed the debate. The two studies have in common a large coverage of the ordinal mammalian diversity and concatenation of several genes leading to nearly 10,000 nucleotide sites analyzed. Although based on different datasets, these papers came to the same conclusion concerning the recognition of four supraordinal clades among the 18 placental orders of placental mammals recognized so far. With regard to the position of whales and dolphins, both papers support unambiguously (i) *Hippopotamus* as the sister taxon of Cetacea, thus confirming paraphyly of Artiodactyla and, incidentally, polyphyly of Suiformes (see also Chapter **2** of this volume); (ii) the inclusion of Cetacea + Artiodactyla in the Laurasiatheria supraordinal clade, together with Perissodactyla (horses), Carnivora (cats), Pholidota (pangolins), Chiroptera (bats), and Eulipotyphla (hedgehogs).

Different molecular markers, such as SINEs (short interspersed repetitive elements), have also been used, because they are an alternative type of evolutionary marker. SINES are mobile genetic elements that have been integrated into a host genome by retroposition, which is by integration of a reverse-transcribed copy of RNA. SINEs are powerful phylogenetic markers because of minimal homoplasy due to the random nature of integration and the absence of elimination from the host genome. Hence, taxa sharing the same SINE at a locus are likely to have a common origin. Concerning cetacean origin, Okada's team (Shimamura *et al.* 1997; Nikaido *et al.* 1999) has identified different SINEs that are shared by Cetacea and *Hippopotamus* to the exclusion of all other artiodactyl lineages (Ruminantia, Suina and Tylopoda).

These results definitively demonstrated a sister group relationship between cetaceans and hippos.

In our analyses performed on a dataset of 27 taxa representing 4 mammalian orders and 15 Cetacea (see paragraph 3.3.2 for detailed analyses), the resulting phylogenetic tree (Fig. 3.1) unsurprisingly supports *Hippopotamus* as the closest living sister taxon of Cetacea. Estimated divergence time (Fig. 3.2) performed using a Bayesian relaxed molecular clock (see paragraph 3.3.2) give 50 Ma (43-59) for the split between Cetacea and *Hippopotamus*, in accordance with the oldest fossils of Cetacea (*Pakicetus*) dated at 55 Ma (Early Eocene; McKenna and Bell 1997).

The name Cetartiodactyla was first given by Montgelard *et al.* (1997) to this new placental order including Cetacea among former Artiodactyla. Waddell *et al.* (1999) defined Whippomorpha for the association of Cetacea with Hippopotamidae, Cetruminantia for the clade Whippomorpha + Ruminantia, and Artiofabula for the grouping Suidae + Cetruminantia. The clade Cetacea + Hippopotamidae also was named Cetancodonta by Arnason *et al.* (2000). Cetancodonta seems to be preferable to Whippomorpha—a contraction of the English Whale and latin Hippomorpha— because it uses the suffix Ancondonta that includes present and fossil (Anthracotheridae) hippopotamid lineages (Simpson 1945). It is interesting to note that, with the exception of Insectivora and Artiodactyla, all other mammalian orders previously defined by morphologists are monophyletic. Insectivora appear polyphyletic (at least two clades: Afrosoricida + Macroscelidae, and Eulipotyphla), but this order was long ago recognized as a wastebasket group (Simpson 1945). In fact, Cetartiodactyla was the only eutherian order unrecognized by morphological characters. The reason for this lack of recognition is clearly because of the rapid evolution of the morphology of cetaceans due to their special adaptation to aquatic life, relative to the Hippopotamidae that have retained numerous ancestral characters (Montgelard *et al.* 1998).

In conclusion, all these molecular datasets and their correlative interpretations are congruent and validate the same hypothesis about the systematic position of Cetacea, and, more generally, about the supraordinal relationships among mammals. It does not mean, however, that there are no more issues to be tackled. It is, on the contrary, the beginning of a revival in the interpretation of evolution of morphological characters that are known to be subject to convergence. This coincides with a peculiar moment where paleontological data (even if they are still fragmentary) are much more complete than 30 years ago. A more accurate phylogeny also will favor a better interpretation of the present disjointed geographical distributions of Cetacea.

3.2.3 Contribution of Paleontological Data. Toward a Resolution of the Conflict?

Progress brought about by molecular analyses changed the way to search for cetacean relationships, and two paths were opened: a reappraisal of morphological characters by morphologists in the light of molecular

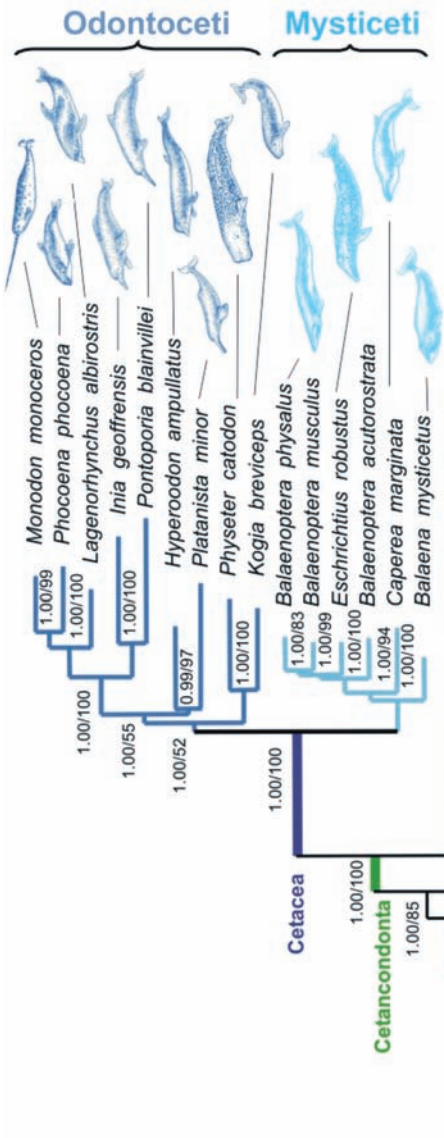

Fig. 3.1 Suprafamilial relationships among Cetacea. Bayesian phylogram obtained by combination of 15 mitochondrial and 3 nuclear genes (17145 characters) for 27 taxa including 15 Cetacea. The analysis was performed using a GTR + I + G model on each of the 13 partitions (see text). Posterior probabilities of the Bayesian analysis and bootstrap percentages after 100 replications in maximum likelihood (with the model GTR + I + G without partition) are indicated at nodes, from left to right, respectively. Copyright CNRS-Laurence Meslin for drawings.

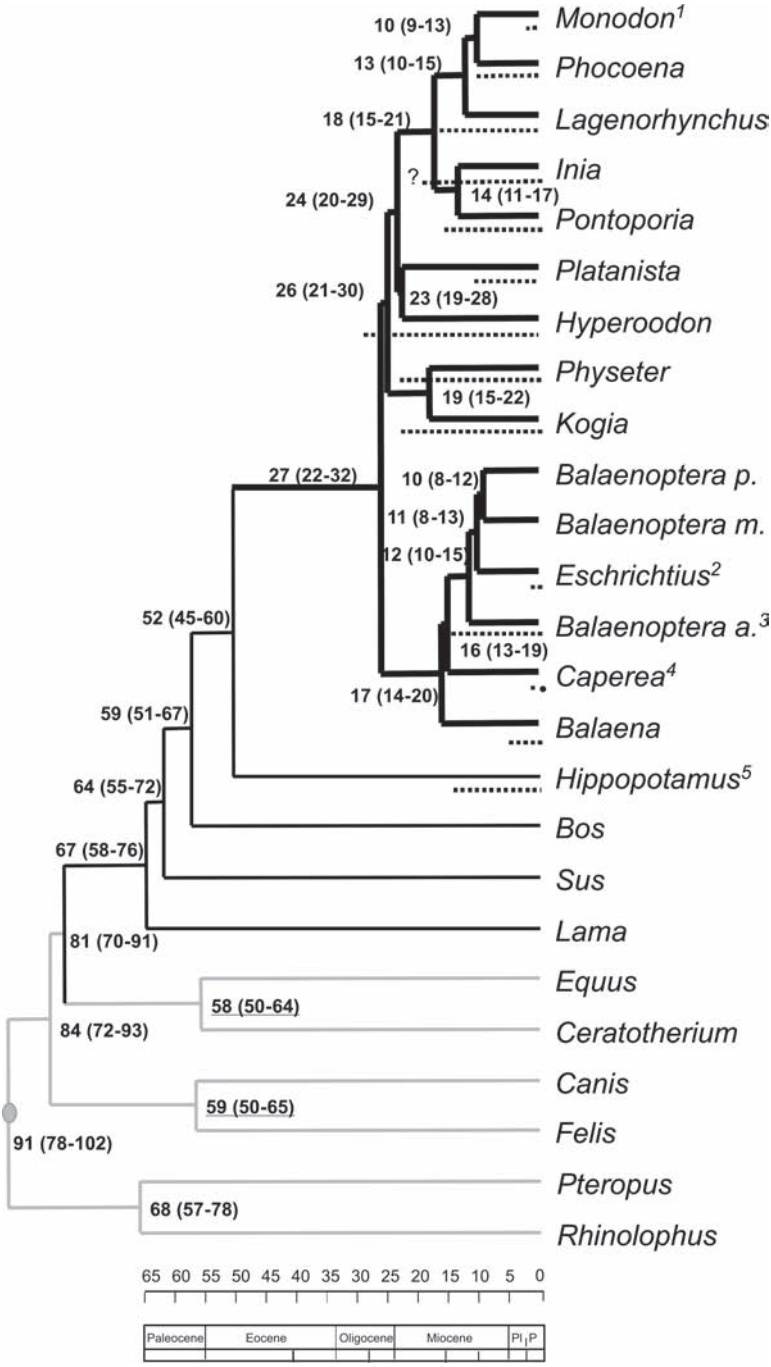

Fig. 3.2 Chronogram of the evolution of Cetacea and Cetartiodactyla. Branch lengths are proportional to the age of their ascending nodes, as estimated under

Fig. 3.2 Contd. ...

hypotheses (see paragraph above), and a look to fossils that may shed light on the question. The working hypothesis being that molecular conclusions are correct (Artiodactyla-Cetacea association), the question is which morphological characters can be considered as true synapomorphies for the group? Fossils, on the other hand, reveal extinct species with original combinations of characters that can be very different from present taxa (a demonstrative example is *Odobenocetops*, a Pliocene toothed whale with walrus-like adaptations; de Muizon 1993). It can thus be expected that new finds support some of the hypotheses derived from molecular analyses. This is exactly what happened during the last 30 years of fieldwork, mainly conducted in Pakistan. Many Eocene cetaceans were unearthed, and the oldest ones definitively demonstrate a link between cetaceans and artiodactyls (Gingerich and Russell 1981; Gingerich *et al*. 1983; Gingerich *et al*. 1994; Thewissen *et al*. 1994; Thewissen *et al*. 1998; Thewissen and Madar 1999; Thewissen *et al*., 2001; Gingerich *et al*. 2001). The connection was, however, not direct because some early cetaceans (also known as archaeocetes) exhibit some characteristics that link them to artiodactyls, while others link them to modern cetaceans (see Thewissen and Williams 2002). Some archaeocetes are characterized by the presence of posterior limbs with the diagnostic ankle bone – the double-pulley astragalus – considered up to now as the exclusive synapomorphy of the order Artiodactyla (Gingerich *et al.* 2001; Thewissen *et al.* 2001). This means that the ancestor of cetaceans was a terrestrial mammal, the body weight of which was borne between toes III and IV. Among characteristics of archaeocetes indicating a relationship with modern cetaceans are those of the auditory region of the skull and of the lower jaw. These characteristics represent apomorphies of cetaceans (see above) and are associated with underwater hearing. It must be pointed out that evolution of whale hearing occurred early, during the Eocene (Nummela *et al.* 2004). Teeth are also indicative of the relationship of modern cetaceans to primitive ones. Some extinct forms of toothed whales have definitively modern skeletons that still exhibit teeth of triangular configuration. This characteristic defines an

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Fig. 3.2 Contd. ...

a Bayesian relaxed molecular clock approach on 17145 characters divided into 13 partitions (see text). Values on nodes are mean divergence times expressed in million years, with 95% credibility intervals given between parentheses. Gray branches connect non-cetartiodactyl laurasiatherian taxa (Eulipotyphla was the outgroup, pruned from subsequent analyses, and connected to the ingroup root [gray disk]). Black branches connect cetartiodactyls, whereas thick branches connect cetaceans. The two paleontological prior constraints were the *Equus*/*Ceratotherium* and *Canis*/*Felis* splits bounded between 49 Ma and 65 Ma, and their posterior divergence time estimates are underlined. Dotted lines on each branch represent the paleontological range of the corresponding terminal taxon, starting from the oldest fossil (McKenna and Bell 1997) recorded in each cetacean lineage: [1]Pleistocene; [2]Late Pleistocene; [3]age of the genus *Balaenoptera* is given by *B. acutorostrata*; [4]Recent; [5]Middle Miocene for the Hippopotamidae.

intermediate stage of evolution with archaeocetes and consecutively a link with that group. A rich bibliography is now available on these subjects (summaries and original data in many papers such as Thewissen and Williams 2002).

If Cetartiodactyla are now accepted as a monophyletic group, the search for the cetacean sister group within Cetartiodactyla is a much more difficult question. The phylogenetic link between hippos and cetaceans advocated by molecular analyses was at first rejected (see references in Geisler and Uhen 2003). As in the search for cetacean kinship, a similar reasoning has been followed, that is, if the molecular hypothesis is right, there must be hitherto unrecognized morphological characters that support it. Are there also some fossils that may be considered as intermediate between cetaceans and hippos? However in that case, it must be stressed that known fossils have limited value in this respect because of a huge gap between the oldest archaeocetes and the hippos, the latter having a rather short fossil record (Middle Miocene, not earlier than ca 16.5 Ma). Moreover, possible ancestry of hippos among members of the extinct artiodactyl group known as Anthracotheres is hotly debated, the latter being considered as paraphyletic. As hippos still possess characteristics of a terrestrial way of life, most of their characteristics are useless for unravelling their possible affinities with cetaceans. However, a recent analysis by Geisler and Uhen (2003) reassesses the few morphological characteristics that may support the kinship between cetaceans and hippos. The authors recognized some possible dental characteristics as true synapomorphies, but the reasoning is indirect because it is based on comparative tooth anatomy of early archaocetes, artiodactyls and hippos. Some other characteristics are considered as equivocal synapomorphies, such as the mastoid process of the periotic bone that is visible on juvenile skulls, because these characteristics cannot be scored on some fossil cetacean groups (Raoellidae) that are not well known. Despite a recent cladistic analysis (Boisserie *et al.* 2005) based on morphological characters, which confirms the link between Hippopotamidae and the extinct group Anthracotheriidae, new fossils, especially more primitive archaeocete whales, are necessary to further our understanding of cetacean affinities.

3.3 MAJOR LINEAGES AMONG CETACEA

3.3.1 Morphological Data

The order Cetacea is traditionally split into two suborders, namely Odontoceti (toothed whales) and Mysticeti (baleen whales) that differ in many features correlated to their way of life. The Odontoceti include fish and cephalopod eaters and are characterized by the presence of teeth although some have only a very small number (Ziphiidae) or even no teeth protruding from the gums (upper jaw in Physeteridae). Mysticeti include filter feeders of zooplankton and have instead of teeth, whalebones that are skin derivatives extending from the upper jaws.

The skull in the two suborders is different. The skull of baleen whales is very wide at the level of the articulation of the lower jaws in correlation with the opening of the mouth to engulf large amounts of water. The profile of the skull is convex. The brain case is rather small in comparison with the face, and the occipital part of the brain case projects forward. The maxilla forms an infraorbital process and there is no fusion of the lower jaws (no mandibular symphysis). In toothed whales, the spherical brain case is much bigger relative to the rostrum. The upper part of the skull is concave, in correlation with the presence of a 'melon', a soft structure associated with echolocation and through which ultrasounds are emitted. The maxillaries and premaxillaries are elongated backwards as lamellar expansions covering the frontal bones, and the skull appears as telescoped backwards. The toothed whales' skull in dorsal view is more or less asymmetric, a character that appears to be linked to echolocation. There is a mandibular symphysis. The periotic and tympanic bones in both suborders are modified in comparison with other mammalian orders. More or less tightly fused together, they are made of heavy bone and constitute a complex structure isolated from the rest of the skull, a situation that explains why many museum specimens lack these bones. A double blowhole characterizes Mysticeti whereas all Odontoceti have a single blowhole (Jefferson *et al.* 1993).

Monophyly of Odontoceti has been questioned from a molecular point of view, the question bearing on whether sperm whales are more closely related to Mysticeti than to Odontoceti (Milinkovitch *et al.* 1994; Hasegawa *et al.* 1997). The skeletons of the sperm whale (*Physeter*) and of the pigmy sperm whale (*Kogia*) have many peculiarities. At first glance, several characters are clearly indicative of a kinship with Odontoceti: the presence of teeth and of an asymmetric skull, this asymmetry being much more pronounced in *Physeter*. However the occurrence of these characters, and of some others, can also be explained by parallel evolution (see Milinkovitch 1995) toward a way of life similar to the one of toothed whales. Because the spermaceti, an organ peculiar to both Physeteridae and Kogiidae, is located aside a fatty melon, the head of sperm whales is markedly different from that of other toothed whales, as is the case for their skull morphology and their outer nostril (blowhole). The alternative to an independent origin of *Physeter* and *Kogia* (i.e. from other toothed whales), is that they represent an early offshoot of the Odontoceti radiation (see below).

3.3.2 Molecular Analyses

The following data set was analyzed: 15 mitochondrial genes (all mitochondrial genes except the 20 tRNAs and the control region) available from the complete mitochondrial genomes published by Arnason *et al.* (2004) and three nuclear genes: IRBP (Interphotoreceptor retinoid binding protein; Cassens *et al.* 2000), lactalbumin (Cassens *et al.* 2000), and SRY (sex determining region of the Y chromosome; Nishida *et al.* 2003). The whole alignment represents 17145 characters for 27 taxa, among which 15 Cetacea

representing all the different families. The remaining 12 taxa are sampled among the four different artiodactyl lineages (*Hippopotamus* for Ancodonta, *Bos* for Ruminantia, *Lama* for Tylopoda, and *Sus* for Suina), and two taxa for each of the following placental orders: Perissodactyla, Carnivora, Chiroptera and Eulipothyphla. Accession numbers for cetacean sequences are given in Table 3.1. Accession numbers for other complete mitochondrial genomes are: *Hippopotamus amphibius* (AJ010957), *Bos taurus* (V00654), *Sus scrofa* (AJ002189), *Lama pacos* (AJ566364), *Equus caballus* (X79547), *Ceratotherium simum* (Y07726), *Canis familiaris* (U96639), *Felis catus* (U20753), *Pteropus capulatus* (AF321050), *Rhinolophus pumilus* (AB061526), *Sorex unguiculatus* (AB061527), *Talpa europaea* (Y19192). Accession numbers for IRBP are: *Hippopotamus amphibius* (AF108837), *Bos taurus* (M20748), *Sus scrofa* (U48588), *Lama glama* (AF108836), *Equus caballus* (U48710), *Canis lupus* (AY170074), *Felis catus* (Z11811), *Pteropus hypomelanus* (Z11809), *Sorex palustris* (U48587), *Scalopus aquaticus* (AY170089). Accession numbers for lactalbumin are: *Hippopotamus amphibius* (AJ007813), *Bos taurus* (X06366), *Sus scrofa* (M80520), *Lama guanicoe* (AJ007814). Accession numbers for SRY are: *Bos taurus* (Z30327), *Sus scrofa* (U49860), *Lama guanicoe* (U66068), *Equus caballus* (Z26908), *Canis familiaris* (U15160), *Felis catus* (AB099654).

Phylogenetic analyses were performed with two different probabilistic approaches (Bayesian and maximum likelihood methods) using the most general model of sequence evolution: GTR + I + G. The GTR (general time-reversible) model incorporates unequal base frequencies and six different rates of nucleotide substitutions. Rate variation among sites was approximated by a gamma distribution (G) and a proportion of invariable sites (I). Bayesian analysis was performed with MRBAYES 3.0b4 (Huelsenbeck and Ronquist 2001), using 4 Markov chains Monte Carlo (MCMC), 10^6 generations, trees sampled every 50 generations, and a burn-in (trees generated before likelihood stationarity) of 1500 trees. The default priors were used, i.e., dirichlet priors for base frequencies (1,1,1,1) and for GTR parameters (1,1,1,1,1) scaled to the G-T rate, a uniform (0.05, 50.00) prior for the G shape, and an exponential (10.0) prior for branch lengths. All topologies were *a priori* equally probable. Three partitions of the dataset have been tested to take into account different evolutionary patterns: one partition for the whole dataset, 18 partitions (one per gene), and 13 partitions, i.e., one partition for each non-protein coding gene (12S rRNA, 16S rRNA, Lact, SRY), one partition for each codon position of the L-strand coding mitochondrial genes, one partition for each codon position of the H-strand ND6 mitochondrial gene, and one partition for each codon position of the nuclear IRBP gene. Maximum Likelihood (ML) analyses have been performed on the whole dataset with the program PHYML (Guindon and Gascuel 2003) with robustness of nodes assessed with 100 bootstrap replications under the same model of sequence evolution.

Molecular dating has been performed under the hypothesis of a Bayesian relaxed molecular clock with the software MULTIDIVTIME (Thorne and Kishino 2002). The molecular dating was run in two steps by using the

maximum posterior probability topology and by partitioning the mitochondrial + nuclear data into 13 subsets (see above). First, the program ESTBRANCHES recalculated branch lengths of the reference topology and the corresponding variance-covariance matrix for each of the 13 partitions and under a F84 + G model of nucleotide substitution. Second, the program MULTIDIVTIME used the 13 variance-covariance matrices to run MCMC and calculate divergence times of nodes and their 95% credibility intervals (Cred. I.). After a "burn-in" stage of 100,000 cycles, the MCMC was sampled 10,000 times every 100 cycles. We used the following priors of Gamma distributions for the model of rate autocorrelation: 80 Ma (SD = 40 Ma) for the expected number of time units between tips and the laurasiatherian root (Springer *et al.* 2003) if there has been no constraint on node times, 0.004 (SD = 0.004) for the rate at root node for the nucleotide substitutions, and 0.0125 (SD = 0.0125) for the Brownian motion constant that described the degree of rate autocorrelation along the descending branches of the tree. We chose the Paleozoic-Mesozoic boundary (240 Ma) for the highest possible number of time units between tip and root. Two paleontological prior calibrations were used (Garland *et al.* 1993): *Equus/Ceratotherium* and *Canis/Felis* splits bounded between 49 Ma (Early Eocene) and 65 Ma (Paleocene).

3.3.3 Phylogenetic Relationships

The estimated log-likelihood values (harmonic mean) obtained in Bayesian analysis with one, 18, and 13 partitions are –180664.78, –177813.32, and –174286.01, respectively. These values show that the likelihood is increased when different character partitions are considered, and that the model with 13 codon-based partitions is more appropriate than the 18 gene-based partitions for modeling the underlying evolutionary processes.

Whatever the partitions used, all nodes among Cetartiodactyla are supported by posterior probabilities [PP] of 1.00, to the exception of the grouping *Hyperoodon+Platanista* (PP=0.99 with 13 partitions and PP=1.00 for one and 18 partitions), and *Sus scrofa* as sister taxa of Cetancondonta+*Bos* (PP=0.84 with 1 partition and PP=1.00 for 13 and 18 partitions). The phylograms obtained (Fig. 3.1) show monophyly of both Mysticeti and Odontoceti, but supports for these two clades are quite different. Mysticeti appears strongly supported (posterior probability [PP] of 1.00 and ML bootstrap [BP] of 100%), whereas Odontoceti is not (PP = 1.00 but BP = 52%). Several molecular studies addressed this question with a substantial number of nucleotides of mitochondrial and/or nuclear genes (Gatesy 1998; Gatesy *et al.* 1999; Cassens *et al.* 2000; Arnason *et al.* 2004). In all studies, support for monophyly of Odontoceti is at the best only moderate, major problems being the discordance between mitochondrial and nuclear datasets (Gatesy 1998), as well as the position of the cetacean root that appears unstable (Cassens *et al.* 2000). Finally, only the study of Nikaido *et al.* (2001), based on the identification of three SINEs loci, convincingly supports a monophyletic Odontoceti. It is amazing to note the facility with which Odontoceti is

unambiguously morphologically identified (see previous paragraph) with the difficulty to define this clade from a molecular point of view (not fully resolved with 17,000 characters). These contrasting patterns reveal once more that molecular and morphological rates of evolution can be decoupled, leading to very different estimated rates of homoplasy.

Our molecular estimations (Fig. 3.2) led to the date of 27 Ma (22-32) for diversification of Cetacea, whereas divergence time estimated for modern Odontoceti and Mysticeti is 26 Ma (21-30) and 17 Ma (14-20), respectively. These estimations seem a little young with regard to the oldest fossils recorded for Odontoceti and Mysticeti at about 34 Ma (Early Oligocene; Gingerich and Uhen, 1998). This discrepancy could be related to the lack of resolution of molecular data in the deepest nodes among Cetacea. These dates are also very different from estimations obtained by Cassens *et al.* (2000), Nikaido *et al.* (2001), and Arnason *et al.* (2004). The importance of the calibration point, which appears different in all studies, should be noted.

Within the Odontoceti, our analyses support, although very moderately (PP=1.00 but BP= 55%), Physeteroidea (Physeteridae and Kogiidae) as the first emergence, a relationship confirmed by the insertion of two SINE loci (Nikaido *et al.* 2001). Until this study, the phylogenetic position of sperm whales has always been a difficult problem to solve because results were either inconclusive or grouped sperm whales with Mysticeti (Milinkovitch *et al.* 1994; Hasegawa *et al.* 1997). The reason is probably to be found in the fact that as an ancient lineage, much of evolution has occurred in the branch. The split within Physeteroidea is dated at 19 Ma (15-22), a date fully congruent with the oldest physeterid fossils identified in the Early to Middle Miocene (23-16.5 Ma).

The most recent clade among Odontoceti is represented by a group joining Delphinidae as sister taxa of Phocoenidae + Monodontidae. These relationships have been established by numerous molecular studies (Milinkovitch *et al.* 1994; Arnason and Gullberg 1996; Waddell *et al.* 2000; Cassens *et al.* 2000; Nikaido *et al.* 2001; Arnason *et al.* 2004). This clade received the superfamily rank, Delphinoidea, and would have diversified about 13 (10-15) Ma. In our tree, the sister taxon of Delphinoidea is the group *Inia* + *Pontoporia*, thus recovering the Infraorder Delphinida as defined by de Muizon (1988). Separate studies based on several markers (Cassens *et al.* 2000; Nikaido *et al.* 2001; Arnason *et al.* 2004) also came to this conclusion. Delphinida are dated at 18 Ma (15-21).

Our tree identified a sister group relationship between *Hyperoodon* (Ziphiidae) and *Platanista* (Platanistidae) that is rather well-supported (PP=0.99 and BP=97%). This relationship is, however, not recovered in the analysis performed on a much more complete dataset including 61 species of Odontocetes for two mitochondrial genes (see paragraph 3.5 below). Moreover, Nikaido *et al.* (2001) identified two SINE loci supporting Ziphiidae as the sister group of Delphinida (Delphinidae, Phocoenidae, Monodontidae, Iniidae, and Pontoporiidae) and two others for *Platanista* as the second split

among odontocetes after Physeteroidea. It is thus possible that the association *Hyperoodon-Platanista* recovered here but also in other studies performed on a great number of molecular characters (Cassens *et al.* 2000; Arnason *et al.* 2004), may partly result from a lack of sample representativeness in the ziphiid group.

3.4 INTRA-MYSTICETI RELATIONSHIPS

Four families are included in Mysticeti: Balaenidae, Balaenopteridae, Eschrichtiidae and Neobalaenidae, of which the last two are monospecific. Balaenidae includes 3 species, whereas Balaenopteridae is the most diversified family with at least 9 species described since *Balaenoptera bonaerensis* was raised to species rank (see Arnason *et al.* 1993) and *B. omurai* and *B. brydei* were described as new species by Wada *et al.* in 2003. Because little is known about some of these taxa it is possible that other new species will be identified as more molecular studies are performed (see for example Rosenbaum *et al.* 2000).

Phylogenetic relationships among Mysticeti are, in fact, poorly known and only four studies (Arnason *et al.* 1993; Arnason and Gullberg 1994; Rychel *et al.* 2004; Sasaki *et al.* 2005) include most or all representatives of the mysticetes. Intra-Mysticeti relationships are here based on 7775 characters, representing four mitochondrial (control region, cytochrome *b*, 12S rRNA, and ND4L/ND4) and three nuclear (lactalbumin, SRY, and IRBP) markers (see Table 3.1 for accession numbers). Twelve out of 14 described species of mysticetes are represented in our dataset because, with the exception of the mitochondrial control region (Wada *et al.* 2003), no sequence has been obtained for the two newly described species of Balaenopteridae (*Balaenoptera omurai* and *B. brydei*). Three odontocetes (*Delphinapterus leucas*, *Phocoena phocoena* and *Physeter catodon*) and two Artiodactyla (*Hippopotamus amphibius* and *Bos taurus*) were used as outgroups. Because of alignment ambiguity due to high sequence divergence, the control regions of *Physeter*, *Hippopotamus* and *Bos* were not included in the dataset.

Analyses have been performed with MrBayes using the GTR + I + G model on the whole dataset with either one partition, or 7 partitions (one per gene) and 10 partitions (one partition for each non protein coding gene: control region, 12S rRNA, SRY, Lactalbumin; one partition for each IRBP codon position, and one partition for each codon position of the mitochondrial genes: cytochrome *b*, ND4L/ND4). Five hundred bootstrap replications have been performed in Maximum Likelihood with PHYML on the whole dataset. Likelihood values (harmonic mean) obtained with MrBayes under the different partitioned datasets are –34467.12 with one partition, –33922.03 with 7 partitions, and –33904.90 with 10 partitions. These results indicate that likelihood value is improved when data are partitioned and that the most complex model with 10 partitions is slightly more suitable than a model treating each gene separately. Major differences between the different

partitioned analyses concerned relationships among the *Eschrichtius* – Balaenopteridae group (see Fig. 3.3). Our analyses (Fig. 3.3) and previous studies (Arnason and Gullberg 1994; Rychel *et al.* 2004) agree in showing Balaenidae (*Eubalaena australis, E. glacialis* and *Balaena mysticetus*) as the first offshoot among Mysticeti, and Neobalaenidae (*Caperea marginata*) as the sister taxon of a Balaenopteridae + Eschrichtiidae clade. Among Balaenopteridae, different clades appear strongly supported: *Balaenoptera acutorostrata* + *B. bonaerensis*, *B. musculus* as sister taxa to the *B. borealis*-*B. edeni* group, and *B. physalus* + *Megaptera novaeangliae*. These groupings question the validity of the genus *Megaptera* that appears deeply nested within the paraphyletic genus *Balaenoptera*. It is likely that the genus *Megaptera* was defined on particular characters (e.g. robust body, extremely long flippers) and not on common ancestry that should have included it in the genus *Balaenoptera*.

The study of Wada *et al.* (2003), based on the control region, is the only one enabling discussion of the systematic position of the two new species of *Balaenoptera* that are lacking in our analysis (*B. omurai* and *B. brydei*). According to this study, *B. brydei* is sister taxon of *B. borealis* whereas *B. omurai* is in an intermediate position between a clade *B. physalus* + *B. musculus* and a clade *B. edeni* + *B. brydei* + *B. borealis*. Knowing that hybridisation is possible between *B. physalus* and *B. musculus*, Rychel *et al.* (2004) suggest that *B. omurai* might possibly have resulted from hybridisation between *B. physalus* or *B. musculus* and *B. borealis* or *B. brydei*.

The essential question among Mysticeti concerns the position of the Gray whale (*Eschrichtius robustus*). Morphological studies include *Eschrichtius* and *Balaenoptera* in the superfamily Balaenopteroidea (Geisler and Sanders 2003) but the position of *Eschrichtius* relative to extant species of *Balaenoptera* is not specified. In all our analyses, *Eschrichtius* appears nested in Balaenopteridae (see Fig. 3.3), making this family paraphyletic. This is also the result obtained by previous studies on the control region (Arnason *et al.* 1993), cytochrome *b* (Arnason and Gullberg 1994), ND4 and Lact (Rychel *et al.* 2004). However, none of these studies clearly resolved relationships among the *Eschrichtius* – Balaenopteridae group, and the sister taxon of *Eschrichtius* is not identified. Even the most recent study of Sasaki *et al.* (2005) based on complete mitochondrial genome sequences of 12 mysticete species did not resolve the position of *Eschrichtius*. The posterior probability for a monophyletic Balaenopteridae family is 0.20 with 7 partitions and 0.08 with 10 partitions in Bayesian analysis, and the bootstrap support is 35% in ML. It can be concluded that the systematic position of *Eschrichtius* is not totally settled. Rychel *et al.* (2004) conclude their paper in stating that *Eschrichtius* as sister taxon of a monophyletic Balaenopteridae is the preferred hypothesis because it implies fewer assumptions concerning morphological evolution, as compared to a paraphyletic Balaenopteridae that would imply reversion for several morphological characters that are thought to be ancestral in the *Eschrichtius* lineage. It must be mentioned, however, that the feeding mode of the Gray whale (scraping the bottom-sediments for sucking up prey) is unique

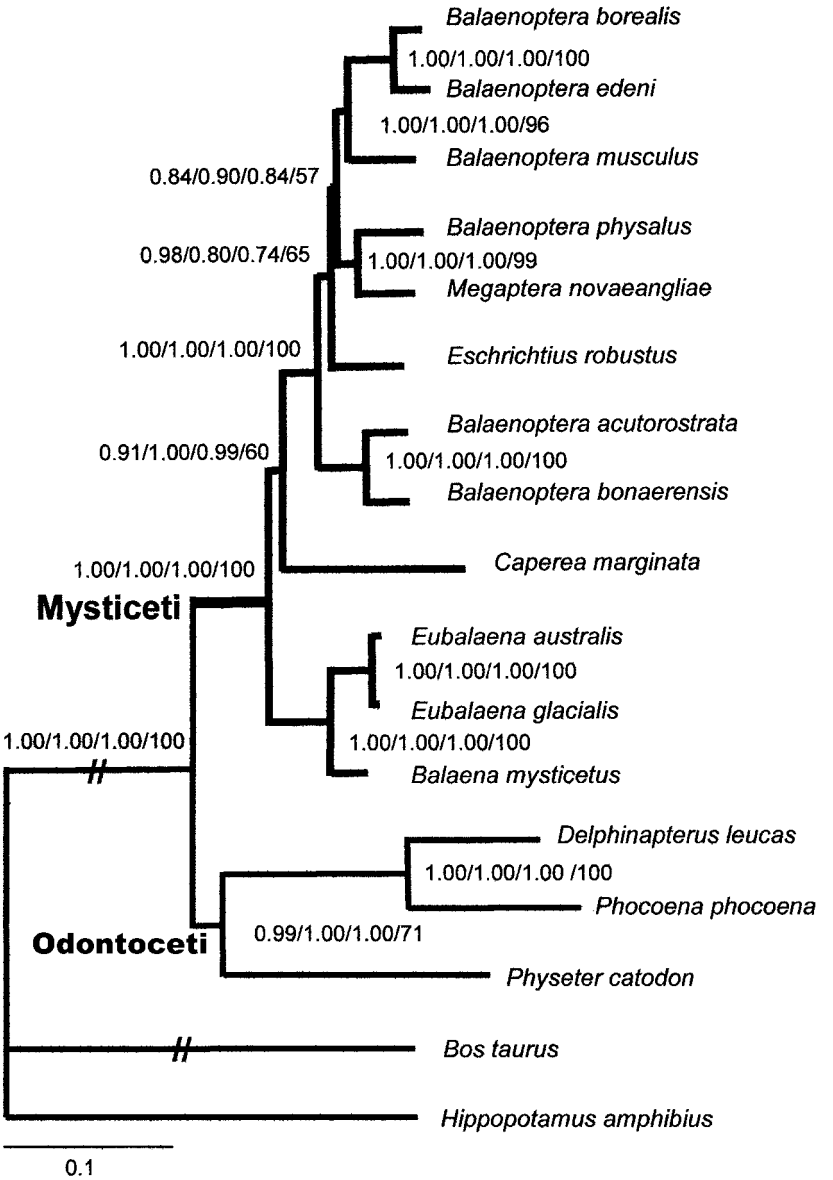

Fig. 3.3 Phylogenetic relationships among Mysticeti. Bayesian phylogram obtained by concatenation of four mitochondrial and three nuclear genes (7775 characters) for 17 taxa, including 12 mysticetes. Tree was recovered using a GTR + I + G model on each of the predefined partitions (see text). Bayesian posterior probabilities for one, seven and ten partitions, and maximum likelihood bootstrap percentages (500 replications) are given from left to right at each node, respectively. Two slashes on a line indicate that the branch has been reduced twice.

among Cetacea and could have had a major impact on evolution of morphological characters. In conclusion, it seems that more molecular data (more genetic and taxonomic sampling), would be necessary to clarify phylogenetic relationships within the Balaenopteridae + Eschrichtiidae lineage.

3.5 INTER-FAMILIAL AND INTRA-FAMILIAL RELATIONSHIPS AMONG ODONTOCETI

Odontoceti contain 73 species in 10 families but most of the diversity (78%) is distributed in two families, Delphinidae (36 species) and Ziphiidae (21 species).

Relationships among Odontoceti have been assessed using two mitochondrial genes: the complete cytochrome *b* (1140 bp) and about 514 nucleotides of the 5′ portion of the control region (see accession numbers in Table 3.1). The dataset for cytochrome *b* includes a total of 61 Odontoceti sequences, whereas 40 sequences were available for the control region (*Physeter catodon* has not been used because the sequence is too divergent as compared to other Odontoceti). Analyses have here been performed on the combination of both markers for the whole dataset (61 species of Odontoceti) to which six species of Mysticeti have been added as outgroups (*Balaena mysticetus, Eubalaena glacialis, Balaenoptera musculus, B. physalus, B. acutorostrata, Caperea marginata*), hence representing a total of 1654 characters for 67 species. A four-partition analysis has been used with the Bayesian analysis: one partition for the control region and one partition for each codon position for cytochrome *b*. The maximum likelihood tree has been inferred with the program PhyML and robustness of nodes was assessed with 100 replications of bootstrap. For both reconstruction methods, the GTR + I + G model has been applied.

The two analyses indicate that the six families of Odontoceti represented by more than one species (Monodontidae, Phocoenidae, Platanistidae, Kogiidae, Delphinidae and Ziphiidae) are clearly monophyletic (Fig. 3.4A, B). We have seen in paragraph 3.3 that a number of suprafamilial relationships have been identified with the multigenic dataset of 17,145 nucleotides. It is interesting to note here that essentially the same relationships are recovered with the data set of 1654 mitochondrial characters (approximately ten times less), although some groupings are poorly supported. It is possible that the decrease in the character numbers has been compensated by the increase in taxon sampling from nine Odontoceti in the global analysis (Fig. 3.1) to 61 (on 67 extant species). In fact, the topology of the tree (Fig. 3.4A) is exactly the same as the relationships established from the SINE flanking sequences (Nikaido *et al.* 2001).

3.5.1 Relationships among River Dolphins

River dolphins are represented by five species distributed in four genera (*Inia*, *Pontoporia*, *Lipotes* and *Platanista*). All species inhabit fresh river or coastal

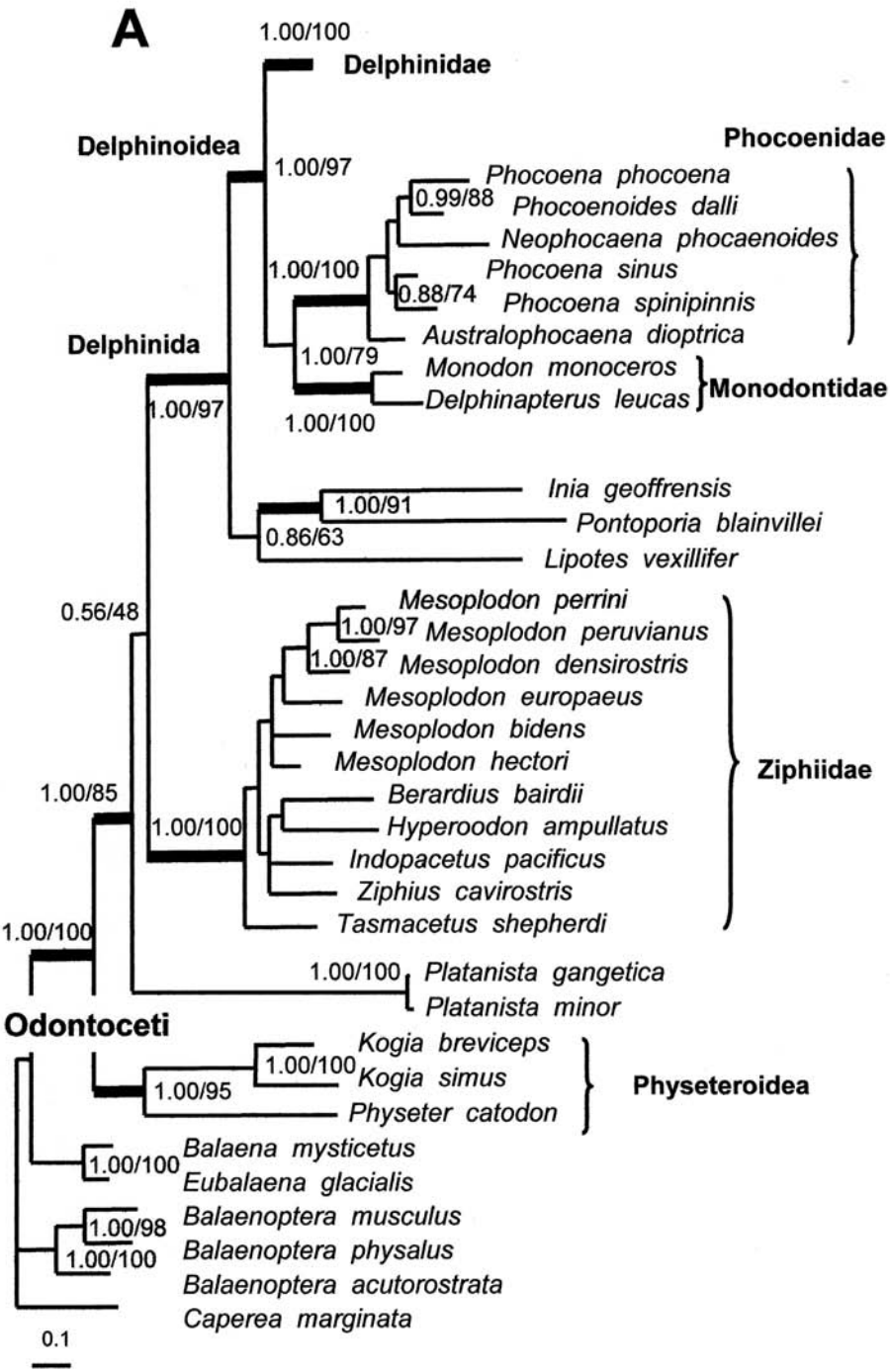
A
1.00/100
Delphinidae
Delphinoidea
Phocoenidae
1.00/97
Phocoena phocoena
0.99/88
Phocoenoides dalli
Neophocaena phocaenoides
1.00/100
Phocoena sinus
0.88/74
Phocoena spinipinnis
Australophocaena dioptrica
Delphinida
1.00/79
Monodon monoceros
Delphinapterus leucas
Monodontidae
1.00/97
1.00/100
Inia geoffrensis
1.00/91
Pontoporia blainvillei
0.86/63
Lipotes vexillifer
0.56/48
Mesoplodon perrini
1.00/97
Mesoplodon peruvianus
1.00/87
Mesoplodon densirostris
Mesoplodon europaeus
Mesoplodon bidens
Mesoplodon hectori
Ziphiidae
1.00/85
Berardius bairdii
1.00/100
Hyperoodon ampullatus
Indopacetus pacificus
Ziphius cavirostris
Tasmacetus shepherdi
1.00/100
1.00/100
Platanista gangetica
Platanista minor
Odontoceti
Kogia breviceps
1.00/100
Kogia simus
1.00/95
Physeter catodon
Physeteroidea
1.00/100
Balaena mysticetus
Eubalaena glacialis
1.00/98
Balaenoptera musculus
1.00/100
Balaenoptera physalus
Balaenoptera acutorostrata
Caperea marginata
0.1

Fig. 3.4 Contd. ...

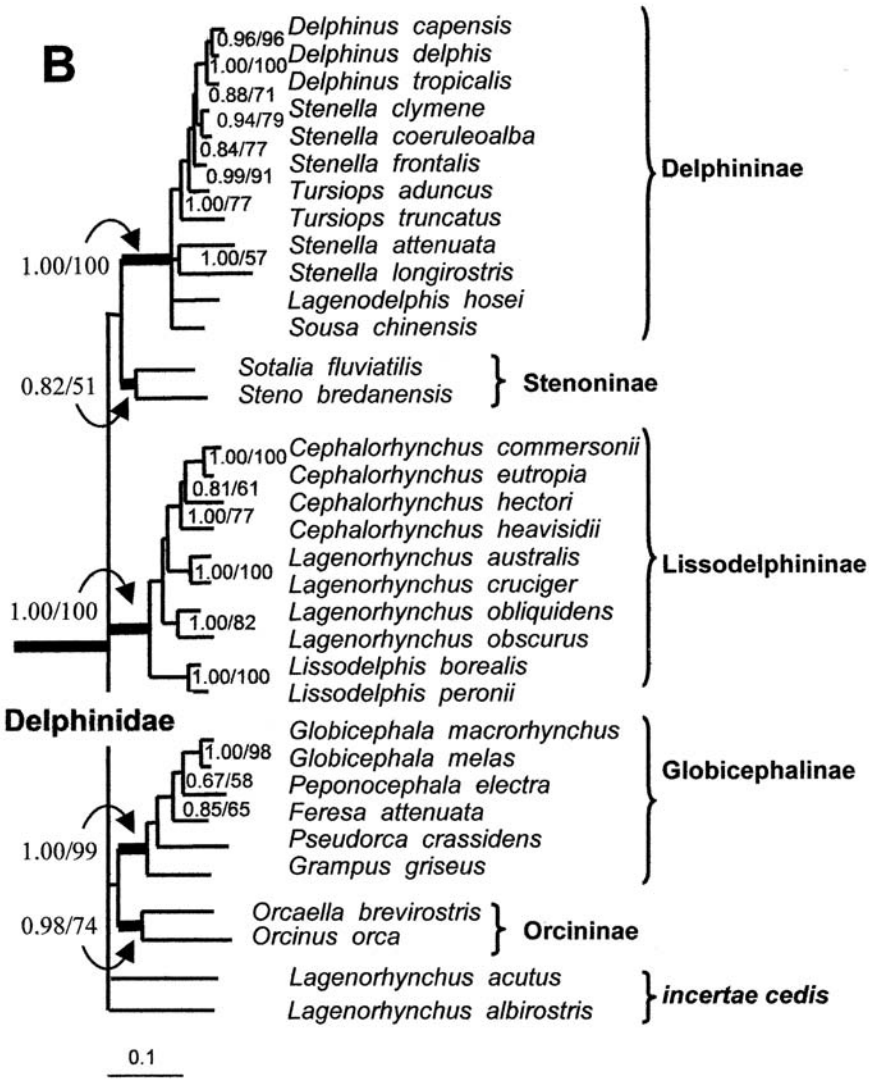

Fig. 3.4 Phylogenetic relationships among Odontoceti (**A**) and Delphinidae (**B**). Bayesian phylogram obtained for the combination of the complete mitochondrial cytochrome *b* (1140 bp) and partial control region (514 bp) for 67 taxa, including 61 Odontoceti. The analysis has been performed using a GTR + I + G model on each of the four partitions: three codon positions and the control region. Values for the Gamma shape parameter are 0.60, 0.16, 0.06, and 8.00 for the control region and the first, second and third positions of the cytochrome *b*, respectively. The proportion of invariable sites is 0.18, 0.17, 0.33 and 0.02 for the same partitions. Posterior probabilities in Bayesian analysis and maximum likelihood bootstrap proportions after 100 replications are indicated at nodes when supported by more than 50% bootstrap, from left to right, respectively.

waters but their distributions are geographically disjunct (Ridgway and Harrison 1989). All river dolphins were once grouped in a single group (Platanistoidea) but naturalness of the group has long been questioned from morphological (de Muizon 1988) and more recently from molecular data (Arnason and Gullberg 1996).

The inclusion of the four genera in our analyses leads to the conclusion that two or three lineages can be recognized among river dolphins, but that their phylogenetic affinities with other odontocetes are still not clearly established. The association between *Inia* and *Pontoporia* represents the most strongly supported clade in all analyses (see Figs. 3.1, 3.4A). This grouping also appears fully congruent with their South American past and present distributions, and this clade (unnamed for the moment) can be considered as endemic to South America (Hamilton *et al.* 2001). In our analysis of cytochrome *b* plus the control region (Fig. 3.4A), *Lipotes* is grouped with *Inia*+*Pontoporia* but not robustly (PP= 0.87 and BP= 63%). The same grouping is also retrieved by Nikaido *et al.* (2001) from the SINEs flanking sequences, whereas Cassens *et al.* (2000) and Hamilton *et al.* (2001) suggest *Lipotes* as sister taxa of the group Delphinoidea+*Inia*+*Pontoporia*. These three river dolphins are also characterized by long branches on the phylogram (Fig. 3.4A), and for this reason, a long branch attraction phenomenon cannot be excluded. Concerning the last genus, *Platanista* appears as the second split among Odontoceti after the emergence of Physeteroidea, but this position is not well supported (PP=0.56 and BP=48%). The same result has been found by Hamilton *et al.* (2001) on about 2000 mitochondrial characters, by Cassens *et al.* (2000) in some of their trees and by Nikaido *et al.* (2001), who identified two SINE loci supporting *Platanista* at this position. Finally, there is no support in any of these studies for the sister group relationships between *Platanista* and Mysticeti, which was suggested by Verma *et al.* (2004). Pending further studies allowing clarification of relationships between the four genera of river dolphin, each genus has here been attributed to its own family rank (see Table 3.1).

3.5.2 Relationships among Phocoenidae

Four genera and six species are included in Phocoenidae (porpoises), which have been divided into two sub-families by Barnes (1984) on the basis of morphological characters. Genera *Phocoena* and *Neophocoena* are classified into the Phocoeninae, whereas *Australophocoena* and *Phocoenoides* are in the subfamily Phocoenoidinae. However, the molecular analyses do not support this classification, as has already been mentioned by Rosel *et al.* (1995) on the basis of the same markers (cytochrome *b* and control region). In our analysis, both reconstruction methods support a close phylogenetic relationship between *Phocoena sinus* and *P. spinipinnis* on the one hand, and between *P. phocoena* and *Phocoenoides dalli* on the other, making the genus *Phocoena* paraphyletic (Fig. 3.4A). Analyses of Rosel *et al.* (1995), and our ML analysis as well, support *Australophocoena* as the sister taxa of the clade *Phocoena sinus*-

P. spinipinnis. However, this result is not recovered by the Bayesian analysis that strongly supports *Australophocoena* as a basal emergence within Phocoenidae (PP = 0.95). We agree with Rosel *et al.* (1995) in stating that the use of the sub-familial level should be avoided pending further studies that would allow more accurate revisions.

3.5.3 Relationships among Ziphiidae

Ziphiidae (beaked whales) is the least known family among Cetacea, probably due to their deep-diving way of life and the difficulty in identifying living specimens that do not spend much time at the surface. Some species are known only by skeletal material and new taxa will probably be identified as more data become available. At the present time, 21 species are described and distributed in five genera (*Berardius, Hyperoodon, Mesoplodon, Tamacetus* and *Ziphius*). Among them, 14 species are now included in the genus *Mesoplodon*, including two recently discovered new species, *M. perrini* Dalebout *et al.* (2002) and *M. traversii* (ex *M. bahamondi*) van Helden *et al.* (2002). In our analysis, of the 11 odontocete species lacking the cytochrome *b* dataset, nine belong to the Ziphiidae. Most *Mesoplodon* have been sequenced for about 350 bp of the control region (Henshaw *et al.* 1997; Dalebout *et al.* 1998, 2002) but eight were not included in our dataset because they were not sequenced (even partially) for the cytochrome *b*. The combined analysis (Fig. 3.4A) does not give much resolution among the 11 ziphiid species, with the exception of *M. densirostris*, which was identified as the sister taxon (PP=0.97, BP=83) of the clade *M. peruvianus-M. perrini* (PP=0.97, BP=99). Monophyly of the genus *Mesoplodon* is not supported (PP=0.81, BP=49) and no intergeneric relationships are clearly evidenced. This analysis leads to us to envisage an explosive evolutionary radiation, giving rise to the different genera. It is clear that much work remains to be done to answer these questions and decipher taxon validity and systematic and phylogenetic relationships within this family. Further studies will probably lead to the description of several new species of Ziphiidae. An example is, *Mesoplodon hectori*, for which the high divergence observed between populations of South Australia and North Pacific (7% for 350 nucleotides of the mitochondrial control region) suggests the presence of two species (Dalebout *et al.* 1998).

3.5.4 Relationships among Delphinidae

Delphinidae (dolphins) is the most speciose of the cetacean families, with at least 36 extant species distributed in 17 genera. Among them, however, only three genera (*Cephalorhynchus, Lagenorhynchus,* and *Stenella*) contain more than three species. With the exception of two species of *Sousa* (*S. plumbea* and *S. teuszii*), all other 34 remaining Delphinidae are represented in the cytochrome *b* dataset, whereas 21 species have been sequenced for the control region (see Table 3.1).

The different delphinid clades recovered in our analyses (Fig. 3.4B) are essentially the same as those described in LeDuc *et al.* (1999) and we will here

adopt those authors' subfamilial classification. Three well-supported families are identified: Delphininae, including genera *Delphinus, Stenella, Tursiops*, *Lagenodelphis* and *Sousa*; Lissodelphininae, with genera *Cephalorhynchus, Lissodelphis,* and *Lagenorhynchus* (with the exception of *L. acutus* and *L. albirostris*); and Globicephalinae, including genera *Globicephala*, *Peponocephala*, *Feresa, Grampus,* and *Pseudorca*. Two other subfamilies: Stenoninae, containing the genera *Sotalia* and *Steno*, and Orcininae, containing the genera *Orcaella* and *Orcinus*, are doubtful because they are much less supported. More molecular data will be necessary to assess the naturalness of these two subfamilies, as well as, to establish relationships between the diverse delphinid subfamilies. Finally, two species attributed to the genus *Lagenorhynchus* (*L. acutus* and *L. albirostris*) fall apart in a basal position in the delphinid tree, making the genus *Lagenorhynchus* polyphyletic. These two genera do not appear to be closely related and do not show any particular affinity with other members of the Delphinidae, making their systematic position *incertae cedis*. This result is not without consequence on the definition of the genus *Lagenorhynchus* itself, since *L. albirostris* was the type-species of the genus (see discussion in Leduc *et al.* 1999). Two other genera, *Stenella* and *Tursiops*, also appear as artificial groupings, whereas monophyly of the genera *Cephalorhynchus, Delphinus, Globicephala,* and *Lissodelphis* is well-supported. In conclusion, reassessment of a number of genera, both from molecules and morphology, is necessary in order to clarify systematics and classification among Delphinidae (see also Ridgway and Harrison 1994).

3.6 ACKNOWLEDGMENTS

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CHAPTER 4

Functional Anatomy of the Cetacean Reproductive System, with Comparisons to the Domestic Dog

Sentiel A. Rommel[1], *D. Ann Pabst*[2] *and William A. McLellan*[2]

4.1 INTRODUCTION

Marine mammals[1] have streamlined body shapes, hypertrophied axial musculoskeletal systems, and thick fatty layers that are morphological features, which reduce the energetic costs of both swimming and whole body thermoregulation (reviewed in Pabst *et al*. 1998). Interestingly, some of these morphological features would appear to threaten the temperature-sensitive reproductive tissues of these marine mammals. For example, male dolphins possess ascrotal testes – a condition identified as an adaptation for body streamlining (e.g., Howell 1930; Slijper 1936, 1979). As a consequence of streamlining and axial swimming style, the testes and epididymides are literally juxtaposed against or between thermogenic axial and abdominal locomotor muscles (Boice *et al*. 1964; Arkowitz and Rommel 1985; Pabst *et al*. 1998) and their reproductive tissues could potentially be exposed to core or above-core body temperatures. Cetaceans have core temperatures between 35 and 38 ºC, which are within the range of most other mammals (Costa and Williams 1999; Williams *et al*. 2001). These temperatures can effectively block spermatogenesis (Cowles 1958; Van Demark and Free 1970) and abdominal

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[1]Our morphological descriptions are based on dissections of carcasses of animals that had either stranded or had been killed incidentally in commercial fishing operations. Illustrations of cetacean features are based on our dissections and were generated using EasyCAD (Evolution Computing, Tempe, AZ).

temperatures can detrimentally affect long-term storage of spermatozoa in the epididymis (Bedford 1977). In many mammals, viable sperm production and epididymal storage require temperatures 2-6 °C below core temperatures (Moore 1926; Cowles 1965; Bedford 1977).

The scrotum is unique to mammals and is common among terrestrial species (Setchell 1978). The scrotum provides a "thermal window" through which heat may be exchanged with the environment (arrows, Fig. 4.1A). Physical separation (e.g., scrota and cremaster sacs) from the body core or from the nearby thermogenic muscles reduces the thermal load on the testes. Additionally, countercurrent heat exchange within the spermatic cord, between the venous pampiniform plexus and the testicular artery (Harrison 1948), helps maintain below core temperatures in the mammalian testes. Interestingly, although cetaceans possess high internal body temperatures, they lack a physical separation between testes and body core and they lack pampiniform plexuses (Figs. 4.2A, 4.3A, F).

Body streamlining and axial locomotor style also impact the thermoregulation of female reproductive systems of cetaceans. Thermogenic muscle and insulating blubber surround female reproductive tissues (Fig. 4.2B); this arrangement suggests elevated temperatures at the uterus that could detrimentally affect fetal development. Because the mammalian fetal metabolic rate may be as much as twice that of maternal tissues (Power *et al*. 1984), heat must be continuously transferred from the fetus to the mother in order to maintain a stable fetal temperature (reviewed in Rommel *et al*. 1993). Any physiological or anatomical condition that limits the ability of the fetus to transfer its metabolic heat to the maternal environment will cause potentially harmful increases in fetal temperature. Such increases are known to cause detrimental effects including low birth weights (Shelton 1964), retarded fetal growth (Alexander *et al*. 1987; Bell 1987), skeletal and neural developmental anomalies (reviewed in Lotgering *et al*. 1985), and ultimately acute fetal distress and death (Morishima *et al*. 1975; Cephalo and Hellegers 1978;).

Under steady-state conditions in experimental terrestrial mammals, approximately 85% of the heat produced by the fetus is convectively transported to the placenta (Power *et al*. 1984; Gilbert *et al*. 1985; Gilbert and Power 1986). This heat is then transferred to the internal maternal environment and subsequently lost to the external environment. The remaining 15% of fetal heat is transported away from the fetal skin surface, via the amniotic and allantoic fluids, to the uterine wall and to the maternal environment (Gilbert *et al*. 1985; Bell 1987). This heat is subsequently lost to the external environment through the maternal abdominal wall (Hart and Faber 1985; Gilbert and Power 1986).

The relatively thin muscles and skin of the maternal ventral abdominal wall function as a maternal "thermal window" (Hart and Faber 1985; Gilbert and Power 1986) (arrows on the left, Fig. 4.1B). These tissues of the abdominal wall thermal window are cooler than the maternal core temperature and cooler than other organs with which the uterus is in contact. Terrestrial

mammals typically locomote with their non-axial, appendicular limb muscles. This muscle arrangement allows some of the heat generated by locomotion to be lost through the skin directly to the environment (Schmidt-Nielsen 1990) and thus not contribute to the thermal load of the fetal environment (arrows on the right, Fig. 4.1B). The presence and large mass of heat-producing axial and abdominal locomotor muscles and insulating blubber suggest that cetaceans lack such maternal thermal windows (Fig. 4.2B).

Thus, the streamlined body shapes of cetaceans appear to increase thermoregulatory threats to the reproductive systems of both males and females. How do cetaceans regulate the temperature of their reproductive tissues to avoid hyperthermic insult? In both male and female cetaceans, we have described novel vascular arrangements that function as reproductive countercurrent heat exchangers (CCHEs) deep within the caudal abdominal cavity (Rommel *et al*. 1992, 1993, 1994; Pabst *et al*. 1995, 1998). These vascular structures bring cool venous blood returning from the superficial surfaces of the dorsal fin and flukes to a position juxtaposed to the arterial supply of the reproductive tissues. In contrast to the indirect reproductive CCHE mechanism of cetaceans, seals and manatees cool their reproductive systems directly by bringing cooled superficial blood deep within the body core and juxtaposing this cooled blood to the reproductive tissues rather than to the arteries supplying the tissues (Rommel *et al*. 1994; Pabst *et al*. 1995).

We will describe the cetacean reproductive CCHE and offer physiological evidence that they function to regulate the temperature of reproductive structures in both males and females. Our specific goals are to describe the gross morphology of the reproductive systems and the vascular structures associated with these systems. We also review some of the physiological evidence to support our model of reproductive thermoregulation.

4.2 CETACEANS REPRODUCTIVE MORPHOLOGY

We briefly review reproductive morphology, focusing mainly on odontocete cetaceans, and we compare the odontocetes with the dog (see Boyd *et al*. 1999 for a general review of marine mammal reproduction and Matthews 1950 and Perrin *et al*. 1984 for reviews of cetacean reproduction). The structures we describe for odontocete cetaceans also have been observed in mysticetes and are believed to function in much the same way.

4.2.1 Males

In adult cetaceans, the testes (Fig. 4.2A) lie within the caudal abdominal cavity, a position we define as intra-abdominal (also called cryptic, endorchid, and testicond) (e.g. Slijper 1936, 1966; DeSmet 1977; Rommel *et al*. 1992). Each mature testis rests on the ventral abdominal floor. The testes are large. In mature individuals, the testes may fill the entire cross section of the abdominal cavity (Boice *et al*. 1964). The testis is attached to the dorsolateral abdominal wall by a mesorchium that wraps around the lateral margin of the testis

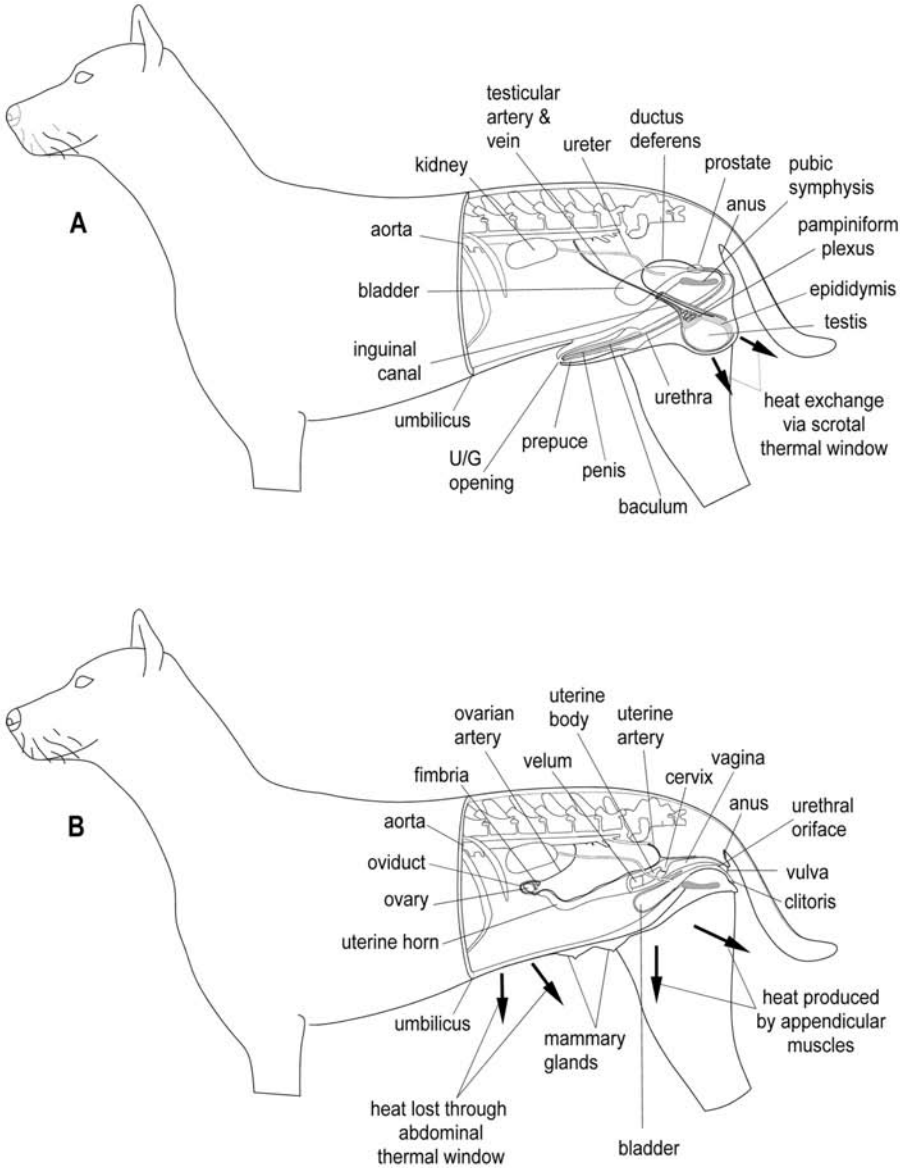

Fig. 4.1 Schematic representation of the left lateral aspect of the reproductive structures and their vascular supplies in sexually mature domestic dogs (*Canis familiaris*). A male dog is illustrated at the top and female dog at the bottom of the figure. The caudal abdomen and pelvic regions have been exposed from the level of the umbilicus. The pubic symphysis, some vertebrae, and the left ribs caudal to the umbilicus are illustrated for positional reference. **A**. In males, the penis is anchored to the caudolateral aspects of the pubic symphysis by paired crura. The

Fig. 4.1 Contd. ...

(Fig. 4.3F). The epididymis lies along the ventromedial aspect of the testis. The proximal epididymis (caput) extends as a slight bulge onto the cranial aspect of the testis. The distal epididymis (cauda) consists of a large lobe, which is made up of convolutions of the epididymal duct (Fig. 4.2A). The epididymal duct continues beyond the cauda epididymis as the ductus deferens. The ductus deferens joins the urethra distally via the ejaculatory duct (Harrison 1969).

The only accessory gland that has been described in cetaceans is the prostate (Meek 1918; Slijper 1936, 1966, 1979; Matthews 1950; Harrison 1969; Simpson and Gardner 1972; Collet and Robineau 1988), which lies at the base of the penis between the pelvic vestiges (Fig. 4.2A). The prostate gland is surrounded by a very powerful prostate compressor muscle (Matthews 1950). The penis is anchored to each of the pelvic elements by a crus (plural crura); these crura fuse in the body of the penis to form a single corpus cavernosum. The urethra travels through a poorly developed corpus spongiosum (Simpson and Gardner 1972; termed corpus cavernosum urethra by Slijper 1966). The large, bilaterally paired ischiocavernosus (erector penis) muscles surround the crura and corpus cavernosum (Meek 1918; Collet and Robineau 1988; Meek

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Fig. 4.1 Contd. ...

penis contains an os penis (baculum). The testes have descended into a scrotal sac and reside outside the abdominal cavity. An artifact of descensus is the loop that the ductus deferens makes around the ureter on the dorsolateral aspect of the bladder. The testis is supplied by a testicular vein. The scrotal sac provides a "thermal window" through which heat is transferred to the environment in order to keep the testes below core body temperature. Additional thermoregulation is via the heat transfer from the relatively warm testicular artery to the relatively cool veins of the pampiniform plexus. **B.** Each ovary is juxtaposed to the fimbriae on the margins of the funnel of each uterine tube (oviduct). The uterine tube extends between the fimbriae to the distal end of the uterine horn. The bicornuate uterus terminates at a muscular cervix. There is a distinct uterine body between the cervix and the uterine horns; a velum divides the cranial midline of the uterine body. The vaginal canal terminates at the vulva. Mammary glands are ventrolateral; the number varies with breed. There is a distinct urethral orifice just dorsal to the clitoris on the cranial aspect of the vulva. Female dogs have relatively thin abdominal walls when compared with those of marine mammals. As the abdominal wall stretches and becomes thin during pregnancy, it functions as an abdominal wall "thermal window" through which heat from the fetus may be transferred to the environment. Additionally, the thermogenic locomotory muscles are appendicular and therefore do not surround the developing fetus. These muscles lose heat directly to the environment and thus do not contribute to the thermal burden of pregnancy. The arterial supply to the reproductive tissues is made up of an ovarian artery anastomosing with a uterine artery. Figure drawn after Figs. 9-18 and 9-37 in Evans, H. E. and Christensen, G. C. (1993). Saunders, Philadelphia; Figs. 313-B381-C in Schaller, O. (1992). Stuttgart: Ferdinand Enke; Schummer, A., Nickel, R., Figs. 136 and 140 in Sack, W. O. (1979). Verlag Paul Parey, Berlin., and Fig. 539 in Schummer, A., Wilkens, H., Vollmerhaus, B. and Habermehl, K.-H. (1981). Verlag Paul Parey, Berlin.

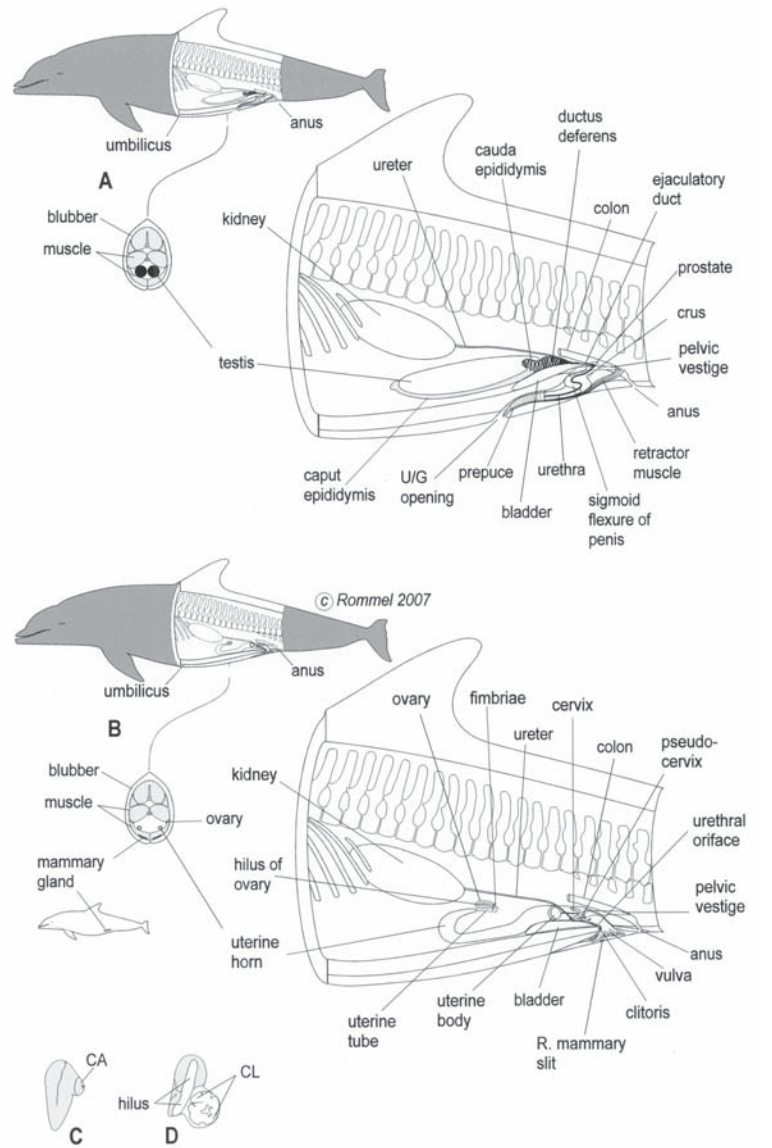

Fig. 4.2 Schematic representation of the reproductive structures and their vascular supplies in sexually mature bottlenose dolphins (*Tursiops truncatus*). The caudal abdomen and pelvic regions have been exposed between the level of the umbilicus and just caudal to the anus. The left pelvic vestige, some vertebrae, and the left ribs caudal to the umbilicus are illustrated for positional references. Cross sections are at the level of the reproductive tracts. A male is illustrated at the top and a female at the bottom. Note that the reproductive tissues are surrounded by thermogenic locomotory muscles and insulated by blubber. **A**. The testes of cetaceans are relatively large and are positioned within the ventrocaudal abdomen

Fig. 4.2 Contd. ...

1918). The erector penis muscles attach to the pelvic vestiges. The penis is made erect by voluntary contraction of these muscles and can be manipulated at will (Boyd *et al*. 1999).

The cetacean penis (Fig. 4.2A), which can be retracted within the body wall (into the prepuce), is fibroelastic like those of most artiodactyls (Meek 1918; Matthews 1950; Slijper 1966, 1979; Collet and Ronineau 1988). There is no os penis in cetaceans (Harrison 1969). The non-erect cetacean penis is curved to the left into a sigmoid flexure within the body wall (Schummer *et al*. 1979). Upon erection, the cetacean penis extends forward as it becomes turgid and straightens but it does not dramatically change its absolute length or diameter. A retractor penis muscle originates on the superficial aspect of the colon near the anus. This muscle inserts onto the ventral aspect of the penis, just distal to the sigmoid flexure at the caudal aspect of the prepuce (Meek 1918; Matthews 1950; Slijper 1966, 1979; Harrison 1969; Collet and Robineau 1988). This anatomical arrangement also is seen in ruminants (Schummer *et al*. 1979). The retractor penis ostensibly functions to maintain the position of the nonerect penis within the prepuce. Interestingly, Slijper (1966) suggests that the retractor penis may function "as a brake in regulating the stretching of the penis during erection."

4.2.2 Females

The position and general form of the female reproductive tract in cetaceans are similar to those of the female reproductive tracts in terrestrial mammals

Fig. 4.2 Contd. ...

ventral to the kidneys. Thus the testes are intra-abdominal. The penis is fibroelastic and is anchored to the pelvic vestiges by bilaterally paired crura. The non-erect penis is curved into a sigmoid flexure within the body wall. A retractor penis muscle originates on the superficial surface of the rectum and attaches to the ventral surface of the penis, just distal to the sigmoid flexure. The prostate gland and ischiocavernosus muscles lie between the pelvic vestiges. **B**. The urogenital opening of female cetaceans is on the ventral midline just cranial to the anus. Cetacean uteri are bicornuate and the ovaries are positioned within the ventrocaudal abdomen, slightly caudal to the kidneys. The distal tips of the horns curl dorsocaudally in older individuals. There is a short uterine body between the cervix and the uterine horns. In the vagina, just distal to the true cervix, the wall of the vaginal canal exhibits annular folds, termed pseudocervices. There is a distinct clitoris at the cranial aspect of the genital slit. The small urethral orifice is dorsocranial to the clitoris. The mammaries are inguinal. Each ovary has a distinct hilus on its lateral aspect. **C**. The medial aspect of a left ovary that has a single corpus albicans (CA). Corpora albicantia are permanent ovarian scars that indicate the number of pregnancies associated with that ovary. **D**. The lateral aspect of a right ovary with a corpus luteum (CL) of pregnancy. CL of pregnancy may approach the mass of the rest of the ovary. The CL has been sectioned. The gross anatomy is modified from Rommel, S.A. and Lowenstein, L.J. 2001. CRC Press, Boca Raton, FL., Fig. 4 (copyright Rommel), the ovarian anatomy is after Harrison, R.J., Brownell, R.L. and Boice, R.C. 1972. Academic Press, New York.

(Fig 4.2B). The vagina opens cranial to the anus and leads to a bicornuate uterus. The body of the uterus is found on the midline and is juxtaposed to the dorsal aspect of the urinary bladder. The uterine horns (cornua) extend from the uterine body towards the lateral aspects of the body cavity. Implantation of the fertilized egg and subsequent placental development are in the mucosa (endometrium) of the uterine horns. The dimensions of the uterine horns vary with reproductive history and age. Often the fetus may expand the pregnancy horn to the point that it fills a substantial portion of the abdominal cavity. Each horn terminates abruptly, narrowing and extending as a uterine tube (fallopian tube) to the ovary. The uterus and its lateral components are held in place in the abdominal cavity by the broad ligaments.

Uterine and ovarian scarring may provide information about the reproductive history of the individual. The cetacean uterus, like that of most other mammals, is temporarily scarred immediately after the postpartum loss of the deciduous placenta. The ovaries of mature female cetaceans may have one or more white or yellow-brown scars, called corpora albicantia or corpora lutea, respectively. The corpora lutea of cetaceans can be used to infer the reproductive history of individuals. Corpora albicantia (Fig. 4.2C) are regressed corpora lutea. A corpus albicans from each pregnancy persists throughout the remaining lifetime because the corpus luteum of pregnancy greatly enlarges (approaching the mass of the rest of the ovary; Fig. 4.2D) and when it regresses there is extensive fibrosis and hyalinization (Harrison *et al*. 1972).

The ovaries are paired, relatively flat, oval organs (Slijper 1966; Harrison 1969). Each ovary resides in an ovarian bursa (not illustrated). Each ovary is juxtaposed to the fimbriae on the margins of the funnel of each uterine tube (Fig. 4.2B). The uterine tube (oviduct, fallopian tube) has a small lumen diameter that provides a path from the fimbriae to the distal end of the uterine horn (Meek 1918; Pycraft 1932; Wislocki 1933; Slijper 1936, 1966, 1979; Wislocki and Enders 1941; Harrison 1969). The ovary, uterine tube, and uterine horn are held in place by an extensive mesentery termed the broad ligament. The broad ligament has three regions, the mesovarium, mesosalpinx, and mesometrium. The mesovarium attaches the ovary to the dorsolateral abdominal wall – folds in the mesovarium are the (cranial) suspensory ligament and the (caudal) round ligament. The attachment site of the mesovarium at the hilus of the ovary leaves a distinct longitudinal crease on the lateral aspect of the ovary (Fig. 4.2B, D). The mesosalpinx folds around the ovary forming an ovarian bursa and bending the oviduct in to a U shape. The mesosalpinx attaches the uterine tube to the lateral abdominal wall. The mesometrium attaches the uterus to the abdominal wall (Fig. 4.3G).

The bicornuate uterus terminates at a muscular true cervix (Fig. 4.2B). There is a short body between the cervix and the uterine horns. The fetus develops in one of these uterine horns. Unlike the dorsoventrally folded fetuses of other mammals, cetacean fetuses are folded ventrolaterally (Wislocki and Enders 1941; Slijper 1966; Etnier *et al*. 2004). The cetacean placenta is diffuse and

epitheliochorial, as are the placentas of many artiodactyls (Wislocki and Enders 1941; Schummer *et al*. 1979; Benirschke and Cornell 1987).

Caudal to the true cervix, the wall of the vagina exhibits one or more annular folds (Fig. 4.2B), termed pseudocervices in the marine mammal literature (Harrison 1969), which have been mistakenly identified as unique to cetaceans (Slijper 1979; Schroeder 1990). Schroeder (1990) calls the presence of pseudocervices a "remarkable anatomical adaptation for breeding in the marine environment." Slijper (1979) states that "these peculiar folds ... are not found in any other mammal." However, the proximal vaginal canals of both cows and sows exhibit annular folds (vaginal ridges; Schaller 1992) that are particularly evident in younger individuals (Schummer *et al*. 1979). Thus, this character is shared with artiodactyls and is not unique to cetaceans. The vaginal canal terminates at the vulva, which lies within a slit-shaped aperture (urogenital or U/G slit) in the ventral body wall that is shared with the anus and mammary slits. Mammary glands are ventrolateral and relatively caudal (Fig. 4.2B). In cetaceans there is a pair of mammary slits located within the urogenital slit (note that the presence of mammary slits should not be used to determine gender because some male cetacean species have distinct mammary slits). There is a distinct urethral orifice just dorsal to a well-developed clitoris on the cranial aspect of the U/G slit.

4.3 REPRODUCTIVE VASCULAR STRUCTURES

We will describe the reproductive vasculature separately for males and females. Our descriptions are of the left side, but the vascular structures associated with the reproductive systems in both males and females are bilaterally symmetrical. All arteries and veins are schematized – they are more complex and convoluted than presented herein[2].

4.3.1 Males

4.3.1.1 Testicular vascular plexuses - TAP and TVP

Blood to the testis is provided via an arterial plexus, which is supplied by the dorsal aorta (Rommel *et al*. 1992). Rather than a single testicular artery, as is found in most other mammals (Fig. 4.1A), approximately 20-40 individual arteries leave the aorta to supply the cetacean testis (Fig. 4.3A). Each of these arteries is convoluted as it leaves the aorta, but straightens as it courses laterally and ventrally toward the testis. The arteries form a flat plexus of closely spaced, parallel vessels – the testicular arterial plexus (TAP).

Near the ventrolateral margin of the TAP, the arteries coalesce and form a cone-shaped mass of vessels. These vessels anastomose into fewer larger-diameter arteries as the cone tapers caudally. At the caudal terminus of the cone, a single testicular artery enters the tunic of the testis (Fig. 4.3A). A few

[2]In some of our previous articles some of the terminology was inconsistent, mixing human and veterinary terminology. Currently we are making every effort to be consistent with the Illustrated Veterinary Anatomical Nomenclature by O. Schaller (1992).

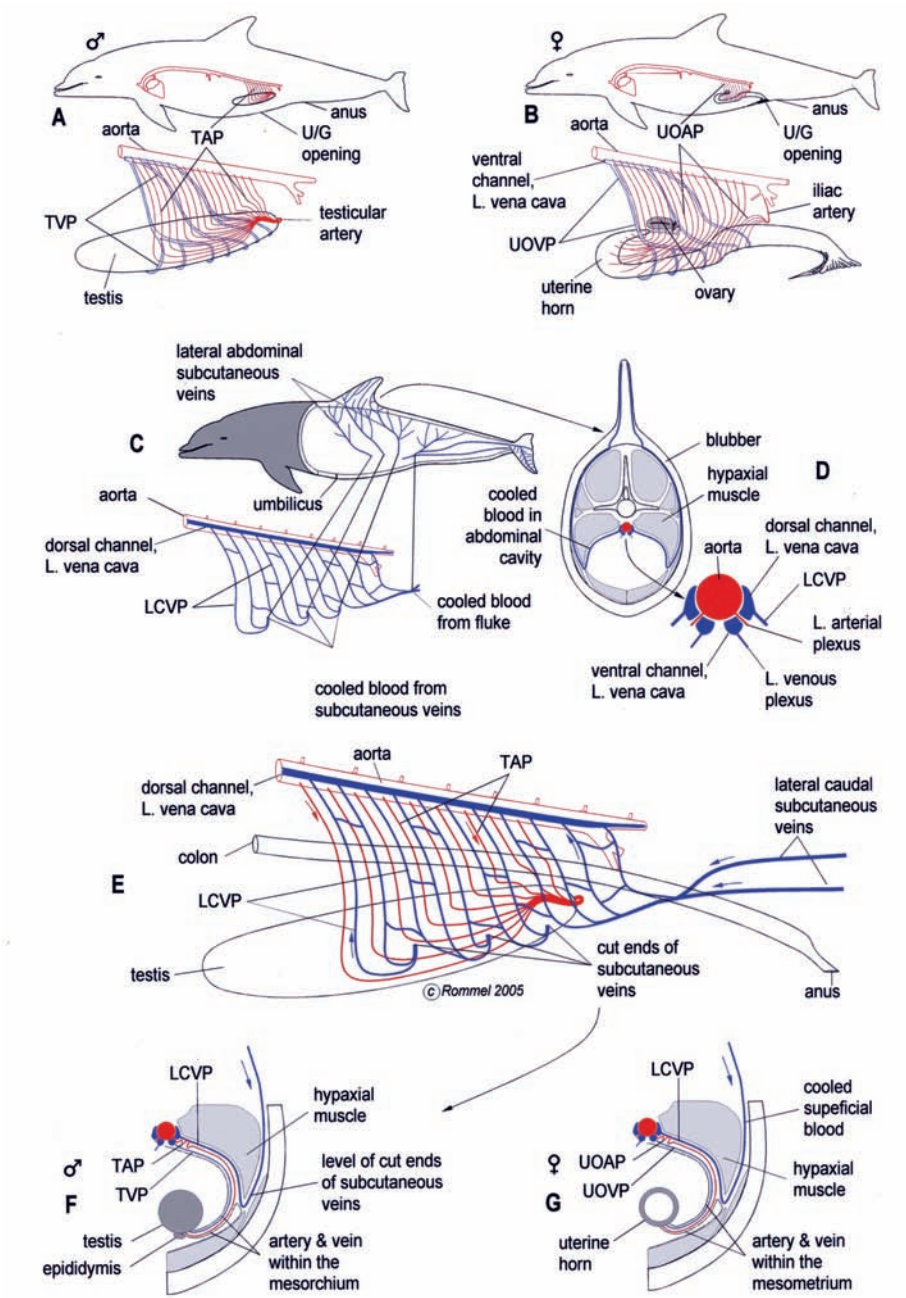

Fig. 4.3 Representation of the vascular structures that form the reproductive countercurrent heat exchanger (CCHE) of bottlenose dolphins (*Tursiops truncatus*). **A**. Topography of the superficial veins that supply the lumbocaudal venous plexus

Fig. 4.3 Contd. ...

branches of the testicular arterial plexus bypass the cone and feed the epididymis directly. The TAP is less well developed in sexually immature males than in mature males, enlarging as the animal approaches sexual maturity and the reproductive tissue demands increase.

The testis is drained by an array of veins, the testicular venous plexus (TVP, Fig. 4.3A). The veins of the TVP emerge from the body of the testis and course along the ventromedial aspect of the TAP toward the ventral channel of the

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Fig. 4.3 Contd. ...

(LCVP). Blood in the superficial veins of the dorsal fin and flukes is cooled by heat transfer to the surrounding water. These extremities are drained by thick-walled, large diameter veins that remain superficial until they coalesce and course inwards to enter the abdominal cavity. These veins feed directly into the lateral and caudal margins of the LCVP. Thus, relatively cool blood can be introduced into the caudal abdominal cavity near the reproductive tissues. **B**. Cross section of the body at the level of the dorsal fin and reproductive tissues. The trajectories of the cooled superficial blood are just deep to the blubber layer and superficial to the axial muscles. The path is a relatively long return to the dorsal vena cava. In the caudal abdomen, the venae cavae are bilaterally paired and further subdivided into dorsal and ventral channels. **C**. Topography of the testicular arterial plexus (TAP) arising from the dorsal aorta and supplying the testis. The TAP is a unique arrangement of arteries that extend ventrolaterally from the dorsal aorta. The vessels are organized into a single layer and are oriented roughly parallel to each other. At the distal margin of the plexus, the arteries coalesce to form a cone-shaped structure, from which a single testicular artery enters the caudal pole of the testis. Medial to the TAP is an irregular plexus of veins, the testicular venous plexus (TVP), that returns blood from the testis to the ventral channel of the left vena cava. The TAP is located between the LCVP dorsolaterally and the TVP ventromedially. **D**. Topography of the uterovarian arterial plexus (UOAP) arising from the dorsal aorta and supplying the uterus and ovary. Medial to the UOAP is an irregular plexus of veins, the uterovarian venous plexus (UOVP), that returns blood from the reproductive tissues to the ventral channel of the left vena cava. **E**. Left lateral view of the reproductive countercurrent heat exchanger (CCHE) in a male dolphin. Arrows indicate directions of flow. Juxtaposition of the LCVP to the TAP produces a CCHE that uses the extrinsic blood from the superficial veins to cool the intrinsic blood supply of the reproductive tissues. **F**. Partial cross section of a male dolphin at the level of one testis illustrating the juxtaposition of the LCVP on the ventral aspect of the hypaxial muscle. Dorsally, the TAP is sandwiched between the LCVP and the TAP. The TAP and TVP extend to the testis within the two layers of peritoneum of the mesorchium. **G**. Partial cross section of a female dolphin at the level of one distal uterine horn illustrating the juxtaposition of the LCVP on the ventral aspect of the hypaxial muscle. Juxtaposition of the LCVP to the TAP produces a CCHE that uses the extrinsic blood from the superficial veins to cool the intrinsic blood supply of the reproductive tissues. The UOAP and UOVP extend to the uterus within the two layers of peritoneum of the mesometrial part of the broad ligament. Illustration modified after Rommel, S. A., Pabst, D. A., McLellan, W. A., Mead, J. G. and Potter, C. W. 1992. Anatomical Record 232: 150-156, Figs 1, 2, 3; Rommel, S. A., Pabst, D. A. and McLellan, W. A. 1993. Anatomical Record 237: 538-546, Figs 2, 4, 6.

ipsilateral vena cava[3] (Fig. 4.3D). The arteries of the TAP and the veins of the TVP are sandwiched between the two layers of peritoneum that make up the mesorchium (Fig. 4.3F). The testes are fed by the arteries of the TAP and are drained by the veins of the TVP (Fig. 4.3A). These vessels are homologous to the reproductive vasculature found in other tetrapods.

4.3.1.2 Lumbocaudal Venous Plexus – LCVP

There is a novel venous structure associated with the cetacean testis – the lumbocaudal venous plexus (LCVP, Fig. 4.3C). The derived lumbocaudal venous plexus and superficial venous components are similar in both genders. The LCVP is formed by a single layer of irregularly anastomosed thin-walled vessels embedded in a connective tissue matrix (Rommel *et al*. 1992). This plexus is affixed to the ventral aspect of the hypaxial muscle and lies against the dorsolateral aspect of the TAP (Fig. 4.3D, E, F). This juxtaposition of the TAP and the LCVP places arteries and veins in close proximity and is well suited for heat exchange because of the countercurrent nature (flows in opposite directions) of blood flow within the two structures (arrows, Fig. 4.3E).

On its lateral aspect, the LCVP is supplied with blood from the superficial veins that drain the dorsal fin and the flukes (Fig. 4.3C). An extensive system of large-diameter superficial veins drains the dorsal fin; these lateral abdominal subcutaneous veins (Slijper 1936) remain just deep to the blubber layer as they course ventrally (Fig. 4.3D). Along their course, the lateral abdominal subcutaneous veins coalesce into three or four larger-diameter veins that remain superficial until they reach the lateral border of the caudal abdominal wall. Here, these veins become deep, continue to follow the ventrolateral surface of the hypaxial muscle (Fig. 4.3D), and feed into the lateral aspect of the lumbocaudal venous plexus (Fig. 4.3C, F). Similarly, the dorsal and ventral superficial veins that drain the surfaces of the flukes coalesce into the dorsal and ventral lateral caudal subcutaneous veins (Slijper 1936). These two veins (arrows on the right, Fig. 4.3E) coalesce, dive deep at the pelvic vestige, and join the caudal aspect of the LCVP. Thus, cooled blood from superficial veins from the dorsal fin, superficial caudolateral body, and flukes is distributed along a portion of the dorsolateral wall of the abdominal cavity by the LCVP. The LCVP, in turn, is drained via the dorsal channel of the ipsilateral vena cava (Fig. 4.4D).

4.3.2 Females

4.3.2.1 Uterovarian vascular plexuses, UOAP and UOVP

In contrast to the two or three arteries found in most other mammals (Fig. 4.1B; e.g., Schummer *et al*. 1981), cetaceans have 20-40 vessels that form an uterovarian arterial plexus (UOAP, Fig. 4.3B) (Walmsley 1938; Rommel *et al*.

[3]In marine mammals the lumbar venae cavae are paired (e.g. Slijper 1936; Walmsley 1938; Harrison and Tomlinson 1956; Rommel and Caplan 2003).

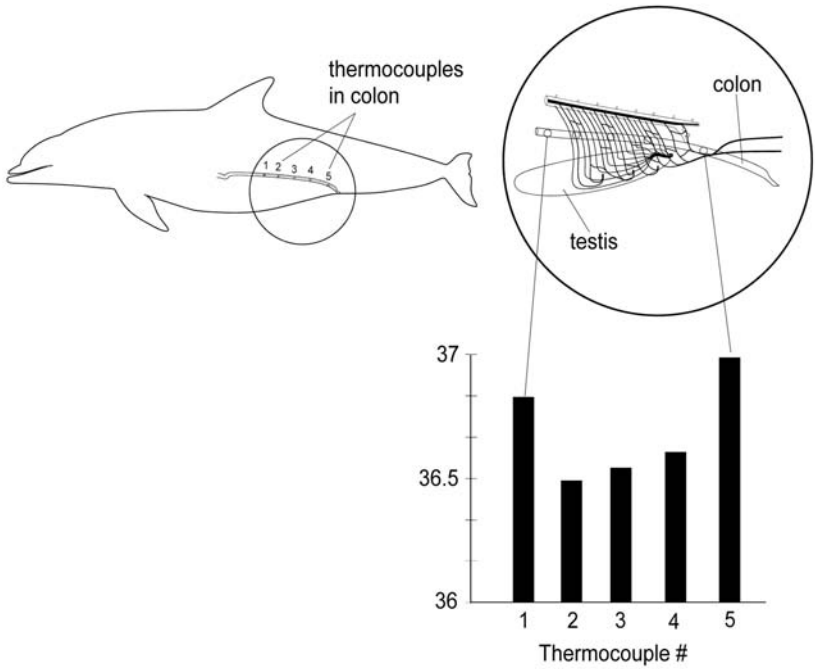

Fig. 4.4 Regional colonic temperature differences in a resting bottlenose dolphin *(Tursiops truncatus*). In cetaceans, the distal colon follows the midline between the anus and the base of the dorsal fin. Distal colonic temperatures reflect temperature at the vascular plexuses. Thermocouple #1 is cranial to, thermocouples #2-4 are within, and thermocouple #5 is caudal to the reproductive countercurrent heat exchanger. After Pabst, D. A., Rommel, S. A., McLellan, W. A., Williams, T. M. and Rowles, T. K. 1995. Journal of Experimental Biology 198: 221-226, Fig 4.

1993). The arteries of the UOAP exit the ventrolateral aspect of the lumbar aorta and pass between the dorsal and ventral channels of the ipsilateral vena cava (Fig. 4.3D). A few branches from the common iliac artery (Walmsley 1938) make up the caudalmost portion of the UOAP (Fig. 4.3B).

The UOAP of females – like the TAP of males – is distinguished by two relatively different regions: a region proximal to the dorsal midline and a more distal region within the mesometrium (Fig. 4.3G). The proximal region is juxtaposed to the LCVP described above. In this proximal region, the arteries are ordered in parallel channels with few branches or anastomoses. This arterial plexus is loosely attached to the dorsolateral wall of the abdominal cavity. In the mesometrial region of the UOAP, there are more branches and the arrangement of vessels becomes increasingly irregular (Fig. 4.3B). The mesometrium wraps around the lateral margin of the uterus and attaches along ventrolateral and medial aspects of the uterine horn (Fig. 4.3G). Thus, the UOAP is positioned between the wall of the uterus and the abdominal muscles, much the mesorchium and TAP wrap around the testis in the male.

Immature specimens have both arterial and venous plexuses, but in a less developed form than those found in pregnancy or postpartum specimens.

On the ventromedial aspect of the UOAP lies an uterovarian venous plexus (UOVP, Fig. 4.3B). This plexus provides venous return from the uterus to the vena cava. The veins of this plexus enter the ventral channel of the ipsilateral vena cava ventromedial to the UOAP. The arteries of the UOAP and the veins of the UOVP are sandwiched between the two layers of peritoneum that make up the mesometrium (Fig. 4.3G).

4.4 FUNCTION OF THE REPRODUCTIVE CCHE

The reproductive CCHE flanks a region of the colon (Figs. 4.2A, B, 4.3E) and influences colonic temperatures, thus permitting indirect assessment of the introduction of relatively cool venous blood deep within the abdominal cavity via the lumbocaudal venous plexus. Using a linear array of multiple copper-constantan thermocouples housed in a rectal probe, we measured colonic temperatures in bottlenose dolphins at positions cranial to, within, and caudal to the region of the bowel flanked by the CCHE. We investigated colonic temperatures of both peripubescent and adult male bottlenose dolphins while resting and of peripubescent males just before and after vigorous swimming (Rommel *et al*. 1994; Pabst *et al*. 1995).

In bottlenose dolphins under resting conditions, colonic temperatures measured in the region of the reproductive CCHE are cooler than temperatures measured cranial or caudal to this region (Fig. 4.4). The influence of the CCHE on colonic temperatures is dependent on a number of variables. For example, temperatures at the reproductive CCHE were 0.2-0.7EC cooler than positions cranial and/or caudal to the CCHE in peripubescent males, and were 0.9-1.3EC cooler in a sexually mature male (Rommel *et al*. 1994; Pabst *et al*. 1995). Temporary heating of the dorsal fin and flukes increased temperatures at the reproductive CCHE but had little or no effect on temperatures caudal to its position.

Our observations demonstrate that, under resting conditions, cooled blood is introduced into the deep abdominal cavity in a position to regulate the temperature of arterial blood flow to the dolphin testis. The similar morphology in female cetaceans implies a similar thermoregulatory function employed for the uterus and this has been verified by field measurements of wild dolphins in Florida.

Temperatures in the region of the colon flanked by the reproductive CCHE decrease with exercise. When the dolphin was allowed to rest for longer than 4-6 min after exercise, colonic temperatures at the region of the CCHE slowly increased. These data suggest that the CCHE has the ability to cool the arterial blood supply to the testes and may thermally isolate the testes from adjacent locomotor muscles when the dolphin is swimming vigorously (Pabst *et al*. 1995). We hypothesize that the maximal cooling observed is the result of increased flow of cooled venous blood through the CCHE during exercise.

The venous blood flowing through the LCVP of the reproductive CCHE is from the surfaces of the dorsal fin and flukes. These extremities have an additional venous return. Periarterial venous channels (Elsner *et al*. 1974) are found deep within the fin and have been hypothesized as a heat-conserving countercurrent heat exchanger (Scholander and Schevill 1955). The superficial venous system is considered a shunt to bypass the deeper countercurrent heat exchange system because blood routed through the superficial veins would be cooled by heat transfer to the surrounding water (Scholander and Schevill 1955; Kanwisher and Sundes 1966). Scholander and Schevill (1955) suggested that the mechanism for routing blood though these venous systems was "semiautomatic." If the dolphin needed to conserve heat, the rate of blood flow through the fin would be slow, at lower pressure, and venous blood would be preferentially returned via the deep periarterial venous channels. If, on the other hand, the animal needed maximal cooling, blood flow and blood pressure through the fin would increase. The increased flow would swell the nutrient arteries, occlude the surrounding periarterial veins, and force blood through the superficial venous system.

Heart rates of exercising dolphins are increased relative to resting dolphins (Williams *et al*. 1992) suggesting that blood flow through the radiating surfaces of the dorsal fin and flukes would be increased during exercise. Heat loss from blood in the superficial veins would increase by increased convective heat exchange (Schmidt-Nielsen 1990) at higher speeds of swimming. Thus, changes in blood flow patterns through the dorsal fin and flukes, coupled with increased convective heat loss from venous blood returning through the CCHE, may allow maximal cooling of the intra-abdominal testes during exercise.

4.5 CONCLUSIONS

The streamlined body shape, hypertrophied axial musculoskeletal system, and thick blubber layer of cetaceans are aquatic specializations that could pose thermoregulatory threats to temperature-sensitive reproductive tissues. Cetaceans possess a reproductive CCHE deep within the caudal abdominal cavity that functions to deliver cool venous blood returning from the superficial surfaces to thermoregulate the arterial supply to the reproductive tissues.

In male cetaceans, a reproductive CCHE is formed by the TAP and the juxtaposed LCVP (Fig. 4.3F). In female cetaceans, the reproductive CCHE is formed by the proximal region of the UOAP and the juxtaposed LCVP (Fig. 4.3G). Thus, the arterial supplies to the reproductive tissues are positioned next to vessels carrying cooled venous blood returning from the superficial surfaces of the dorsal fin and flukes.

The vascular structures that supply blood to and drain blood from the reproductive tissues of cetaceans are homologous to those of other tetrapod mammals (Pabst *et al*. 1998). However, the LCVP, which is unique to cetaceans,

is independent of, but juxtaposed to, the vascular supply of the reproductive tissues. Together, the LCVP and the reproductive arterial plexus (TAP or UOAP) vessels form a reproductive CCHE that delivers cooled venous blood deep within the abdomen in a position to cool the arterial supply to the testes in males and the uterus in females.

In healthy terrestrial mammals, colonic probes usually show relatively uniform core temperatures. In contrast, bottlenose dolphins display regional heterothermy in colonic temperatures – stable but different temperatures at different locations along their colons. Observed temperature differences are related to vascular adaptations that lower temperatures at thermally sensitive reproductive tissues. We have shown that dolphins possess vascular structures that shunt superficially cooled blood to positions deep within their bodies to avoid reproductive hyperthermic insult. These marine mammals divert cooled, venous blood to tissues surrounding their reproductive organs before it is mixed with the core circulation – co-opting extrinsic venous circulation that is separate from the intrinsic circulation of their reproductive tissues. Temperatures within the region of the heat exchanger are cooler than temperatures in front of and behind this region in bottlenose dolphins.

It is reasonable to speculate that temperature profiles, unique to individual dolphins, may provide information about differences in venous returns from the dorsal fin and flukes. These two regions of the body have different blood supplies and thus temperature differences within the profile will reflect not only differences in surface blood flow from the two regions but blood supplies to them.

4.6 ACKNOWLEDGMENTS

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CHAPTER 5

Anatomy with Particular Reference to the Female

Stephanie Plön[1]* *and Ric Bernard*[2]

5.1 INTRODUCTION

Historically, most knowledge on reproductive anatomy in cetaceans is derived from the necropsy of animals either killed in whaling operations (Mackintosh and Wheeler 1929; Matthews 1948; Chittleborough 1954; Best 1967; Gambell 1968; Lockyer and Smellie 1985), animals incidentally caught and killed (by-caught) in fishing gear (Karakosta *et al.* 1999) and from strandings (Ross 1979a; Beckmen 1986). However, a number of difficulties are encountered when analyzing animals from the wild as no knowledge of the prior reproductive history is available to aid in interpretation of the findings (Harrison 1977). Even in captive animals records of mating behavior and sexual activity often are insufficient (Harrison 1977), although recent studies have managed to account for most of these incidents (Brook *et al.* 2002). Recent advances in research techniques promise progress in the study of the reproductive status of wild cetaceans. Biopsy samples, commonly taken for the study of genetic relationships and pollution levels of free-ranging specimens, can now be used to provide information on the progesterone levels and thus reproductive status of the female (Mansour *et al.* 2002; Kellar *et al.* 2006).

5.2 CLITORIS, VAGINA, VAGINAL FOLDS

External genitalia are rarely mentioned in the recent literature, but Mackintosh and Wheeler (1929) describe the clitoris of *Balaenoptera physalus* (Fin whale) as being an incurved, keeled structure about 8 cm long. In *Phocoenoides dalli* (Dall's porpoise) and *Phocoena phocoena* (Harbour porpoise) the clitoris is

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described as being prominent (Meek 1918; Morejohn and Baltz 1972) (see also Fig. 5.1A). In most immature *B. physalus* the genital groove is closed so that little or nothing can be seen of the genitalia, but Mackintosh and Wheeler (1929) found the vulva of two females with near term fetuses as greatly swollen and the genital groove stretched open to some extent.

Cetaceans belong to the few mammals (Artiodactyla, see Chapter 4; Perissodactyla and some species of Insectivora, Ommanney 1932) that exhibit vaginal folds (Meek 1918; Morejohn and Baltz 1972). Harrison (1949) gives a short overview of this phenomenon. While the outer part of the vagina is smooth with some longitudinal folds (Slijper 1962), the inner part has a number of prominent annular folds, which look like a chain of successive funnels with the mouth pointing towards the cervix (Slijper 1962). The number of folds varies between different species of cetaceans, and also within some species. Beckmen (1986) reported considerable individual variation in size, location, and configuration of these folds in both *Kogia* (Pygmy and Dwarf sperm whale) species, with the number of folds varying from one to five or more (Beckmen 1986). The function of this peculiar morphology is unclear but speculations include serving as a barrier to water, and providing extra room in the birth canal (Slijper 1962). Additionally, it is thought that these folds act as a stimulus to the penis, promoting the release of seminal fluid from the male reproductive tract during intercourse. The seminal fluid then would collect in the chambers formed by the vaginal folds and likely prevent seawater from collecting in the folds (Harrison 1949; Slijper 1966). The fluid would then be transported towards the cervix and into the uterus by means of muscular contraction. Surprisingly, vaginal folds are absent in immature *Phocoenoides dalli* and only partially developed in mature animals of the species (Morejohn and Baltz 1972).

5.3 VAGINAL MUCUS

In cytological preparations of vaginal smears from immature and anestrous mature *Balaenoptera musculus* (Blue whale) and *B. physalus* (Fin whale), Mackintosh and Wheeler (1929) report small clumps of epithelial cells along with many scattered, singly-occurring epithelial cells, some from the mucosal surface, others from the submucosal glands in the mucus. In immature mysticetes, a few erythrocytes are seen on cytology, and in pregnancy whales, erythrocytes are the dominant cell type and leucocytes are few in number. Mackintosh and Wheeler (1929) further note that the vaginal mucus of a whale in late gestation is very thick and contain few cells including epithelial cells and erythrocytes. In contrast, the vaginal smear from an ovulating female contain many singly-occurring epithelial cells admixed with many other uncharacterized cells but presumably no erythrocytes or leukocytes. Finally, vaginal smears from lactating whales contain epithelial cells admixed with few erythrocytes (Mackintosh and Wheeler 1929).

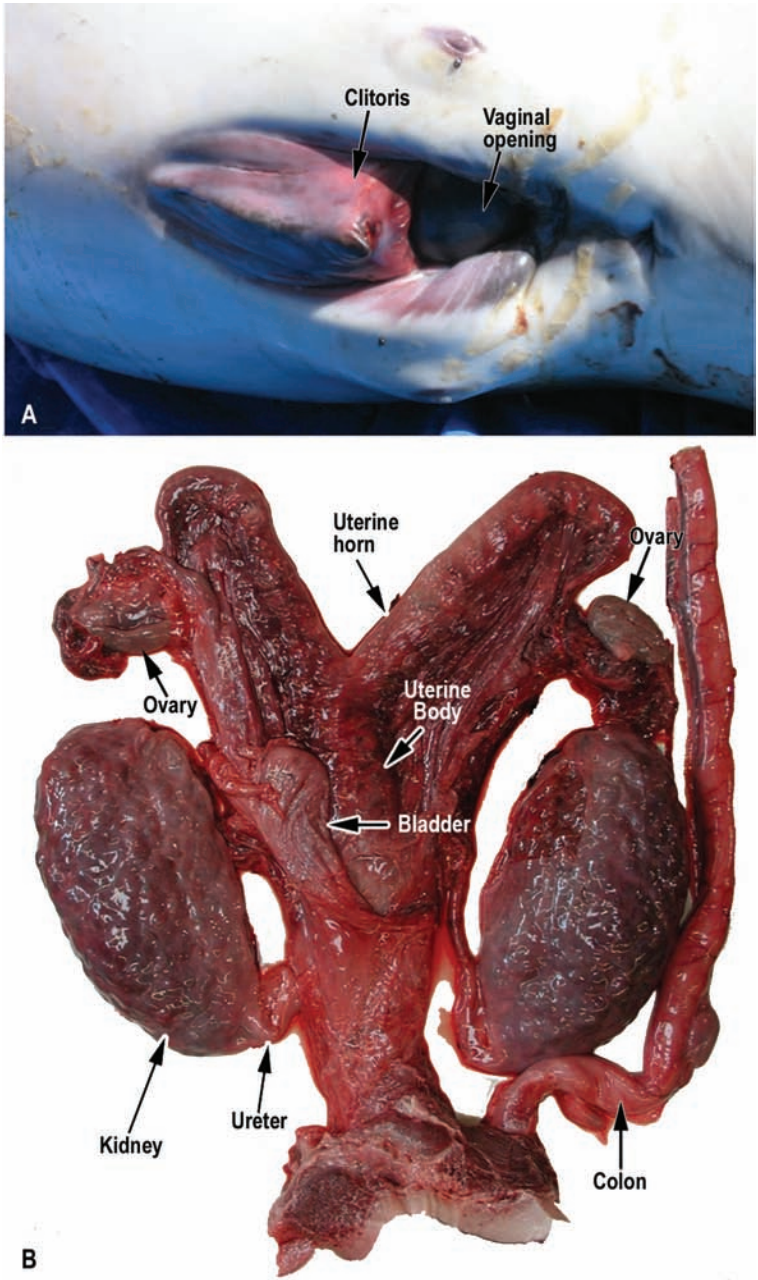

Fig. 5.1 External and internal reproductive organs of female cetaceans. A: Clitoris and vaginal opening of a harbour porpoise (*Phocoena phocoena*) (photo courtesy of Albert B. Shepard, San Juan County Marine Mammal Stranding Network, San Juan Island, WA, USA); B: Uro-genital apparatus of a female bottlenose dolphin (*Tursiops truncatus*) (photo courtesy of Bruno Cozzi, University of Padova, Italy).

5.4 VAGINAL BANDS

Mackintosh and Wheeler (1929) first describe vaginal bands in cetaceans. These are analogous to the hymen in humans and are present in 31% of immature and 14% of mature *Balaenoptera physalus* (Mackintosh and Wheeler 1929). Only once has a vaginal band been reported in a *B. musculus* (Mackintosh and Wheeler 1929). The vaginal bands are attached behind the urethra and stretch across the opening of the vagina (Mackintosh and Wheeler 1929). The band starts as a small projecting mass of tissue with papilliform appendages at the cranial vagina, then stretches along the vagina as a 7-8 cm long strand, ca. 1 cm wide (Mackintosh and Wheeler 1929). The band is mainly composed of fibrous connective tissue with a few small blood vessels (Mackintosh and Wheeler 1929). There are numerous small, convoluted ducts randomly distributed throughout the tissue. Along the luminal surface, the band is covered with papillae but at the cranial and distal ends the band is smooth (Mackintosh and Wheeler 1929). Ohsumi (1969) reviews the presence of vaginal bands in mysticetes, where it is intact in most prepubertal animals but broken in most females that have copulated or given birth. He also examines a number of odontocete species but no vaginal bands were found in the latter. An exception is *Phocoenoides dalli*, for which vaginal bands have been reported (Morejohn and Baltz 1972).

5.5 CERVIX

As in terrestrial mammals, the uterus of most cetaceans protrudes into the vagina by means of a snout-like cervix (Fig. 5.2). The cervix is composed of a very thick and rigid wall, essentially occluding the passage to the uterus (Slijper 1962). The thick wall of the cervix is composed of a mucous membrane, a connective tissue lamina propria and an underlying smooth muscle with vascular area that resembles erectile tissue (Simpson and Gardner 1972). Narwals are reported to lack a definite cervix (Slijper 1962).

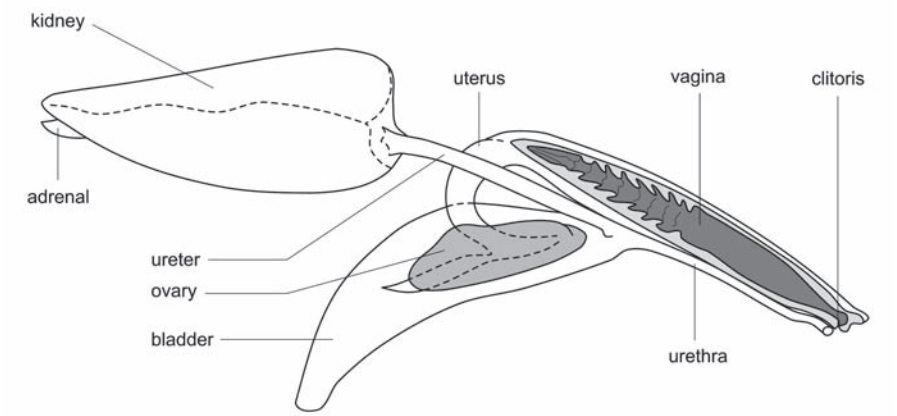

Fig. 5.2 Schematic representation of the uro-genital apparatus in the female. After: Meek, A., 1918. Journal of Anatomy 52: 186-210, Fig. 15.

5.6 UTERUS AND OVIDUCTS

The uterus of odontocetes is similar to that found in *Equus caballus* (the horse) (Rommel *et al.* 1993). It is a short corpus dividing into two uterine horns (cornua), which run parallel for a short part of their length and then bend to the left and right, respectively, curving first up and then downwards to continue as the oviducts (Slijper 1962) (see also Fig. 5.1B). As in other mammals, the cetacean uterine wall is made up of the outer serosa, a muscular myometrium (consisting of an inner circular and an outer longitudinal smooth muscle layer), and the inner endometrium (Simpson and Gardner 1972; Lockyer and Smellie 1985). The endometrium lines the uterus forming the mucosal layer that nurtures the placenta (*decidua*) and the fetus during pregnancy. The endometrial stroma is loose connective tissue containing variably-sized but often large arterioles (Simpson and Gardner 1972). The endometrium is connected to the myometrium of the uterus by loose connective tissue in the sub-mucosa, which is well vascularized (Lockyer and Smellie 1985). The mucosal layer itself can be divided into functional zones as follows: a) the thinner, sub-epithelial layer (or *stratum compactum*), which contains the openings of the ducts from the underlying glands, which are located in the thicker, deeper b) *stratum spongiosum* (Matthews 1948; Lockyer and Smellie 1985). The glands in the stratum spongiosum are highly convoluted and their diameter increases towards the mucosal surface (Matthews 1948; Lockyer and Smellie 1985). Both strata of the mucosa contain blood capillaries but the *stratum compactum* becomes highly vascularized, especially during pregnancy (Lockyer and Smellie 1985). The epithelium covering the mucosa is made up of a single layer of cuboidal to columnar epithelial cells (Simpson and Gardner 1972). Researchers have reported that it is rarely intact (Mackintosh and Wheeler 1929; Matthews 1948; Lockyer and Smellie 1985), and in immature mysticetes it is usually lost, except for glandular ostia of the luminal epithelium (Mackintosh and Wheeler 1929); however, this is most likely a postmortem artifact (Matthews 1948; Lockyer and Smellie 1985).

The uterine glands are tubular and are lined by cuboidal to slightly columnar epithelium (Simpson and Gardner 1972). The glands in a resting uterus are generally unbranched and extend to the myometrium (Simpson and Gardner 1972).

Few studies have examined the histology of the uterine mucosa in detail, possibly due to a lack of suitable samples. Matthews (1948) examined a series of *Balaenoptera musculus*, *B. physalus* and *Megaptera novaeangliae* (Humpback whale) in various stages of maturity. The changes in the uterine mucosa during these stages from adolescence, through estrus, pregnancy and lactation to anestrus show alterations in mucosal thickness, glandular and vascular size and abundance, and epithelial characteristics (Fig. 5.3). Matthews (1948) noted individual variations in these categories but stated that mean variations were even greater.

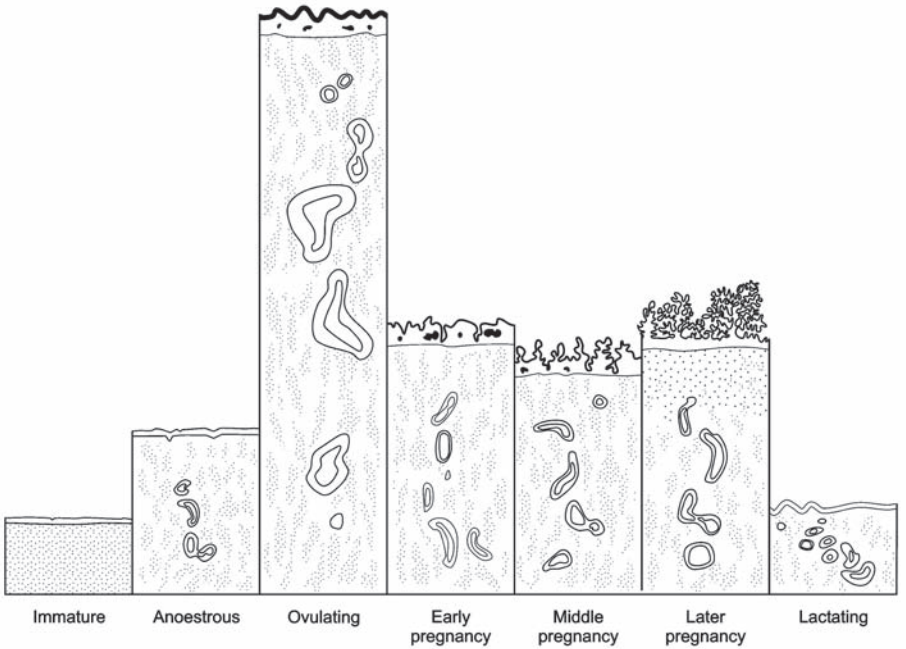

Fig. 5.3 Variation of the uterine mucosa with reproductive state. After: Matthews, L. H., 1948. Journal of Anatomy 82: 207-232, Fig. 4.

In immature mysticetes, both strata of the mucosa are thin and the glands have small or no lumens (Matthews 1948). The glands are confined to the *stratum spongiosum* and appear very coiled and branched, with few openings onto the smooth surface (except for rare depressions at glandular osia) (Matthews 1948). Blood vessels are few and small. The mucosa increases in thickness from immature to mature and ovulating animals. In the latter, the mucosa increases nearly 3.5 times that of mature animals, probably as a result of the influence of the progesterone produced in the newly formed *corpus luteum* (CL) (Matthews 1948).

In the anestrus adults, the mucosa is more than twice as thick as in immature animals but the *stratum compactum* is only ca 1.5 times as thick (Matthews 1948). Similarly, the glandular diameter in mature animals is ca 1.5 times that of immature animals. Blood vessels are larger and more numerous, especially at the base of the *stratum spongiosum* where many of them have thick walls (Matthews 1948). In mature animals, the mucosal surface is smooth with occasional depressions noted at glandular ostia (similar to immature animals) and the glands are greatly coiled and branched (Matthews 1948).

After ovulation, there is a slight decrease in the thickness of the mucosa. The mucosa remains essentially the same thickness until lactation, when it once again decreases. Similarly, postovulatory glands are numerous, closely

packed, and with patent lumens (Matthews 1948). The glands are larger in the superficial *stratum spongiosum* but few ducts traverse the *stratum compactum* to open onto the surface. Many depressions are found on the surface of the mucosa. These indentations appear less prominent in *Globicephala melas* (Long-finned pilot whale) (Harrison 1949). The mucosa contains a close network of capillaries lying immediately below the epithelial cells. This capillary system is present throughout pregnancy and is very pronounced at this stage (Matthews 1948).

In contrast the diameters of the glands located in the deeper parts of the *stratum spongiosum* show a different cycle. Here, the glands increase in diameter with approaching ovulation, decrease in early gestation, then increase slightly during mid gestation up until late gestation when their maximum diameters are reached (Matthews 1948). Thus two peaks are seen regarding the changes of glandular diameter throughout the gestational stages, and are defined by the appearance of lumens and increases in the thickness of the glandular epithelium. After parturition glandular diameter decreases rapidly. In anestrus females, pronounced decrease in glandular diameter is noted, with the smallest diameters occurring deep in the mucosa (Matthews 1948).

After parturition, involution of the uterus takes place with surprising rapidity. In fact, Mackintosh and Wheeler (1929) noted that involution was complete in almost all lactating females examined. The change in uterine size is primarily due to vascular alterations throughout the uterine mucosa (Mackintosh and Wheeler 1929).

Rice and Wolman (1971) demonstrate cyclical changes in the uterine wall of *Eschrichtius robustus* (Gray whale), which closely parallel those described by Matthews (1948) for *Balaenoptera musculus*, *B. physalus* and *Megaptera novaeangliae* and Lockyer and Smellie (1985) for *B. physalus* and *B. borealis* (Sei whale). In the absence of any ovarian data or a fetus the reproductive status of females may be identified using anatomical and histological criteria (Benirschke *et al.* 1980; Lockyer and Smellie 1985; Slooten 1991). In addition, macroscopic anatomical changes (e.g. uterine cornua width, mammary gland thickness, and uterine myometrial thickness) have been reported by Lockyer and Smellie (1985) to vary with reproductive status in *B. physalus* and *B. borealis*, and thus may be used for reproductive staging.

Cetacean placentae, like those of ungulates, are classified as diffuse and epitheliochorial (Wislocki 1933; Matthews 1948; Zhemkova 1967; Pabst *et al.* 1999), the former referring to the fact that villi are formed over the whole surface of the chorion and the latter meaning that the fetal chorion is in contact with an intact maternal uterine epithelium (Enders and Carter 2004). During pregnancy both the amniotic cavity and placenta extend to the distal tips of both the pregnancy and non-pregnancy uterine horns (Wislocki 1933; Rommel *et al.* 1993). In the majority of cetaceans examined the fetus lies in the left uterine cornu (Wislocki 1933). Rommel *et al.* (1993) describe a

counter-current heat exchange system located in the dorsal fin and flukes of odontocetes that functions to regulate the thermal environment of the uterus and the developing fetus (see also Chapter **4** of this volume). A detailed study on the morphology and histology of the placenta (including the umbilical cord) are presented by Wislocki (1933) for *Phocoena phocoena*. Details on cetacean placental structure will be presented in Chapter **11**.

Ova pass from the ovaries through the oviducts and into the uterus. The oviducts, or fallopian tubes, are straight tubes in some species but twisted to varying degrees in others (Slijper 1962). Few studies describe the histology of the oviducts. However, an account by Honma (2004) describes the oviduct of an immature harbour porpoise as a highly intricate folding of mucosal glands, with the fimbriae of the tube and part of the ovary being enveloped by a loose connective tissue bursa. The duct is made up of simple columnar epithelium but in the ampullar portion, the mucosal epithelium is tall columnar and intricately folded (Honma *et al*. 2004). The serous membrane and loose connective tissue stroma are highly vascularized.

5.7 OVARIES, FOLLICLES, CORPORA LUTEA, CORPORA ALBICANTIA, CORPORA ATRETICA

Cetacean ovaries are situated on the dorso-lateral side of the abdominal cavity just behind the kidneys, placing them roughly in the same position as the testes in the male (Slijper 1966). The literature on the ovaries of odontocetes has been extensively reviewed by Slijper (1966), Harrison (1969, 1972) and Harrison and McBrearty (1977). These works deal mainly with the macroscopic examination of the ovaries and the different types of corpora.

Limited information is available on gonadal development in neonatal and juvenile animals. Early researchers describe the macroscopic features of mysticete ovaries and the developmental changes taking place from the late fetal stage to maturity, including the development of follicles, based on samples obtained in the whaling operations of large baleen whales: Chittleborough (1954) for *Megaptera novaeangliae*, Mackintosh and Wheeler (1929) for *Balaenoptera musculus* and *B. physalus*. Similar data are rare for the smaller odontocetes. Ovaries of immature *Tursiops truncatus* (Bottlenose dolphin) are devoid of large follicles and corpora, and there is a marked reduction in the number of oocytes in the cortex over the first and second years of life (Harrison 1977). Karakosta *et al*. (1999) present morphological and histological data on the gonadal development of neonatal and juvenile *Phocoena phocoena* of both sexes. Previous studies found no difference in the appearance and size of the two ovaries in immature animals, but reported a significant increase in the size of the left ovary at the onset of sexual maturity (Fisher and Harrison 1970). Karakosta *et al*. (1999) report a significant asymmetry of the number of naked ova (that is oogonia that have no enclosing epithelium) in neonatal ovaries. This discovery supports previous studies on ovarian symmetry in this species, and presents evidence that this asymmetry is present from an even earlier age than previously thought.

Macroscopically, odontocete ovaries are similar to those of other mammals, while mysticete ovaries resemble those of birds (Slijper 1962). Immature ovaries of mysticetes are flat and have a varying number of grooves; however, in adults they look like a bunch of grapes (Slijper 1962). These protrusions represent follicles and/or luteal bodies of previous cycles, which vary from ca 3.2 to 5.1 cm in diameter (Laws 1961; Slijper 1962). From rather rounded, soft structures the ovaries of mysticetes develop into pale, flat, compact organs and never weigh more than 0.9 or 1.4 kg in immature *Balaenoptera physalus* and *B. musculus*, respectively (Mackintosh and Wheeler 1929). In contrast, in mature *B. physalus* and *B. musculus* the ovaries are elongated bodies measuring usually between 20 and 40 cm in length (Mackintosh and Wheeler 1929). The *corpus luteum* (CL) can easily account for half the weight of the ovary in pregnancy females (a 25.3 m pregnancy blue whale had ovaries that weighed 29.5 kg each) and the ovaries of mature, non-pregnancy females weigh up to 6.8 kg and 4.1 kg, respectively, in *B. musculus* and *B. physalus* (Mackintosh and Wheeler 1929).

In contrast, odontocete ovaries, like mammalian ovaries, do not resemble bunches of grapes but are uniformly round: they have a large number of ova, each individual one lying in a primary follicle of its own (Slijper 1962). Ovaries of immature odontocetes are elliptical (Harrison 1949) (Fig. 5.4). The ovaries become less flattened and progressively darker with increasing age (Harrison 1949; Beckmen 1986). In *Kogia* the color of the ovaries of both species changes from beige or white in immature ovaries to dark gray, dark brown or black in mature ovaries (Beckmen 1986; Plön 2004). While the ovaries of adult odontocetes are generally spherical (Harrison *et al.* 1972), those of adult *Kogia* are ovoid (Beckmen 1986; Plön 2004) (Fig. 5.4).

Ovary weights have been provided for a number of species and generally increase with increasing length and age of the animal (Chittleborough 1954; Best 1967; Harrison and Brownell 1971; Ross 1979a, 1984; Marsh and Kasuya 1984; Cockcroft and Ross 1990; Hohn *et al*. 1996), although in some species considerable variation in ovarian weight is found among animals of similar age or length (Marsh and Kasuya 1984). Considerable overlap in ovarian weights between immature and mature animals was reported for *Physeter macrocephalus* (sperm whale) (Best 1967). This overlap did not reflect variation due to CLs of pregnancy because pregnancy females were placed in a separate category. Chittleborough (1954) reports that ovary weights of *Megaptera novaeangliae* decrease slightly just after ovulation and increase slightly during late pregnancy, the latter being due to the large size of the CL. Ovarian weight is not a good indicator of attainment of sexual maturity in *Kogia sima* (Dwarf sperm whale) as it varies substantially depending on the stage of regression of the latest CL (Plön 2004). Both the macroscopic and microscopic examination of the ovaries for corpora remain the most reliable indicators for the onset of sexual maturity in cetaceans.

The microscopic anatomy of the ovaries of cetaceans is generally similar to that of terrestrial mammals (Simpson and Gardner 1972; Harrison 1977) and

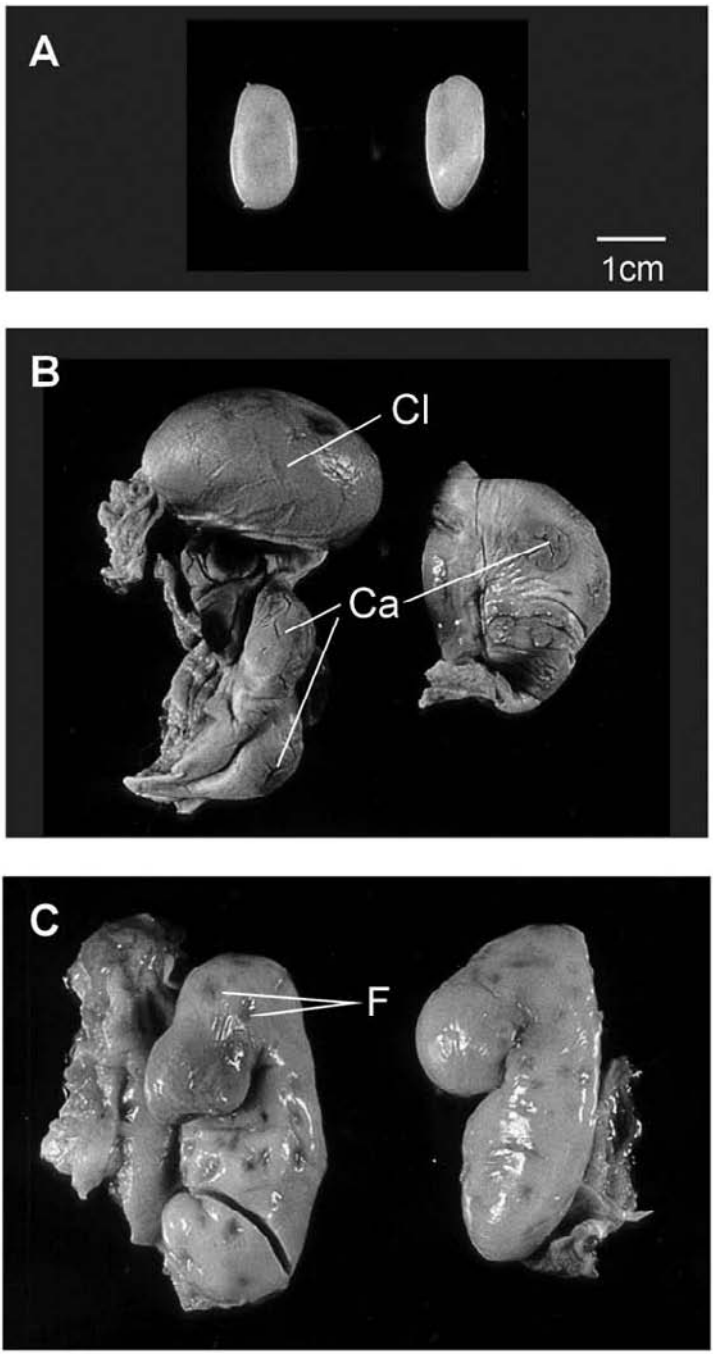

Fig. 5.4 Ovaries of *Kogia*. A: Immature ovaries. B: Mature ovaries. CL: *Corpus luteu*m, CA: *Corpus albicans*. C: Mature ovaries. F: Follicles.

has been examined in detail for a number of species (Sergeant 1962; Slijper 1966; Best 1967; Fisher and Harrison 1970; Harrison 1977; Collet and Harrison 1981; Ivashin 1984; Collet and Robineau 1988; Claver *et al.* 1992). Simpson and Gardner (1972) provide a number of detailed histological sections. The typical mammalian ovarian histology consists of the lining germinal epithelium, which consists of low columnar or squamous epithelial cells (Harrison 1977), tunica albugenia, cortex, and medulla (Simpson and Gardner 1972). Follicles in various stages of development and involution are present within the cortex (Simpson and Gardner 1972), each of which in the late fetal stage contains an oocyte 60-80 μm in diameter surrounded by a single layer of primitive granulosa cells and embedded in stromal tissue (Harrison 1977). In the porpoise ovary a fairly prominent basophilic cortical stroma is present, which is composed of compact intertwining connective tissue cells (Simpson and Gardner 1972). Compared to the cortex, the medulla is less cellular but more vascular and collagenous (Simpson and Gardner 1972). Ultrastructural studies of cetacean corpora have been carried out by Bryden *et al*. (1984) and Harrison (1977).

When a female is not in season, the follicles are immature and thus there is no antral space, which results in the follicle walls coming into close contact with the ovum (Slijper 1962). However, as the follicle matures it becomes distended by the accumulation of fluid within the antrum and moves outward to the surface of the ovary, from which it begins to protrude (Slijper 1962). Eventually the wall of the follicle bursts and the ripe ovum is released (Slijper 1962). In polytoccus mammals (i.e. litter-producing species) a number of follicles mature and protrude from the ovary simultaneously, while in unitoccus species one ovum matures at a time. While cetaceans usually have a single young, they have a number of protruding follicles, even when they are not in season (Slijper 1962). Usually only one follicle develops during one season. In *Balaenoptera musculus* and *B. physalus* this mature follicle is usually found in the anterior part of the ovary, where the wall is thinnest (Slijper 1962). However, more than one follicle can mature simultaneoulsy or one follicle can discharge a number of ova, resulting in twinning or even triplets if the eggs are fertilized (Slijper 1962).

Unlike the gradual process of sexual maturation in male cetaceans (see Chapter **8** of this volume), female maturation is rapid and the onset of sexual maturity is generally defined as the age at which a female has ovulated at least once as evidenced by the presence of at least one corpus in her ovaries (Perrin and Donovan 1984). The corpora, reflecting the past reproductive history of the female (Perrin and Donovan 1984), can be related to the age of the animal in order to determine the age of first ovulation, the birth interval, and her reproductive lifespan. This in turn, can lead to further indications as to the reproductive ability of the whole population.

Inward growth and hypertrophy of the follicular epithelium with the formation of luteal tissue begins very soon after ovulation, so that the antral spaces remaining after rupture of the Graafian follicle are soon filled with

luteal tissue. Thus the CL is simply a ruptured follicle whose wall has greatly increased in size and formed new tissue (Mackintosh and Wheeler 1929; Chittleborough 1954; Slijper 1962). This tissue produces progesterone, a hormone that stimulates and strengthens adhesion of the fertilized ovum to the uterine wall (see also Chapters **6**, **7** and **10** of this volume) (Slijper 1962). Occasionally, a translucent substance occupies the centre of the newly formed CL and the surface of the CL contains an annular structure, representing the site of follicular rupture (Chittleborough 1954). A newly developed CL can readily be distinguished from later stages, because the outer cell layer is very thin and contains numerous blood vessels visible immediately beneath it (Chittleborough 1954). The luteal tissue is soft, pale, and the cells are vacuolated in contrast to the firmer yellow tissue in late pregnancy (Chittleborough 1954); in older, regressing CLs the vacuoles disappear (Mackintosh and Wheeler 1929; Harrison 1977). The initial size of a CL after formation is dependent on the size of the follicle at ovulation, but once formed, the CL continues to increase in size (Chittleborough 1954). In pregnancy mysticetes it can take on the size of a small football (Slijper 1962). The weight of a functional CL in *Megaptera novaeangliae* ranges from 305 g to 2185 g and it ranges in diameter from 86 mm to 164 mm (Chittleborough 1954). However, the *corpus luteum* (yellow body) in mysticetes is something of a misnomer, because it is pink (Slijper 1962). In contrast, odontocetes and many other mammals have a yellow CL, which often is larger than the rest of the ovary (Slijper 1962). The CL of *Tursiops* is a compact gland, subdivided into lobules by fibrous septa containing large blood vessels (Harrison 1977). It is often more or less pedunculated but rarely has a central cavity (Harrison 1977). There are conflicting reports in the literature as to whether the CLs of the two *Kogia* species are pedunculate (Harrison *et al.* 1972; Ross 1979b) or not (Beckmen 1986). Examination of the ovaries examined by Ross (1979b) along with additional samples revealed that, although the CLs protrude from the surface of the ovary, they are not truly pedunculate (Plön 2004). In *T. truncatus* the CL has an average diameter between 25 and 32 mm (Harrison 1977; Harrison and McBrearty 1977). In general the CL of pregnancy does not change size throughout gestation (Matthews 1938a; Kasuya *et al.* 1974; Harrison *et al.* 1981; Marsh and Kasuya 1984; Perrin and Donovan 1984; Dans *et al.* 1997), but may increase slightly in early pregnancy until the fetus has reached a certain length and then regresses again slightly (Sergeant 1962; Best 1967; Miyazaki 1984). However, in some species wide individual variation in the CL volume (or index) against fetal length have been observed (Harrison *et al.* 1981; Plön 2004).

A functional CL (i.e., during pregnancy) inhibits the growth of ova and thus ovulation (Mackintosh and Wheeler 1929; Slijper 1962). Therefore, the follicles seen in pregnancy ovaries are those that would later have ovulated if fertilization had not occurred; these follicles do not reach full maturity and are somewhat smaller (Mackintosh and Wheeler 1929; Slijper 1962). The smaller follicles regress and become hidden beneath the surface of the ovary

(Mackintosh and Wheeler 1929; Slijper 1962). During lactation, the larger follicles, having attained a size beyond regression, remain large and retain their alveolar shape but lose the turgidity they had before and during pregnancy (Mackintosh and Wheeler 1929). Degeneration (atresia) also occurs when ovulation is not followed by fertilization and pregnancy. In atresia, the CL starts to regress at about 10 days post-ovulation (Slijper 1962). The glandular yellow (or pink tissue) characteristic of the CL quickly disappears until no more than a fairly degenerate type of white connective tissue remains, the *corpus albicans* (CA). In mysticetes the CAs are generally made up almost exclusively of the thickened elastic walls of the arteries that supplied the CL with blood and which have subsequently been compressed (Slijper 1962). CAs in *Tursiops* vary in shape, size, and histological appearance (Harrison 1977). They can take the shape of rounded protuberances from the body of the ovary, of pedunculated masses joined to the ovary by stalks of varying thickness, of conical papillae projecting from the ovarian surface, or of scars with a wrinkled surface (Harrison *et al.* 1969; Harrison and Brownell 1971; Harrison 1977). In the two *Kogia* species, CAs are usually spherical (with or without a core and trabeculations), button mushroom, or crescent shaped (Beckmen 1986). Histologically, CAs show varying degrees of cellularity and degeneration of the cells, probably in accordance with the age of the CA (Harrison 1977). Pigment granules and glycoproteins are present and few leucocytes and histocytes; the prominent feature is the deposition of hyaline material in place of the luteal tissue (Harrison 1977). Electron microscopic examination reveals bundles of collagenous fibers, granules, and vesicles of various types embedded in the amorphous matrix and degenerated remnants of luteal elements (Harrison 1977). Harrison (1977) cautions that old shrunken CAs should not be confused with atretic follicles reduced to hyalinised fibrous scars.

In all cetaceans, the CL of pregnancy persists throughout gestation (Matthews 1938a; Best 1967; Marsh and Kasuya 1984; Perrin and Donovan 1984) and rapidly regresses after the birth of the calf (Sergeant, 1962; Harrison *et al.* 1969; Fisher and Harrison 1970; Harrison *et al.* 1981; Marsh and Kasuya 1984; Perrin and Donovan 1984). In fact, only the CA remains during the second half of lactation. The rate of regression of CLs and CAs has been covered in detail by Best (1968), Perrin *et al*. (1976) and Kasuya and Marsh (1984), and briefly by Kasuya *et al*. (1974), Cockcroft and Ross (1990) and Read (1990a). Although in most terrestrial mammals and pinnipeds the CA disappears completely after a few years, in cetaceans the scars merely diminish in size and are visible in the ovary for an extended period of time. The accumulation of ovarian scars in a number of species suggests that the corpora of ovulation persist throughout life in cetaceans (Best 1967; Harrison 1969, 1977; Harrison *et al.* 1972; Kasuya 1972; Collet and Harrison 1981; Kasuya and Marsh 1984; Marsh and Kasuya 1984; Perrin and Donovan 1984; Perrin and Reilly 1984; Slooten 1991) and therefore present a reliable record of a females' reproductive history (Slijper 1962; Collet and Saint Girons 1984;

Perrin and Reilly 1984). The CAs do not, however, indicate whether the ovulations were followed by fertilization or pregnancy or whether the fetus was carried to term. An exception to the retention of CAs is found in *Pontoporia blainvillei* (Franciscana dolphin), in which the corpora are completely reabsorbed after four years (Harrison *et al.* 1981). Although some authors claim to be able to distinguish between the scars of ovulations which were infertile and the scars of ovulations that resulted in a pregnancy (Harrison 1969; Harrison and Brownell 1971; Collet and Harrison 1981; Ivashin 1984), the majority of investigators are not able to reliably differentiate two types of scars (Perrin *et al.* 1976; Benirschke *et al.* 1980; Lockyer 1984; Perrin and Donovan 1984; Beckmen 1986; Slooten 1991). Further, a few cetacean species exhibit a number of infertile ovulations at the onset of sexual maturity (Sergeant 1962; Perrin *et al.* 1976; Collet and Harrison 1981; Miyazaki 1984; Perrin and Reilly 1984; Cockcroft and Ross 1990; Read 1990b) and ultimately, CLs have been recorded in animals, which, upon closer examination, were not pregnancy (Benirschke *et al.* 1980). Based on our current understanding, it is likely that earlier estimations of reproductive parameters in various cetacean species were inaccurate (Perrin and Donovan 1984). Given that every ovulation does not necessarily result in the formation of a CL (Brook *et al.* 2002) and that some CAs seem to be resorbed, there is no assurance that the number of CAs present a complete history of past ovarian activity (Perrin and Donovan 1984). It has, however, been suggested that the state of the endometrial histology would be a better guide to distinguish between a current or recent pregnancy and an infertile ovulation (Benirschke *et al.* 1980). Recent studies on the reproductive biology of captive *Tursiops aduncus,* with a known reproductive history, have shown that, although it is possible to correlate CAs with the numbers of past pregnancies, the scars of ovulation do not remain grossly visible on the surface of the ovary in this species (Brook *et al.* 2002) (see Chapter **7** of this volume).

So-called accessory corpora (defined as more than one CL occurring per ovulation in a pair of ovaries) have occasionally been reported for a number of cetaceans (Sergeant, 1962; Harrison and McBrearty 1977), such as *P. macrocephalus* (Best 1967), and occur frequently in *Delphinapterus leucas* (Beluga whale) (Sergeant 1973; Braham 1984) and *Monodon monoceros* (Narwhal whale) (Perrin and Donovan 1984).

In rare instances a *corpus atreticum* can be observed: the granulosa cells in the ovarian cortex degenerate rapidly and leave the collapsed follicle full of thecal cells and vascular tissue (Harrison 1977). In some species *corpora atretica* can apparently be confused with old shrunken CAs (Beckmen 1986).

Today, ovulation can be estimated using ultrasonographic monitoring of ovarian morphology during the ovarian cycle (including folliculogenesis) in live, captive animals (Robeck *et al.* 1998; Brook and Kinoshita 2005). This technique is useful in controlled breeding of captive dolphins but provides little information about endometrial changes (Brook 2001). In addition, follicular development in wild caught *Balaenoptera acutorostrata* (Minke

whales) has been investigated in detail (Fukui *et al*. 1997a, b; Asada *et al*. 2001) and is discussed in Chapter 7 of this volume. Determination of pregnancy and ovarian activity, historically done by postmortem examination of the reproductive organs, can now be done by analysis of milk progesterone levels (West *et al*. 2000), reproductive steroid levels in the urine (Walker *et al*. 1988; Robeck *et al*. 1993), and in blubber biopsies (Mansour *et al*. 2002; Kellar *et al*. 2006).

5.8 OVARIAN SYMMETRY

Some mammals ovulate almost exclusively from one ovary, whereas in other species both ovaries are functional. The latter therefore either exhibit equal ovulation rates or show indications that one ovary is more active than the other (Asdell 1946; Ohsumi 1964). Ohsumi (1964) collected ovaries from mature females of 23 species of Cetacea containing representatives of all families with the exception of the River dolphins. From the examination of the number of corpora present in the left and the right ovary, he concluded that species belonging to the same genus show the same type of corpora accumulation and described three different types of accumulation rates. All species of mysticetes have an equal accumulation rate in the left and right ovary, which Ohsumi (1964) describes as Type I. Based upon one *Kogia* specimen (unidentified to species) having six corpora in the left ovary and seven in the right, Ohsumi (1964) concluded that the entire family had a Type I ovulation pattern. Additionally, *Physeter macrocephalus* and all of the Ziphiidae (beaked whales) were included in the Type I pattern. All the other odontocete families were placed into the Type II or III categories of ovulation patterns (Ohsumi 1964). Both Type II and III categories are characterised by the right ovary maturing somewhat later than the left. This results in a higher accumulation rate of corpora in the left ovaries of young animals. Further, this number is exceeded by the accumulation rate of corpora in the right ovaries of older animals (Ohsumi 1964). The main distinction between the Type II and Type III ovulation patterns is that the disparity in accumulation rate between the left and right ovary is slight in the Type II pattern but pronounced in the Type III pattern (Ohsumi 1964).

Subsequent examinations support Ohsumi's findings that odontocetes ovulate predominantly or exclusively from the left ovary (Best 1967; Fisher and Harrison 1970; Harrison and Ridgway 1971; Perrin *et al*. 1977; Benirschke *et al*. 1980; Collet and Harrison 1981; Cockcroft and Ross 1990; Claver *et al*. 1992; Hohn *et al*. 1996; Dans *et al*. 1997; Read 1990a; Sørensen and Kinze 1994; Read and Hohn 1995) and a Type III accumulation pattern has been described for *Pontoporia blainvillei*, in contrast to a Type I pattern in the other river dolphins (Brownell 1984). However, recent findings indicate that in the genus *Kogia* both ovaries are equally functional in *K. breviceps*, while in *K. sima* the left ovary is more active than the right one (Plön 2004). Thus *K. breviceps* would be classed as having a Type I accumulation rate like *P. macrocephalus* and the

mysticetes, which is in agreement with previous findings (Ohsumi 1964; Harrison *et al.* 1972). However, *K. sima*, although ovulating from both ovaries, would be classed as having a Type II or III accumulation rate. This contradicts earlier findings by Harrison, Brownell and Boice (1972) and Beckmen (1986), who report an equal accumulation rate in both ovaries as well as an equal implantation rate in both uterine horns for both *Kogia* species.

In all odontocetes the fertilized ovum is usually attached to the distended left horn of the uterus (Slijper 1962). In only 7% of the 635 pregnancy dolphins and *Delphinapterus leucas* investigated was the embryo found in the right horn (Slijper 1962), while in *Balaenoptera musculus* and *B. physalus* it was 60-65% for the right ovary and right horn.

Karakosta *et al*. (1999) report a significant asymmetry of the number of naked ova (that is oogonia that have no enclosing epithelium) in neonatal ovaries, supporting previous studies on ovarian symmetry in this species, but presenting evidence that this asymmetry is present from an even earlier age. However, the asymmetry in the number of naked ova observed in neonates of *Phocoena phocoena* disappears in juvenile animals, possibly due to a degeneration of excess oocytes (Karakosta *et al.* 1999). Neonatal and juvenile ovaries contained the same type of follicles and both the left and right ovary contain primordial follicles in the adult animals (Karakosta *et al.* 1999). However, Graafian follicles, CAs and CLs have only been noted from left mature ovaries, indicating that a large number of follicles undergo atresia close to sexual maturity, especially in the right ovary (Karakosta *et al.* 1999). Only few specimens with ovulations from the right ovary have been reported for *P. phocoena*, most of them being at an advanced age (Karakosta *et al.* 1999).

5.9 MAMMARY GLANDS

The mammary glands in cetaceans are two long, fairly small and flat organs, which are inclined to each other at a slight angle and lie internally to either side of the genital slit, longitudinal to the body axis (Slijper 1962).

Measurements for mammary glands of some cetaceans during lactation are provided in Table 5.1. The depth of the mammary gland increases during lactation and thus may be used as an indicator of the reproductive state of the animal (Mackintosh and Wheeler 1929; Oftedal 1997). Other changes include a change in color of the glandular tissue from pink to golden brown and protrusion of the teats from their normally concealed location within the mammary slits (Slijper 1962). The tubuloalveolar glands are divided into countless lobes and lobules by connective tissue septa (Slijper 1962; Simpson and Gardner 1972). The lobules begin as narrow ducts that run longitudinally through the gland into a central lactiferous duct, which becomes strongly distended, forming a large sinus that is connected to the teat by a single canal (Slijper 1962; Oftedal 1997). The teat is usually located at the caudal end of the mammary gland (Mackintosh and Wheeler 1929; Oftedal 1997). During lactation the secretory cells of the gland produce milk, and in dead specimens

Table 5.1 Dimensions of lactating mammary glands in some cetaceans

Species	*Length (cm)*	*Width (cm)*	*Depth (cm)*	*Weight (g)*	*Reference*
B. musculus	150-200	65	20-30	~56250*	Mackintosh and Wheeler 1929; *after Oftedal 1997
B. physalus	150-200	65	20-30		Mackintosh and Wheeler 1929; Laws 1961; Oftedal 1997
M. novaeangliae	170	45	-	177000	Oftedal 1997
E. robustus				115000	Rice and Wolman 1971; Oftedal 1997
B. borealis			13		Gambell 1968; Oftedal 1997
B. acutorostrata			10		Best 1982; Oftedal 1997
E. australis			25		Matthews 1938b; Oftedal 1997
G. melas	45	15	7.5	6000	Sergeant 1962; Oftedal 1997
S. attenuata	27		2.8		Oftedal 1997
S. longirostris	27		2.8		Oftedal 1997
K. sima	50-52	4	3	620-700	Ross 1984; Oftedal 1997
D. delphis	31	7	2	570	Ross 1984; Oftedal 1997
P. phocoena	31	7	2	597	Oftedal 1997

this is generally used as an indication that the animal was accompanied by a suckling calf. Lactation happens by squirting milk into the mouth of the calf.

The division between immature and mature glands is not well defined but in general immature glands are easily recognized (Mackintosh and Wheeler 1929). Immature mammary glands consist largely of connective tissue with few ducts and blood vessels; the ducts are surrounded by clusters of cells forming imperfect alveoli grouped together in small lobules. Mature mammary glands are generally divided into developmental stages corresponding with lactation. Mackintosh and Wheeler (1929) listed four stages that could be identified histologically in mature mammary glands of *Balaenoptera musculus* and *B. physalus*: 1) lactation (milk is actively being secreted), 2) intermediate (glandular lobules are better developed than in the resting stage but not developed as well as in the lactating stage; this condition occurs immediately before lactation and again in the apparently prolonged involution of the gland afterwards), 3) resting (complete involution of the gland) and 4) virgin (gland of an animal that has never been pregnancy).

In the lactating gland, the lobules are markedly swollen with only minimal interlobular connective tissue stroma (Mackintosh and Wheeler 1929). With the lobules, the alveoli are distended such that their outline is rounded and relatively distinct. Secretory material (milk) is filling the alveolar lumens and is clearly seen in the cytoplasm of the alveolar cells, which are swollen and have small, hyperchromatic nuclei (Mackintosh and Wheeler 1929).

The intermediate stage is found in sexually mature animals but their glands are not yet functional nor enlarged (Mackintosh and Wheeler 1929). The lobules in these mammary glands show evidence of either swelling, as in preparation for lactation, or more often, contraction, as happens toward the

end of lactation (Mackintosh and Wheeler 1929). The lobules are less developed than in lactating animals but more developed than in the resting stage (Mackintosh and Wheeler 1929). Similar to the lactating gland, the connective tissue stroma is minimal; however, the alveoli are considerably smaller than in the lactating gland, with poorly defined borders and lumens (Mackintosh and Wheeler 1929). Secretory material is essentially absent from the alveoli. The nuclei of the alveolar lining cells are larger and euchromatic compared to the lactating gland (Mackintosh and Wheeler 1929).

The resting stage occurs in animals which are neither pregnancy nor lactating. Resting mammary glands differ from intermediate glands mainly in the size of the lobules (Mackintosh and Wheeler 1929). Lobules are markedly smaller and more numerous, and alveoli are shrunken and poorly defined.

The fourth stage of mammary gland development is found in young virgin or primiparous whales. This stage of development can be difficult to distinguish from immature glands. Immature mammary glands consist largely of connective tissue with a few ducts and blood vessels; the ducts are surrounded by clusters of cells forming imperfect alveoli grouped together in small lobules. The distinction between immature and mature is not very sharp but it is not difficult recognizing the immature glands (Mackintosh and Wheeler 1929).

In this fourth stage, glandular development is slightly increased from that of immature glands. Further, although stage four glands may retain some immature features, they display glandular development beyond that of the resting stage.

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CHAPTER 6

Endocrinology of Reproduction

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6.1 INTRODUCTION

Understanding reproduction in cetaceans is paramount to our knowledge of dolphins and whales and how they fit into the marine ecosystem they inhabit. Without extensive knowledge of reproductive cycles, it is nearly impossible to manage any species, let alone ones that are pelagic or migratory across vast oceans. Much of the early work on the reproduction of cetaceans was observational (e.g., an abundance of animals with calves were noted at a certain time of year in a given area) or descriptions of the gross morphology of reproductive tracts from whales and dolphins taken through whaling or as by-catch in fisheries. This gross anatomical and morphological work is credited with elucidating the generalized reproductive cycles of whales and dolphins; however, advancements in endocrinology refined the ability of researchers to 1) measure the onset of sexual maturity, pregnancy and other reproductive states and 2) correlate the response of individual whales to changes in their environment.

Advancements in endocrinological techniques have allowed researchers to measure hormones in minute concentrations, and in a variety of biological samples (serum, saliva, urine, feces and blubber). Long-term hormonal monitoring in cetaceans was first described with testosterone concentrations of captive male bottlenose dolphins by Harrison and Ridgway (1971). Using a sensitive competitive protein-binding assay, they measured serum testosterone (one of the main androgens for spermatogenesis in terrestrial mammals) of a captive male *Tursiops truncatus* (Bottlenose dolphin) over a two-year period. This ground-breaking research was followed by a 10-year hiatus, during which time the protein-binding assay was replaced by the more sensitive and specific radioimmunoassay (RIA) technique (Bernson and

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Yallow 1961). Radioimmunoassay made it possible to measure circulating sex steroids in captive animals of various species. In fact, much of what is known about hormone levels and reproductive cycles in cetaceans has been discovered through captive breeding programs, where an individual animal's reproductive state can be closely monitored by behavioral observations and serum/plasma hormone analysis (Atkinson 2000; Atkinson *et al*. 1999; Robeck *et al*. 2001; Boyd *et al.* 1999). Additional information has been opportunistically collected postmortem from free-ranging animals [e.g., *Balaenoptera bonaerensis* (Antarctic minke whales) and North Atlantic *B. physalus* (fin whales)] taken by commercial whaling operations and under special permit by research vessels in the Antarctic Ocean (Kjeld *et al.* 1992; Suzuki *et al*. 2001; Kjeld *et al*. 2003, 2004).

6.2 FEMALE ENDOCRINOLOGY

Progesterone is probably the most commonly measured steroid in cetaceans. Its diagnostic use in differentiating age groups and reproductive status assists with the management of captive dolphins and whales and, in free-ranging cetaceans, its measurement can be used to ascertain the onset of sexual maturity or pregnancy. The other sex steroids that are commonly measured are testosterone and various forms of estrogen. The gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), are typically only measured in cases of assisted reproduction (i.e., artificial insemination or gamete harvesting) or for research purposes. The few studies that have measured adrenal and thyroid hormones provide evidence that these hormones also play a role in reproduction.

The female reproductive states vary more than those for males and therefore require greater detail to describe. In the following sections, female endocrinology is divided into the various reproductive events (pre-puberty and sexual maturity, estrous cycles, gestation, lactation, senescence), and an assessment of the reproductive effects of other hormones (i.e., thyroid hormones). Information for odontocetes and mysticetes is presented separately when suborder-specific data are available.

6.2.1 Pre-puberty and the Onset of Sexual Maturity

In the study of cetacean life history parameters (e.g., age at sexual maturity), accurate determination of sexual maturity of individual animals is important. Steroidogenesis begins in the fetal ovary (Muranishi *et al*. 2004). Growth and differentiation of primordial follicles of *Balaenoptera bonaerensis* was independent of FSH and LH, although this was not true for preantral follicles (Muranishi *et al*. 2004), suggesting different control mechanisms as the follicles develop. As for most mammals, the link between gonadal state and the sex steroids is simple in immature or pre-pubertal cetaceans because progesterone and estrogen have very low circulating concentrations. The onset of sexual maturity (i.e., the occurrence of the first ovulation) is marked by a rise in the circulating concentration of progesterone, which can be

measured in a variety of biological samples (e.g., serum, saliva, urine, feces, blubber). Estrogen is more difficult to discern in pre-pubertal whales because the amounts tend to be very low (picogram concentrations) and to fluctuate around the sensitivities of most assays. In addition, progesterone will typically display a prolonged elevation whereas the pre-ovulatory rise in estrogen may only be elevated for hours or at most a couple of days.

Circulating progesterone concentrations have been used to identify immature (i.e., pre-pubertal) toothed whales. Serum concentrations have generally been less than 1 ng/ml (Table 6.1) in *Stenella coeruleoalba* (Striped dophin; Yoshioka *et al*. 1989), *Phocoenoides dalli* (Dall's porpoise; Temte and Spielvogel 1985), *Tursiops truncatus* (Sawyer-Staffan *et al*. 1983), *Steno bredanensis* (Rough-toothed dolphin; West 2002), *Delphinapterus leucas* (Beluga or White whale; Katsumata *et al.* 2006a) and *Globicephala macrorhynchus* (Short-finned pilot whale; Yoshioka *et al*. 1989). In the baleen whales, similar relationships have been reported between progesterone and sexual maturity. All species for which progesterone concentrations have been reported have had <1 ng/ml in the circulation of immature female whales (Table 6.1), including *Balaenoptera borealis* (Sei whales; Kjeld *et al*. 2003), *Balaenoptera physalus* (North Atlantic fin whale; Kjeld *et al*. 1992), *Balaenoptera acutorostrata* (North Atlantic minke whale; Kjeld *et al*. 2004), *Balaenoptera bonaerensis* (Yoshioka *et al*. 1994), and *Balaena mysticetus* (Bowhead whale; Kenny *et al*. 1981).

Serum concentrations of gonadotropins secreted from the pituitary, i.e., FSH (follicle stimulating hormone) and LH (luteinizing hormone), were measured by Schneyer *et al.* (1985) for captive and free-ranging *Tursiops truncatus*. Both FSH and LH levels were significantly elevated in females (0.24+/−0.08, 0.39+/−0.20 ng/ml) when compared to males (0.20+/−0.07, 0.35+/−0.16 ng/ml), and adults and pubescent animals had higher LH levels than juveniles. Further, Demski *et al.* (1990) demonstrated immunoreactivity for the hypothalamic hormone LHRH (luteinizing hormone releasing hormone) in the terminal nerve cells of the brain of *Tursiops truncatus* and *Delphinus delphis* (Common dolphin) by immunohistochemistry. Unfortunately, information regarding reproductive activity and circulating levels of LHRH is not known for cetaceans.

Plasma and pituitary concentrations of FSH and LH in baleen whales were measured by Suzuki *et al*. (2001) for *Balaenoptera bonaerensis* during the feeding (non-breeding) season. Pituitary weight was not significantly different between sexes; however, pituitary FSH and LH concentrations varied by sex and age. Specifically, pituitary FSH and LH concentrations were higher in females than in males, and higher in mature whales versus immature whales.

6.2.2 Estrous Cycles

6.2.2.1 Spontaneous vs. Induced Ovulations

Some controversy still exists as to whether or not all toothed whales are spontaneous or induced ovulators. Spontaneous ovulators differ from induced

Table 6.1 Reproductive characters of odontocete and mysticete cetaceans

Common name	*Scientific name Genus and species*	*Prepuberty progesterone concentrations ng/ml*	*Age at onset of maturity*	*Estrous cycle length days*	*Seasonally polyestrous*	*Anestrous periods*	*Pseudo-Pregnancy*	*Length of gestation months*	*Pregnancy progesterone concentrations ng/ml*	*Senescence*
Odontocetes										
Spotted dolphin	*Stenella attenuata*		9-11					15		
Rough-toothed dolphin	*Steno bredanensis*	0.38	10-17					?	3-52	
Bottlenose dolphin	*Tursiops truncatus*	<1.0	5-10	17-35	Yes	Yes	Yes	12		
Striped dolphin	*Stenella coeruleoalba*	0.82	9					12	30-40	
Spinner dolphin	*Stenella longirostris*	0.26	4-7		Yes	Yes		10-11		
False killer whale	*Pseudorca crassidens*		8-14			Yes	Yes	15-16		
Short-fin pilot whale	*Globicephala macrorhynchus*	<1.0	7-12		Yes			14-15	3-14	Yes
Dall's porpoise	*Phocoenoides dalli*	0.27	4-5					10-11		
Beluga	*Delphinapterus leucas*		4-7					14-15	3-32	

Table 6.1 Contd. ...

Table 6.1 Contd. ...

Killer whale	*Orcinus orca*	<1.0	8-9	41.6	Yes	Yes	17		Probably
Risso's dolphin	*Grampus griseus*		7-9		Yes		13-14		
Pacific whitesided dolphins	*Lagenorhynchus obliquidens*	0.26	7		Monoestrous	Yes	10-12	8-27	
Common dolphin	*Delphinus delphis*	0.44	5-12		Yes		10-11		
Mysticetes									
Fin whale	*Balaenoptera physalus*	<1.09 nmol/l	5-8				11-12		
Sei whale	*Balaenoptera borealis*	<1.09 nmol/l	8				11		
Minke whale	*Balaenoptera acutorostrata*	0.37 (1.6 ng/g) blubber	6-8				10	In blubber 107.7 ng/g	
Humpback whale	*Megaptera novaeangliae*		5				11-12		
Bowhead whale	*Balaena mysticetus*		12-20				13-14		
Northern right whale	*Eubalaena japonica*								Possibly

ovulators in that the induced ovulators typically require intravaginal physical stimulation for ovulation to occur. The spontaneous ovulators do not require any stimulation other than a necessary LH/FSH surge, followed by a rise in estradiol, with ovulation being the sequel.

Early studies interpreted ovulation in *Tursiops truncatus* and *Pontoporia blainvillei* (La Plata dolphin) to be occurring as a reflex to copulation, i.e., induced-ovulation (Harrison and Ridgway1971; Harrison *et al*. 1972; Harrison 1977; and Harrison *et al*. 1981). Since then, numerous researchers have examined reproductive tracts and measured reproductive hormones in various cetaceans to determine whether a given species is an induced ovulator or a spontaneous ovulator. To date, *Pseudorca crassidens* (False killer whale), *Orcinus orca* (Killer whale), *Grampus griseus* (Risso's dolphin) and *T. truncatus*, have been identified as spontaneous ovulators (Atkinson 2000; Atkinson *et al*. 1999; Robeck *et al*. 1993; Kirby and Ridgway 1984; Sawyer-Steffan *et al*. 1983; Combelles 1995), indicating that earlier studies were probably incorrect. In *T. truncatus* a pre-ovulatory LH surge was detected and 2 to 7 ovulations per annum were measured at approximately 1 mo intervals (Yoshioka *et al*. 1986). A similar LH surge also was detected in *Orcinus orca* with ovulation occurring about 2 d later (Robeck *et al*. 1993). The maximum circulating progesterone concentration is reported to be 40 ng/ml in *T. truncatus ponticus* (Black Sea bottlenose dolphin) within ½ week following spontaneous ovulation (Ozharorskaya 1990).

Relaxin has been identified in at least two cetacean species, *Balaenoptera edeni* (Bryde's Whale) and *Balaenoptera bonaerensis* (Antarcitc minke whale) (Schwabe *et al*. 1989). Relaxin came from active corpora lutea. It was structurally similar in both species and cross-reacted with porcine relaxin antibodies (Schwabe *et al*. 1989).

6.2.2.2 Estrous cycle lengths

Estrous cycle lengths have best been described for *Orcinus orca* and *Tursiops truncatus*. The estrous cycle length of captive *O. orca* was first reported to be 6-7 weeks (Walker *et al*. 1988), but was later refined to a mean of 41.6 d (Robeck *et al*. 1993). Of this period, the luteal phase, which is dominated by progesterone, is 7-19 d in length and the follicular phase, which is increasingly dominated by estrogen, lasts 6-17 d (Robeck *et al*. 1993). The length of the estrous cycle in *T. truncatus* is 17-35 d (Kirby and Ridgway 1984; Yoshioka *et al*. 1986; Robeck et al. 2005a). It is divided almost equally (2 wk each) into follicular and luteal phases, with exact determination of each phase dependant on the frequency of sampling (Yoshioka *et al*. 1986). Estrous cycle length for *Delphinapterus leucas* was 36.7 ± 3.9 days based on body temperature and progesterone concentrations, which were positively correlated (Katsumata *et al*. 2006a).

6.2.2.3 Seasonality

Many toothed dolphins are reported to be seasonal breeders. In *Tursiops truncatus*, progesterone levels consistent with ovulation occurred from early

spring to mid-autumn, with most occurring during the summer months (Ozharovskaya 1990). No ovulations were reported in winter (Yoshioka *et al*. 1986), although births have occurred throughout the year (Kirby 1990). *Pseudorca crassidens* are reported to be seasonally polyestrous with ovulations mainly occurring in spring and summer (Atkinson *et al*. 1999). The exception to the seasonality of estrous is with captive *Orcinus orca* that reportedly ovulate throughout the year (Robeck *et al*. 1993).

6.2.2.4 Anestrus

Anestrus has been detected for most cetacean species for which the sex steroids have been monitored for extended periods of time. Anestrus is characterized by a long period of little or no measurable levels of sex steroids followed by measurable ovarian activity. Anestrus has been documented many times in *Tursiops truncatus* with durations lasting up to 2 yr in captive dolphins (Kirby and Ridgway 1984; Wells 1984, Yoshioka *et al*. 1986). In *Pseudorca crassidens*, anestrous periods were reported to be 3-10 mo in duration (Atkinson *et al*. 1999). In *Orcinus orca*, anestrous periods were reported to vary in length and did not appear to be linked to environmental, social, or nutritional cues (Robeck *et al*. 1993).

6.2.3 Gestation

Progesterone is the predominant hormone responsible for sustaining pregnancy. Circulating concentrations of >3 ng/ml of progesterone are commonly used as a diagnostic indication of pregnancy in *Tursiops truncatus* (Sawyer-Steffan *et al*. 1983). Similarly, estrogen concentrations were found to increase from 23 pg/ml at 8 d post-mating to 90 pg/ml at the end of gestation (Sawyer-Steffan *et al*. 1983). Katsumata *et al*. (2006b) reported an initial rapid increase in serum progesterone concentrations in *Orcinus orca*, followed by a gradual decline throughout the remainder of gestation. Associated with the increased progesterone concentrations were increased basal body temperatures, which subsequently decreased 0.8 ºC in the 5 days prior to parturition (Katsumata *et al.* 2006b).

Although progesterone is credited with being the primary hormone that sustains pregnancy, its primary source is questionable. In many species, the corpus luteum is the primary gland producing progesterone. In addition, the placenta is known to be a steroidogenic organ in many species. The *Orcinus orca* placenta was described in detail by Bernirschke and Cornell (1987), and a *Megaptera novaeangliae* (Humpback whale) placenta opportunistically collected at parturition was analyzed for its progesterone content in the tissue (Silvers *et al*. 1997). Neither of these studies was designed to investigate the steroidogenic abilities of the placental tissue, leaving the question open as to its endocrine role.

Sex steroids are lipophilic and as such may concentrate in fatty tissues. Considering this trait along with the newly gained proficiency for collecting blubber and muscle specimens from free-ranging whales, researchers may have an innovative means for investigating the reproductive endocrinology in

species that are notoriously difficult to study. In *Balaenoptera acutorostrata*, an increase in blubber progesterone concentrations between immature or anestrous whales (1.6 ± 0.3 ng/g blubber) and pregnancy whales (107.7 ± 18.2 ng/g blubber) has been reported (Mansour *et al*. 2002). Further, Yoshioka *et al*. (1994) measured progesterone in serum and muscle of *B. bonaerensis* and reported a 50-fold difference between pregnancy and non-pregnancy whales. Eventually, such measures may be used to determine gestational stage; although, thus far, there are few reports of such attempts. Mansour *et al*. (2002) reported a positive correlation between blubber progesterone concentrations and fetal girth measurements in *B. acutorostrata*. Similarly, Yoshioka *et al*. (1994) attempted to correlate serum or muscle progesterone with fetal growth in *B. bonaerensis*. They chose to let fetal length be the parameter for fetal growth, as it has been most commonly reported; however, they found no relationship.

Pseudopregnancy has been reported in a few cetacean species. In *Tursiops truncatus*, it is reported to last 5-6 mo (Yoshioka *et al*. 1986). In a captive, non-pregnancy *Pseudorca cressidens,* it has been reported to have lasted 3-10 mo (Atkinson *et al*. 1999). Combelles (1995) also reported pseudopregnancy in *Grampus griseus*.

6.2.4 Lactation

The sex steroids can easily be measured in cetacean milk, with most of the progesterone found not in the milk fat, but in the milk solids (West *et al*. 2000). Most of this work was done on captive *Tursiops truncatus* where serum and milk concentrations were highly correlated (West *et al*. 2000). Further, *T. truncatus* may lactate for years and thus researchers endeavoured to determine if dolphins can conceive during lactation. West *et al*. (2000) tracked the gestations of several *T. truncatus* and simultaneously monitored lactation through collection of milk samples. They found that conception can and does occur during active lactation, and during those gestations, milk concentrations of progesterone ranged from 8-46.5 ng/ml.

6.2.5 Senescence

Senescence, or the phase in a female's reproductive life history when the ovaries cease to produce viable ova, occurs in several mammalian species. Senescence is ultimately confirmed by the absence of follicles in the ovaries; however in some species, a change in the form of estrogen can be indicative of senescence. Marsh and Kasuya (1984) reported that senescence probably occurs in *Orcinus orca* and *Globicephala macrorhynchus* (Short-finned pilot whales). Additionally, Hamilton *et al*. (1998) reported that it possibly occurs in *Eubalaena glacialis* (North Atlantic right whales).

6.2.6 Thyroid Hormones

Thyroid hormones have been measured in only a few species of odontocetes (St. Aubin and Geraci, 1989; West 2002; S.A., unpublished data). Thyroid hormones are generally secreted in seasonal patterns with high circulating

concentrations associated with somatic growth and low concentrations with reproductive activity. Preliminary measurements on pregnancy *Tursiops* indicated that some dolphins tended toward hypothyroidism. Atkinson (unpublished data) has found that hypothyroid dolphins are less likely to successfully deliver a breach birth or large calf, or experienced dystocia than dolphins whose circulating thyroid hormone concentrations were within a 'normal' range. Further, hypothyroid dolphins tend to have a greater incidence of stillbirths (S. A., unpublished data).

6.3 MALE ENDOCRINOLOGY

To date, information on male reproductive endocrinology remains sparse unlike their female counterparts. This section focuses on male reproductive endocrinology and will cover: 1) male sexual maturity and hormones and 2) seasonal changes of endocrinological activity in sexually mature males. Spermatozoa are produced by the testes, and cetacean spermatozoan morphology has been previously described by Fleming *et al.* (1981) for *Tursiops truncatus*; Miller *et al.* (2002) for *Orcinus orca* (killer whale), *Lagenorhynchus obliquidens* (Pacific white-sided dolphin) and *Delphinapterus leucas* (beluga), and Kita *et al.* (2001) for 16 cetacean species, including baleen and toothed whales. These published reports, along with new observations, are reviewed in Chapters **8** and **9** of this volume.

6.3.1 Sexual Maturity

Several different methods have been used to determine sexual maturity, including correlation of standard body morphometrics with gonadal morphometrics along with histological examination of tissue samples collected from the testes and epididymes (Perrin and Reilly 1984). Kasuya and Jones (1984), Kasuya and Marsh (1984), and Kato (1986) found relationships among testis weight, diameter of seminiferous tubules and presence of spermatozoa for *Phocoenoides dalli*, *Globicephala macrorhynchus,* and *Balaenoptera bonaerensis*, respectively. With the use of RIA techniques, circulating reproductive hormone levels ranging from picograms (10^{-12} g) to nanograms (10^{-9} g) can be measured and compared to histological characteristics and ultimately correlated with fertility.

6.3.1.1 Toothed whales

Harrison and Ridgway (1971) reported characteristics of testes of immature and mature captive *Tursiops truncatus.* They demonstrated that an immature animal with a body length of 230 cm and testes weights of 29.1 g (left) and 31.1 g (right) had plasma testosterone levels from 0.12 to 0.82 ng/ml during January to September. Additionally, they found that a male with a body length of 260 cm and testes with little spermatogenesis had testosterone hormone levels of 0.27 to 3.92 ng/ml during March to November. Finally, they found that a mature male with testes weights of 532 g (left) and 375 g (right) had 4.91 ng/ml testosterone in January.

The relationships between testicular development (increase in testis weight, diameter of seminiferous tubules, and density of spermatocytes) and testosterone concentrations in serum/plasma and testicular tissue have been studied in several species. Desportes *et al.* (1994) reported that a testis weight of 2 kg or greater in a mature male was positively correlated with plasma testosterone levels in *Globicephala melaena* (Long-finned pilot whales). Contrastingly, Yoshioka (1991) found a linear relationship between testis weight and serum testosterone levels during the immature and early stages of sexual maturation in free-ranging *Phocoenoides dalli*, but no significant relationship once testis weight reached 40 g or more (equating to sexual maturity; Kasuya and Jones, 1984) (Fig. 6.1). Similar findings were reported by Kita *et al*. (1999) and Kita (2001) who analyzed serum and testis testosterone concentrations in free-ranging *Globicephala macrorhynchus* and *Grampus griseus*. Kita *et al*. (1999) first reported testis testosterone concentrations according to reproductive status in cetaceans. Serum and testis concentrations of testosterone in *G. macrorhynchus* ranged from 0.27 to 22.0 ng/ml and from 14.4 to 352.9 ng/g tissue, respectively. In immature animals, testosterone levels were below 1 ng/ml for serum and 14.4 to 154.9 ng/g for testis, whereas in mature males they ranged from 1.40 to 22 ng/ml for serum and 33.0 to 352.9 ng/g tissue for testis, respectively. Kita *et al.* (1999) concluded that both serum and testis testosterone were positively correlated with testis weight and seminiferous tubular diameter in immature and maturing males, but no significant relationships were observed after sexual maturity (Fig. 6.2). Also,

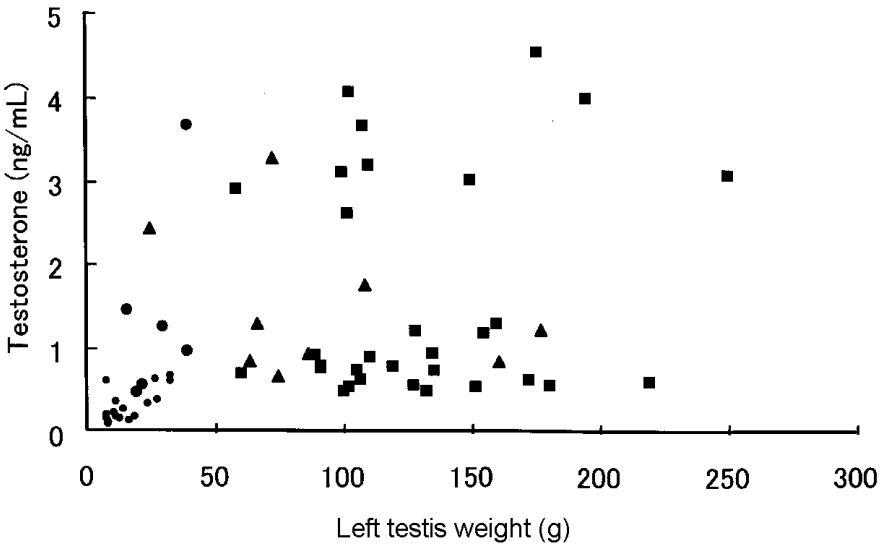

Fig. 6.1 Relationship between left testis weight and serum testosterone levels in *Phocoenoides dalli* taken in the northern North Pacific. Small and large circles, triangle and square indicate immature, early maturing, late maturing and sexually mature, respectively. Original.

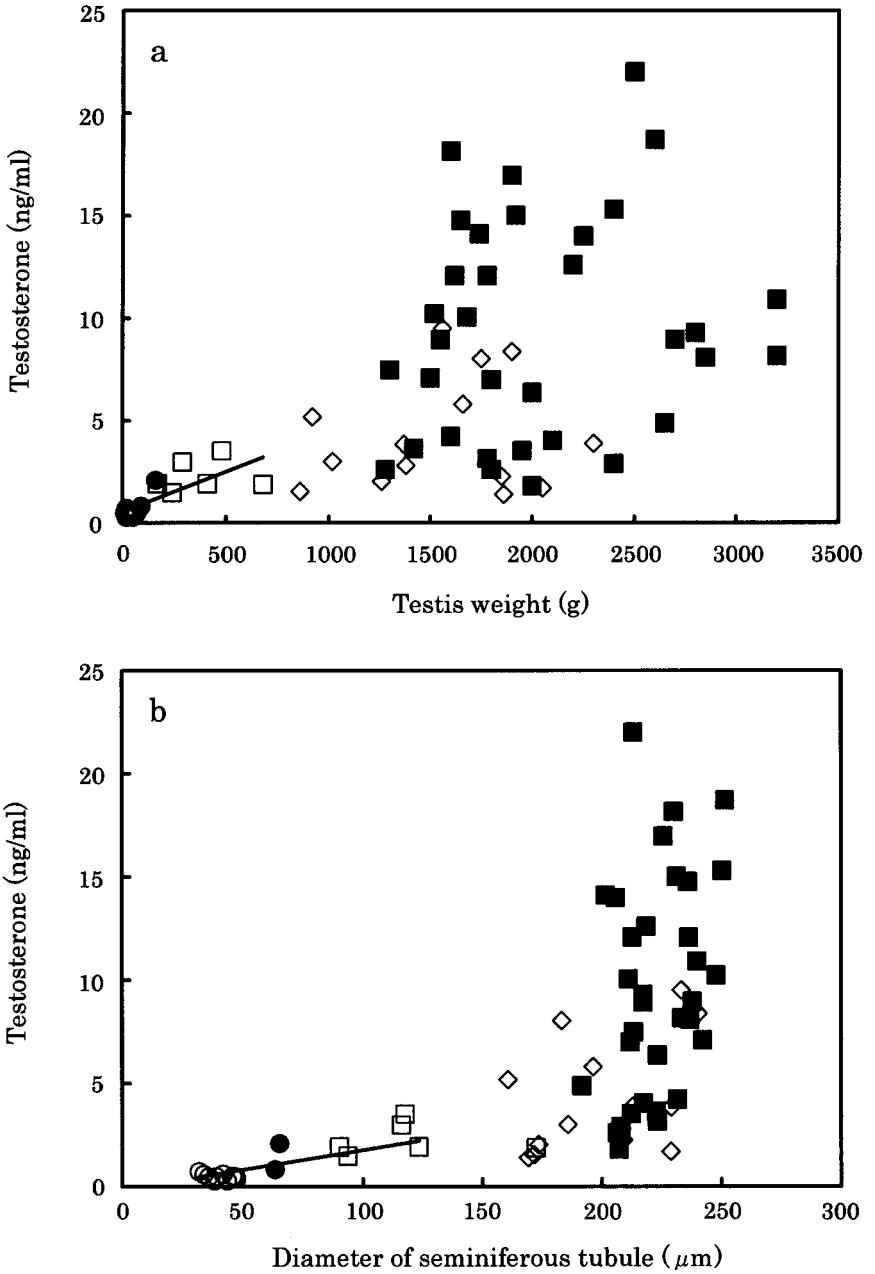

Fig. 6.2 Relationship between serum concentration of testosterone and testis weight and the diameter of seminiferous tubules in southern-form *Globicephala macrorhynchus* (short-finned pilot whale) off the Pacific coast of Japan. Open circle: immature; closed circle: early maturing; open square: late-maturing; closed square: mature (spring); open diamond: mature (fall). S.K., unpublished figure.

testosterone concentrations and density of germ cells (spermatozoa, spermatids and spermatocytes in seminiferous tubules) in mature individuals were not correlated; i.e., testosterone concentration varied among individuals even when testicular size and seminiferous tubular diameter did not.

Høier and Heide-Jørgensen (1994) measured serum testosterone levels in free-ranging *Delphinapterus leucas* from West Greenland. They found that serum testosterone levels in mature males were 4.41 nmol/l (1.27 ng/ml), which were higher than those in immature animals (0.96 nmol/l or 0.28 ng/ml). Similarly, Robeck *et al.* (2005b) measured serum testosterone levels in captive *D. leucas*. They found that the levels of testosterone secretion in older males (> 8 yr of age, 5.0 ng/ml) were elevated compared to those of younger males (< 8 yr of age, 0.95 ng/ml), but only during the interval from January to April.

Robeck and Monfort (2005) characterized male *Orcinus orca* in captivity. They classified reproductive state and age based on serum testosterone levels. Juvenile males (1 to 7 yrs) had serum testosterone levels of 0.13 +/– 0.20 ng/ml, pubertal males (8 to 12 yrs) had levels of 2.88 +/– 3.20 ng/ml, and mature males (=13 yrs) had levels of 5.57 +/– 2.90 ng/ml.

6.3.1.2 Baleen whales

In baleen whales, Yoshioka (1991) reported serum testosterone levels of 196 male *Balaenoptera bonaerensis* during the non-breeding season of the species. Almost all of the males had serum testosterone levels lower than 1 ng/ml, which suggested inactive testes. Figure 6.3 shows distribution of serum testosterone levels related to body length. When body length of *B. bonaerensis* increased over 7 m, higher hormone levels can be seen; however, ca. 67 % of individuals had testosterone levels below the detection limit of the assay (0.06 ng/ml) even when body lengths were greater than 7.9 m (i.e., the length at sexual maturity; Kato, 1990).

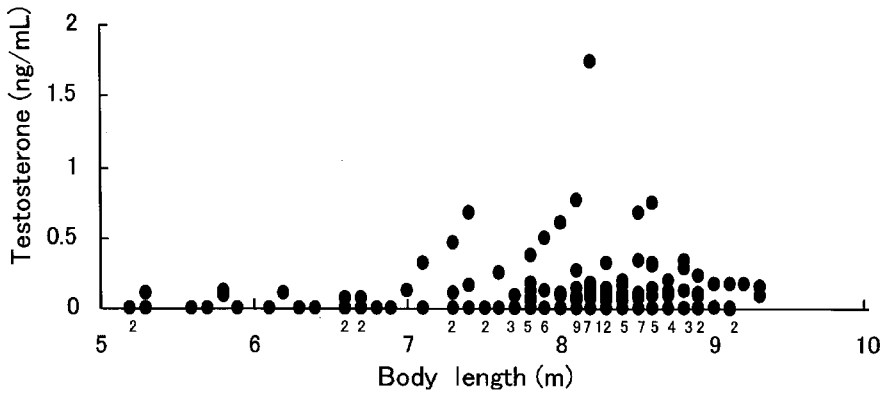

Fig. 6.3 Serum testosterone concentrations and body length in male *Balaenoptera bonaerensis* (Antarctic minke whale). Closed circle on the X axis and figures indicate levels below detection limit of the assay (=0.06 ng/ml) and number of individuals, respectively. Original.

There is limited information regarding urinary steroid hormone levels in male cetaceans. Urine samples from North Pacific *Balaenoptera acutorostrata* were analyzed by T. Iwasaki (pers. comm.) to obtain information on sexual maturity and reproductive status as a supplement to gonad data. Higher (>20 ng/mg Cr) urinary testosterone levels were detected in males of 650-840 cm in body length or males having testis of 250-1700 g.

Plasma and pituitary concentrations of FSH and LH in baleen whales were measured by Suzuki *et al.* (2001) for *B. bonaerensis* during the feeding season. While pituitary weight was not significantly different between sexes, pituitary FSH and LH levels were higher in females than in males, and in mature whales than in immature ones. Plasma levels of FSH and testosterone were not significantly different between immature and mature males but the pituitary FSH and LH levels were positively correlated with maturity. Additionally, testis weight and plasma testosterone levels were positively correlated in mature males.

In both baleen and toothed whales, circulating testosterone levels in sexually mature males are higher than those of immature males, which suggests regulation of testicular function by testosterone, similar to terrestrial mammals. Further, it can be surmised that testosterone is a useful indicator for estimating sexual maturity of male cetaceans. Nevertheless, low testosterone levels do not necessarily equate to sexual immaturity (i.e., when considering variations with breeding season), whereas higher levels are consistent with sexually mature or maturing whales.

6.3.2 Seasonal Patterns

6.3.2.1 Toothed whales

Data on seasonal changes in testosterone concentrations of captive *Tursiops truncatus* have been accumulated from various locations. Harrison and Ridgway (1971) reported a 2-yr profile of plasma testosterone concentrations in one fertile male and showed higher levels during May, June, October, and November with the concentrations ranging from below 1 to 25 ng/ml. Later, Schroeder and Keller (1989) analyzed serum testosterone levels and semen characteristics over 28 mo for a 19-yr-old mature *T. truncatus* trained to ejaculate on command. Testosterone levels ranged from 1.1 to 54.4 ng/ml with seasonal variation and a peak in June. Peak sperm density and volume coincided with the peak period of female breeding activity, but at a time when serum testosterone levels were lowest (60 d after the peak of testosterone). Katsumata *et al*. (1994) also reported seasonal changes in serum testosterone in a sexually mature male *T. truncatus* (body length of 292 cm) throughout a 7-yr study period (i.e., until the animal's death). They noted higher serum testosterone levels in spring compared to fall. Further, it was found that peak serum testosterone concentrations occurred during the spring (maximum levels 59.2 ng/ml), coincident with estrus in the females housed in the same facility (Yoshioka *et al.* 1986).

Interestingly, Brook (2000) found a positive correlation between testis size measured by ultrasonography and serum testosterone levels from January to June in captive *Tursiops aduncus* (Indo-Pacific bottlenose dolphin). She did not find a similar correlation between testis size and sperm production. Likewise, no correlation was noted between serum testosterone levels and sperm production.

Health status of the animal affects testosterone levels in male dolphins. Plasma testosterone concentrations in captive *Tursiops truncatus* decreased prior to death and during illness, e.g., Lobo's disease (Kirby 1990). A similar decrease was reported by Katsumata *et al.* (1994). They noted that a mature male *T. truncatus* had continuously low serum testosterone concentrations (below 1 ng/ml) throughout the first year in captivity, during which occasional dexamethasone treatments were administered.

Wells (1984) reported a seasonal peak in serum testosterone levels in a captive colony of *Stenella longirostris* (Hawaiian spinner dolphins), suggesting seasonality of reproductive behavior and sperm production in *Stenella* species. Yoshioka (1991) reported that serum testosterone levels in two mature captive *Lagenorhynchus obliquidens* (199 and 217 cm in body length) ranged from 0.22 to 15.3 and from 0.22 to 58.0 ng/ml, respectively, and were elevated only during May to June, which coincided with greater semen volume and higher sperm concentration (M.Y. unpublished data).

For *Orcinus orca*, Robeck *et al.* (1993) noted that serum testosterone levels tended to be higher between March and July and lowest in October. Katsumata *et al.* (1999) measured serum testosterone in two animals in captivity every 2 wk for 4-5 yr. The hormone levels fluctuated throughout the years and seasonal patterns were not consistent, suggesting that *O. orca* were fertile throughout the year. Similar findings were recently reported by Robeck and Monfort (2005).

In *Delphinapterus leucas*, Calle *et al.* (2000) showed a seasonal pattern of testosterone secretion with the lowest mean serum levels in October (0.9 ng/ml) and the highest 6 mo later in March (4.95 ng/ml). Robeck *et al.* (2005b) further investigated seasonal changes in captive *D. leucas* and found that testosterone concentrations in older males (over 8 yr of age, 5.0 ng/ml) were significantly elevated from January-April, with the highest testosterone concentrations (6.2 +/− 4.9 ng/ml) occurring from January-March and the lowest concentrations (1.1 +/− 1.0 ng/ml) from August-September. In contrast to testosterone concentrations observed in other seasonally reproductive cetaceans [i.e., *Stenella longirostris* (Wells 1984) and *Lagenorhynchus obliquidens* (Yoshioka 1991)], but similar to cetaceans that produce spermatozoa throughout the year [e.g., *Tursiops truncatus* (Schroeder and Keller 1989) and *Orcinus orca* (Robeck *et al.* 1995; Katsumata *et al.* 1999; Robeck and Monfort 2005)], monthly testosterone concentrations of *D. leucas* never dropped below 1 ng/ml, suggesting that some degree of spermatogenesis may occur throughout the year (Robeck *et al.* 2005b).

Kita *et al*. (1999) compared serum and testis testosterone levels to hunting seasons (spring vs. fall) in the southern form of *Globicephala macrorhynchus* in

the western North Pacific (Fig. 6.2). They found that testosterone levels were higher in the spring. This would correlate with peak of reproductive activity.

In mammals, testosterone has a diurnal rhythm of secretion. Judd and Ridgway (1977) collected *Tursiops truncatus* blood samples every 20 min for 24 h. However, no diurnal pattern was detected in circulating testosterone concentrations.

Recently, a rapid, accurate, reproducible and less invasive assay using high performance liquid chromatography-mass spectrometry (LC-MS) has been developed by Hogg *et al.* (2005) for measuring testosterone. Using this assay they measured endogenous testosterone from saliva (9.73-23 ng/ml) and blow (14.71-86.20 ng/ml) samples of captive *T. truncatus*. Perhaps future studies can implement this technique to aid in elucidating the cyclicity and rhythmicity of testosterone production.

6.3.2.2 Baleen whales

Seasonality of testosterone levels is poorly defined for baleen whales. This is primarily due to limited blood sampling efforts, all of which have been made during the feeding seasons. Ólafsson and Kjeld (1986) indicated that for *Balaenoptera physalus* (Fin whales) in Icelandic waters serum testosterone concentrations rose significantly during the short summer hunting season. Later, Kjeld *et al.* (1992) accumulated more samples from the same whale population and reported serum testosterone levels for 352 male *B. physalus*. The males were divided into two groups: those with concentrations of 0.1 nmol/l (=0.03 ng/ml) or less and those with mean concentrations of 2.00+/−3.78 nmol/l (=0.58+/−1.09 ng/ml). The mean testosterone concentrations increased more than four-fold between June and August as the hunting season proceeded. Similar seasonal changes have been described in *Balaenoptera borealis* in the North Atlantic Ocean (Kjeld *et al.* 2003).

Mogoe *et al.* (2000) investigated changes in testicular morphology and plasma hormone levels of *B. bonaerensis*. Testosterone, estradiol-17 beta, and LH concentrations that were measured by enzyme-immunoassay (EIA) for 62 males having testes weighing over 400 g each, the threshold testis weight of sexual maturity for the species. They did this to describe seasonal variation in male reproductive activity in whales collected during the feeding season (December-March). A reduction in testicular function was found during the summer season (December to March). During February, body length and body weight remained steady, but decreases in testicular weight, epididymal weight, and testicular volume were recorded and plasma testosterone concentrations declined. Similarly, germ cell numbers significantly decreased throughout the feeding season. These changes reflected the percentage of spermatozoa present in the vasa deferentia. Specifically, motile spermatozoa were observed in December.

Kjeld *et al.* (2004) reported seasonal changes of testosterone in *Balaenoptera acutorostrata* from fresh postmortem blood samples collected from 83 males caught in the North Atlantic during May-September. The frequency distribution of male serum testosterone did not show any group-specific

distribution during the hunting season. The mean serum testosterone value for the males was 0.63 +/– 0.13 nmol/l (=0.18+/–0.04 ng/ml). Contrary to earlier reports on *B. bonaerensis*, serum testosterone levels rose during the hunting season in mature males of *B. physalus* and *B. borealis* in the Atlantic Ocean (Kjeld *et al*. 1992; Kjeld *et al*. 2003).

Watanabe *et al.* (2004) conducted studies to obtain relationships among serum testosterone, estradiol-17 beta, FSH, and LH concentrations and histology of seminiferous tubules in captured *Balaenoptera acutorostrata* (n=39 for blood samples, n=15 for testes) and *B. edeni* (Bryde's whale) (n=14 for blood samples, n=7 for testes) during the feeding season from May to August in the western North Pacific. Serum testosterone concentrations in 35.9% of *B. acutorostrata* and 57.1% of *B. edeni* were below the detection limit (< 2.5 pg/ml). There were no significant differences in the serum concentrations of estradiol-17-beta, FSH, and LH among immature, mature *B. acutorostrata* and *B. edeni*, except that LH levels of immature *B. edeni* were higher than those of *B. acutorostrata*. Spermatozoa were observed in seminiferous tubules in 2/13 (15%) of mature *B. acutorostrata* and 4/4 (100%) of mature *B. edeni* with low or undetectable testosterone levels. These results indicate that low serum testosterone concentrations reflect inactivity of spermatogenesis in both baleen whales during the feeding season.

In summary, our knowledge on male reproductive endocrinology in cetaceans (baleen and toothed whales) is primarily limited to testosterone concentrations and its changing patterns in relation to sexual maturity and season. Higher testosterone levels are found in mature animals and during the breeding season, similar to terrestrial mammals. There remains a lack of information regarding the physiology of cetacean spermatogenesis. Difficulties experienced during attempted sample collection and *in vitro* experiments have resulted in limited knowledge of the primary metabolic pathway of cetacean testicular development.

6.4 ENVIRONMENTAL IMPACTS ON REPRODUCTION

6.4.1 Social Suppression of Reproduction

Numerous situations with captive cetaceans have suggested that social dynamics influence the behavior and possibly the physiology of dolphins and whales. Most of the reports have been somewhat anecdotal and thus have not lent themselves to publication in peer-reviewed journals. A short synopsis of three situations follows and also see Chapters **8** (specifically, 8.5, Male Mating Strategies) and **13** (Courtship and Mating Behaviour).

In one situation two young known-aged male *Tursiops truncatus* were being raised in a large naturalistic pool with several mature female dolphins. While the males were the same age and approximately the same size, they seemed to mature at different rates. One male consistently appeared dominant and, once mature, sired 3 offspring the following year. The other male did not sire offspring for another 2 yr even though there were no obvious nutritional or

environmental correlates to explain this delay. Further, testosterone concentrations were consistently lower in the male with the latent onset of reproductive activity.

A second scenario occurred with the two *Pseudorca crassidens* in the study published by Atkinson *et al*. (1999). The progesterone concentrations varied greatly between the two individuals, suggesting some sort of environmental suppression, such as the subclinical stress of captivity. However, it also may have been social suppression, possibly working through pheromonal mechanisms that accounted for the lowered circulating progesterone concentrations in one of the female false killer whales relative to the other female (Atkinson *et al*. 1999).

The third scenario of reproduction suppression was reported for *Grampus griseus* (Combelles 1995). Two female *G. griseus* were housed together or in adjacent pens in an enclosed bay. One female routinely ovulated 2-4 times per year, as measured in elevated progesterone concentrations. The other female only ovulated once during a 2-yr sampling period. The possibilities of both social suppression and reproductive senescence were suggested, although no definitive conclusions could be made.

6.4.2 Chemical Impacts on Reproduction

Through food web accumulation, cetaceans may accumulate high levels of various toxins resulting from anthropogenic contaminants. This may be true even for those species that predominantly reside offshore, where presumably there is low exposure to high levels of some toxins, such as organochlorines. Many of the contaminants have toxicological effects, including endocrine disruption, with the target organ being the reproductive tract (Fossi and Marsili 2003). Tissues from several cetacean species have been analyzed for various contaminants, although few studies have linked the tissue concentrations to endocrine function. Subramanian *et al.* (1987) and Marsili *et al*. (1998) have shown that in *Phocoenoides dalli,* captured in the western North Pacific, with increased organochlorine (PCBs and DDE) levels, there were reductions in testosterone concentrations sufficient to suppress spermatogenesis. In addition, *Balaenoptera acutorostrata* have had transformation of epididymal and testicular tissues (Ankley *et al*. 1998), and pseudohermaphroditis has been reported in two male *B. mysticetus* (Marsili *et al*. 2000). Further investigation into the affects of these and other environmental pollutants on cetacean male reproductive endocrinology is needed.

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CHAPTER 7

Ovary, Oogenesis, and Ovarian Cycle

Yutaka Fukui

7.1 OVARY

A detailed description of the morphology and histology of dolphin and whale ovaries and reproductive tracts has been reviewed (Harrison 1969, 1977; Green 1977; Tinker 1988; Schroeder 1990). Ovarian changes with respect to age, season, and reproductive activity in cetaceans have not been fully understood. It is thought that cetacean ovaries are similar to those of other mammals. The ovary is the site of oocyte maturation. Maturation results in morphological changes in the ovary and is regulated by several hormones throughout the ovarian cycle. Folliculogenesis and oogenesis are important events preceding the release of the mature oocyte (ovulation), as well as for *in vitro* oocyte maturation, which is necessary for assisted reproductive technologies.

7.1.1 Morphology

In the toothed whales (Odontoceti), the ovaries are usually smooth externally, while in the whalebone (baleen) whales (Mysticeti), they are plicate and ridged externally and somewhat resemble a bunch of grapes. Detailed descriptions of ovarian morphology were reported in *Balaenoptera musculus* (Blue whale) (Mackintosh and Wheeler 1929), *B. physalus* (Atlantic fin whale) (Mackintosh and Wheeler 1929; Laws 1961), *B. borealis* (Sei whale) (Gambell 1968), *Megaptera novaeangliae* (Humpback whale) (Chittleborough 1954) and *Balaenoptera bonaerensis* (Antarctic minke whale) (Best 1982; Lockyer 1987). The ovaries of immature and mature *B. bonaerensis* are shown in Fig. 7.1. The ovaries of immature whales each of which appears as a somewhat compressed, grooved, "bean-like body." They enlarge as the whale matures. *Balaenoptera bonaerensis* ovaries reach lengths of about 20 cm and weights of over 500 g, whereas *B. musculus* ovaries are reported to weigh as much as 16 kg (Tinker 1988).

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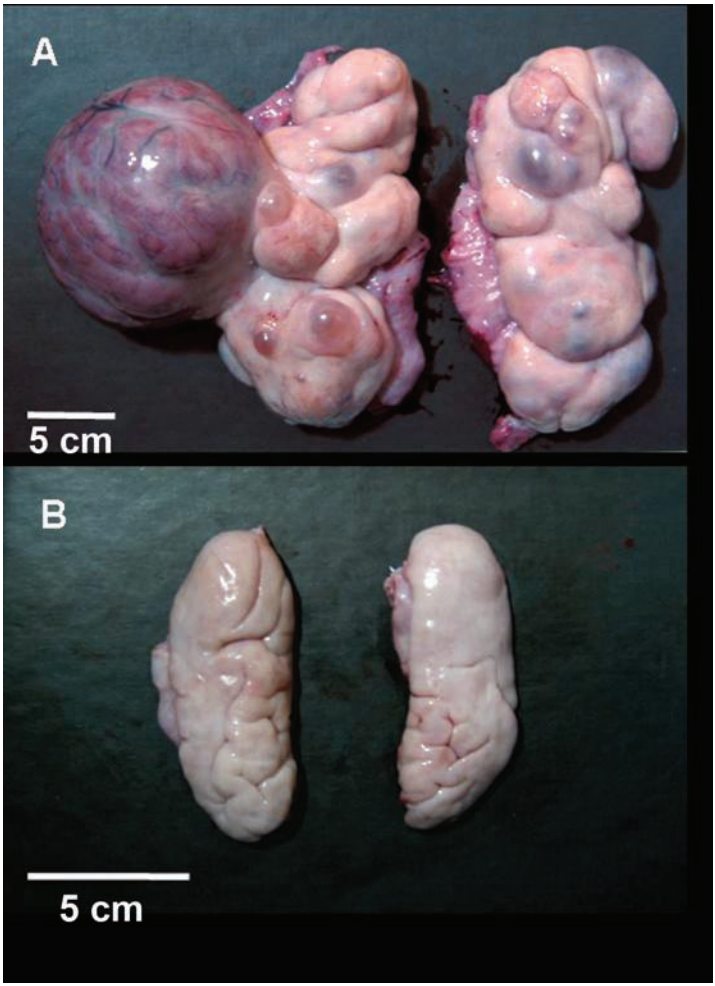

Fig. 7.1 Morphology of *Balaenoptera bonaerensis* (Antarctic minke whale) ovaries. **A**. Mature pregnancy whale. A large corpus luteum in the left ovary and some antral follicles in both ovaries are seen. **B.** Immature whale. No visible follicles are observed. Original.

As in all other mammals, the whale ovary consists of a large number of follicles, each containing a single ovum. A mature spherical follicle (Graafian follicle) may measure 3.8-5.1 cm in diameter. These follicles move slowly to the surface, usually toward the anterior end of the ovary, swell in size, and then release the mature oocyte (ovulate). In whales, the oocytes are released singly. Occasionally two oocytes are released simultaneously which potentially may result in twin young (Tinker 1988). Tetsuka *et al*. (2004) described the morphological and morphometrical changes associated with prepubertal ovarian development in *Balaenoptera bonaerensis*. They described two types of ovarian surface: 1) a smooth flat surface and 2) a surface that has

at least one major furrow and additionally may have a convoluted or wrinkled appearance.

The morphology of paired right and left ovaries was almost identical although preferential growth was noted in some species. Ovarian growth takes place preferentially on the right side in *Balaenoptera bonaerensis* (Tetsuka *et al*. 2004). In mature Mysticeti, both right and left ovaries have been reported to be equally active (Gambell 1968; Lockyer 1987), while in many species of Odontoceti, the left ovary is known to be more active than the right ovary (Ohsumi 1964; Marsh and Kasuya 1992).

7.1.2 Folliculogenesis

Gonadotropins are involved in follicular development and atresia (folliculogenesis). Especially, follicle-stimulating hormone (FSH) is known to induce proliferation and differentiation of granulosa cells from mammalian follicles. During the ovarian cycle in cattle, there are two to three waves of follicular development. Various sizes of follicles are developing and regressing throughout the estrous cycle and even during the non-breeding season in seasonal breeding animals, such as sheep. The dominant follicle (>10 mm in cattle) suppresses the development of neighboring small follicles by secretion of increasing concentrations of estradiol-17β (E_2) and inhibin into the blood vessels (Gibbons *et al*. 1997).

Studies on the relationship between follicular development and hormonal profiles in cetaceans are limited. Ovarian changes with follicular development of *Globicephala macrorhynchus* (Short-finned pilot whales) have been examined in detail by Marsh and Kasuya (1992). They studied follicular development and atresia, corpus luteum (CL) development and regression in 298 specimens. *G. macrorhynchus* begin to ovulate at about 7.5 yr. Ruptured (ovulated) follicles range from 12.5 to 45.0 mm with a mean diameter of 25.1 mm. Large follicles that do not ovulate, degenerate. All follicles studied in *G. macrorhynchus* aged 40 yr or more were atretic (Marsh and Kasuya 1992), similar to what is seen in other mammals. Lockyer (1987) reported that the mean diameter of the largest follicles in immature *Balaenoptera bonaerensis* caught during the feeding season was 6.41 mm. Tetsuka *et al*. (2004) classified ovaries of *B. bonaerensis* into three categories based on follicle type: Type A (25.5%) were ovaries with numerous small follicles less than 5 mm in diameter; Type B (28.7%) were ovaries with 50 to 200 follicles up to 10 mm in diameter; Type C (45.8%) were ovaries where follicles were not visible and only detected by translucent lighting or ovarian palpation, and the diameter of the largest follicle never exceeded more than 10 mm in any ovary. There was a significant association ($P < 0.001$) between body length and incidence of the follicular types.

Real-time ultrasonography is a sophisticated diagnostic imaging method for ovarian morphology, such as follicular development, ovulation physiology, and formation of CL. Robeck *et al*. (1998) used ultrasonography to monitor ovarian follicular changes in *Tursiops truncatus* (Bottlenose dolphin)

after ovulation induction protocols and found that it was possible to serially locate and evaluate superovulated ovaries. Brook (2001) performed ultrasonographic imaging of the ovaries for up to 10 yr in ten female *Tursiops truncatus* and observed small cystic follicles of 2-3 mm diameter in the ovarian cortex. Further, antral follicles up to 4 mm in diameter were occasionally seen during anestrus (Brook 2001). The diameter of follicles just before ovulation has varied among individual *T. truncatus*, ranging from 1.6 to 2.3 cm, but was consistent within individuals. It has been recognized that ultrasonography provides a reliable and repeatable method for examining ovarian changes in dolphins and other Delphinidae including *Delphinapterus leucas* (Beluga) and *Orcinus orca* (Killer whale) (Brook 2001). Robeck *et al*. (2004) determined that follicular growth was slower in *O. orca* compared to *T. truncatus*. Further, Robeck *et al*. (2004) state that endocrine data are essential to determine if ultrasonographically visualized follicles are functional.

Thousands of small follicles, called pre-antral follicles, are contained in mammalian fetal ovaries (Erickson 1966; Tanaka *et al*. 2001). However, information on the regulation of fetal ovarian development is required to understand whale reproductive physiology. The possibility of utilizing small oocytes in primordial follicles for production of mature oocytes by *in vitro* growth culture system has been explored in mice (Eppig 1996), cattle (Miyano 2003) and humans (Abir *et al*. 1997). If successful, a large number of pre-antral follicles in fetal ovaries could be a potential source of oocytes for *in vitro* fertilization (IVF) or other reproductive technologies in whales, as well as in other mammalian species. Muranishi *et al*. (2004) investigated the relationship among the changes in the number of pre-antral follicles (primordial, primary and secondary follicles; Fig. 7.2) and concentrations of FSH, luteinizing hormone (LH) and steroid hormones (P_4, E_2 and androstenedione) in fetal heart, umbilical cord and maternal blood of *Balaenoptera bonaerensis* fetal ovaries. Primordial follicles (mean diameter 36.7 mm), which were smaller than that of primordial follicles (58 mm) in mature *Globicephala macrorhynchus* (Marsh and Kasuya, 1992), had already appeared in a 20 cm fetus, and primary follicles were observed in a 50 cm fetus. Changes in the number of primordial follicles were observed in ovaries of different stage fetuses (fetal length 20-120 cm). In 70 cm fetuses, the number of pre-antral follicles increased rapidly (primordial follicles, 35,840; primary follicles, 1,530). Secondary follicles were present in the 75.5 cm fetus (primordial follicles, 39,560; primary follicles, 3,240; secondary follicles, 160). These pre-antral follicles increased with fetal size up to 160 cm in fetal length. Muranishi *et al*. (2004) concluded that the changes in fetal and umbilical cord blood steroid concentrations coincided with increased number of pre-antral follicles at around 70 cm in fetal length, whereas, the growth and differentiation of primordial and primary follicles were independent of FSH and LH. This study was the first report on the relationship between the change in the number of pre-antral follicles and concentrations of sex hormones in *B. bonaerensis* fetuses. More detailed research is needed on follicular development for all age groups (fetal, calf and adult) of marine mammals.

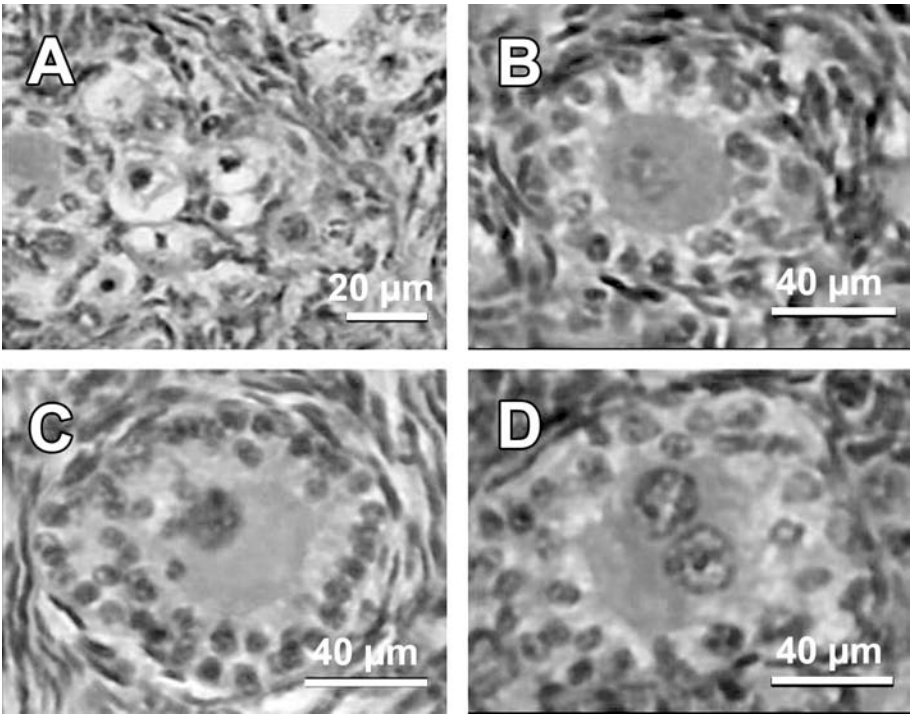

Fig. 7.2 Representative primordial (**A**), primary (**B**), secondary (**C**) follicles in *Balaenoptera bonaerensis* (Antarctic minke whale) fetal ovaries. **D**. A multinuclear follicle. After Muranishi, Y., Sasaki, M., Hayashi, K., Fujihira, T., Ishikawa, H., Ohsumi, S., Miyamoto, A. and Fukui, Y. 2004. Zygote 12: 125-132, Fig. 5.

7.2 OOGENESIS

In mammals, small oocytes grow and reach their final size in the ovary where they mature and are prepared to be fertilized. The process of oocyte maturation is a critical event for the developmental potential of an embryo. In domestic animals, such as cattle and pigs, the proportions of oocytes that exhibit the capacity to resume meiosis and support embryonic development increases gradually with increased oocyte diameter. In bovine oocytes, acquisition of meiotic competence does not occur until the antral follicle stage, when the oocyte diameter is greater than 100 µm. The sizes of immature oocytes (germinal vesicle: GV stage) collected from immature and mature *Balaenoptera bonaerensis* (total oocyte, 198 ± 3.6 and 180 ± 7.9 µm; zona-pellucida, 35.5 ± 2.93 and 32.9 ± 2.9 µm, respectively) were slightly larger than those of bovine immature oocytes (total, 164 ± 4.3 µm and zona-pellucida, 15.5 ± 0.9 µm) (Fig. 7.3). The oocytes first acquire the capacity to undergo germinal vesicle breakdown (GVBD). In metaphase I (M-I), the majority of bovine oocytes exhibit full meiotic competence and can reach metaphase II (M-II) at a diameter of approximately 110 µm. As the follicular diameter increases to

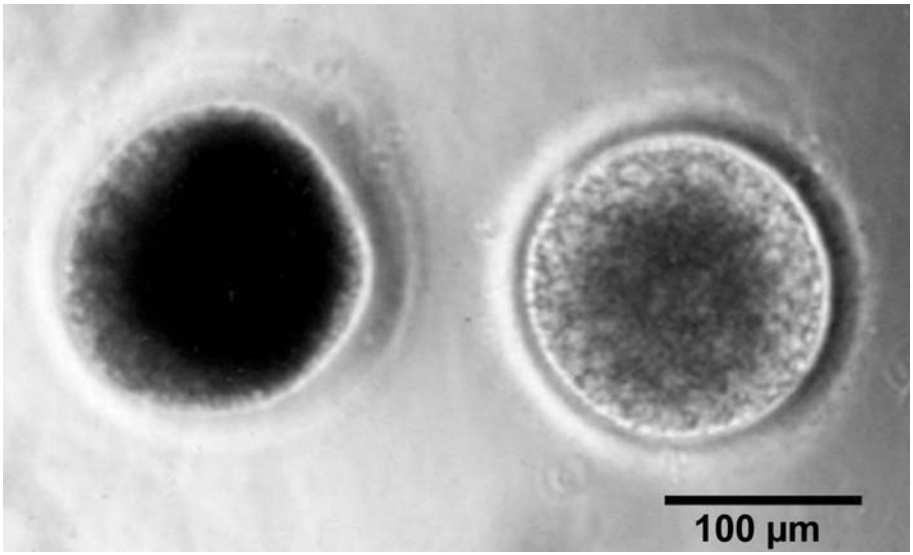

Fig. 7.3 Immature oocytes from prepubertal (left) and adult (right) *Balaenoptera bonaerensis* (Antarctic minke whale). The former is dark and opaque, and the latter's cytoplasm is bright and transparent. After Fujihira, T., Kinoshita, M., Sasaki, M., Ohnishi, M., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2004. Journal of Reproduction and Development 50: 525-532, Fig. 1.

approximately 2 mm and the oocytes increase in diameter from 110 to 120 µm, developmental competency is acquired and the majority of oocytes become capable of supporting fertilization and embryonic development. In follicles larger than 6 mm in diameter, the greatest proportion of oocytes is developmentally competent (Rodriguez and Farin 2004).

Such relationships between follicular and oocyte sizes relating to acquisition of meiotic competence of whale oocytes have not been studied in detail. More information on oogenesis in dolphins and whales is needed for basic research and for application to *in vitro* procedures.

7.2.1 In vitro Maturation of Follicular Oocytes

In mammals, including cetaceans, small oocytes grow and reach their final size in the ovary, where they acquire maturational and fertilizational competence. Most oocytes remain unovulated and degenerate at various stages of follicular development. For fertilization and the subsequent development to embryos, follicular oocytes must have resumed meiosis and reached the M-II stage before ovulation, as in domestic animals. *In vitro* maturation (IVM) of immature follicular oocytes of *Balaenoptera bonaerensis* was first attempted in our laboratory (Fukui *et al*. 1977a). For the IVM culture, several factors such as type of medium, additives (serum, hormones, additional follicular cells) and culture duration were determined. Fukui *et al*. (1977a) estimated oocyte morphology by the degree of attachment of cumulus

cells surrounding the oocyte with different sizes of follicles in immature and mature *B. bonaerensis*. Recovery rates for immature oocytes from follicles of different sizes (small, 1-5 mm; medium, 6-10 mm; large, ≥ 11 mm) were similar in both immature (54.7%) and mature (53.5%) whales, and follicular size did not affect recovery rate. Approximately half the oocytes recovered from small follicles in immature (55.5%) and mature (52.1%) whales were surrounded by at least a few layers of cumulus cells, which could be used for IVM culture. Before IVM culture, 71.7 and 61.3% of oocytes from immature and mature whales, respectively, were at the germinal vesicle (GV) stage. Fukui *et al*. (1977a) also examined the IVM culture conditions [addition of hormones (FSH and E_2), serum types (fetal calf serum and fetal whale serum), and culture duration (3.5 to 5 d)] and reported that the maximum proportion of mature (M-II stage) oocytes after IVM culture was 27.3% by 96 h of IVM culture.

Asada *et al*. (2001) investigated the effects of different concentrations (0, 10 and 20%) of fetal whale serum (FWS) in IVM culture media on nuclear maturation and morphological grade (A or B) of cumulus-oocyte complexes (COC) obtained from prepubertal and adult *Balaenoptera bonaerensis*. Grade A (≥ 5 layers of cumulus cells) COC that were collected from adult whales and cultured in the medium with 20% FWS had 31.8% (n=22) of matured oocytes at M-II stage and 18.2% of the oocytes at anaphase-I (A-I) to telophase-I (T-I) stages. Sexual maturity of the whales and COC grades did not affect the rate of matured oocytes. Furthermore, Asada *et al*. (2001) showed that grade A COC was significantly ($P < 0.05$) higher in cleavage (14.5%) and development to the morula stage (4.2%) after IVF and *in vitro* culture (IVC) than those of grade B COC (2.5 and 0%). Oocytes reaching M-II stage (Fig. 7.4) were fertilized *in vitro* (Fig. 7.5), allowed to develop to the morula stage (Fig. 7.6) and observed (Asada *et al*. 2001). Improvements were achieved by the use of FWS for IVM medium and freshly diluted spermatozoa for IVF to maximize *in vitro* embryo production of *B. bonaerensis* oocytes. Co-culture with cumulus cells or granulosa cells during IVC did not significantly affect cleavage and development after IVF (Fukui *et al*., 1997b; Asada *et al*. 2001). It seems that oocyte quality selected by COC grades is the most important criterion for embryonic developmental capacity of *in vitro* matured and fertilized oocytes. Unfortunately, development to the blastocyst stage has not been observed in our studies. Future studies should focus on the improvement of culture media for whale oocyte maturation and embryonic development *in vitro*.

Recently, Iwayama *et al*. (2005) compared two different hormone-suplemented IVM media (FSH + E_2 and PMSG + hCG) for *Balaenoptera bonaerensis* fresh oocytes using a portable CO_2 incubator. Asada *et al*. (2000) previously investigated the effect of FSH + E_2 and PMSG + hCG in an IVM medium on pronuclear formation and cleavage of *B. bonaerensis* oocytes, but they used frozen-thawed immature oocytes and the influence of the hormones supplemented in IVM media on oocyte maturation was not clarified. Iwayama *et al*. (2005) observed the maximum expansion of cumulus cell mass in the

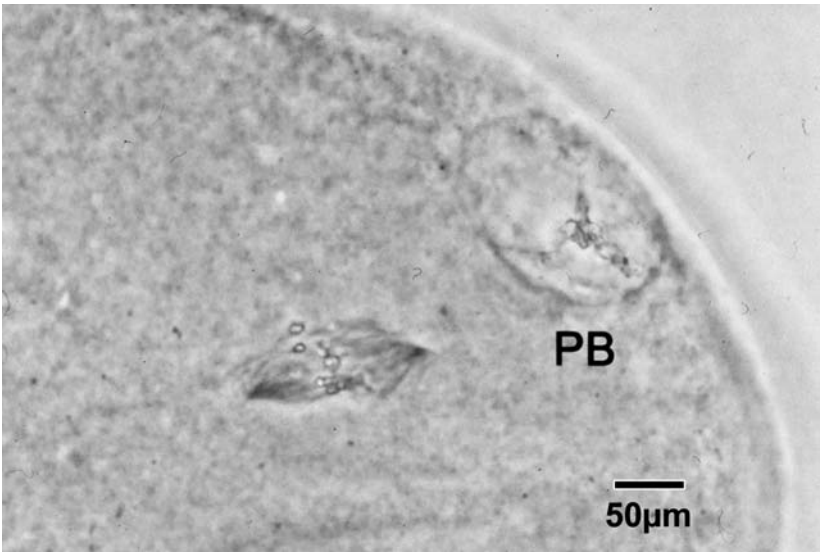

Fig. 7.4. An *in vitro* matured oocyte from an adult *Balaenoptera bonaerensis* (Antarctic minke whale) shows the second metaphase stage with the first polar body (PB) after 120 h culture in the maturation medium containing 20% fetal whale serum. After Asada, M., Tetsuka, M., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2001. Theriogenology 56: 521-533, Fig. 1.

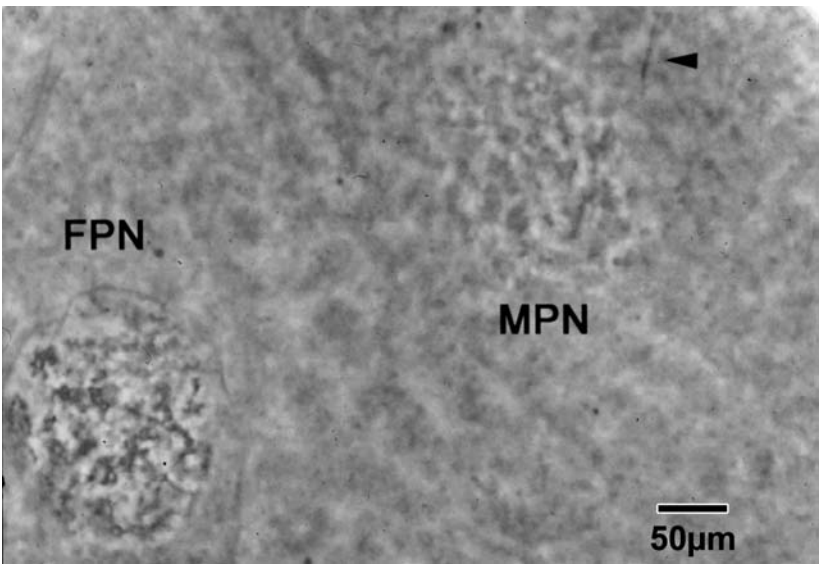

Fig. 7.5 Female (FPN) and male (MPN) pronuclei in the cytoplasm of a *Balaenoptera bonaerensis* (Antarctic minke whale) oocyte observed at 24 h after *in vitro* insemination. A sperm-tail (arrow head) can be seen in the cytoplasm. After Asada, M., Tetsuka, M., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2001. Theriogenology 56: 521-533, Fig. 2.

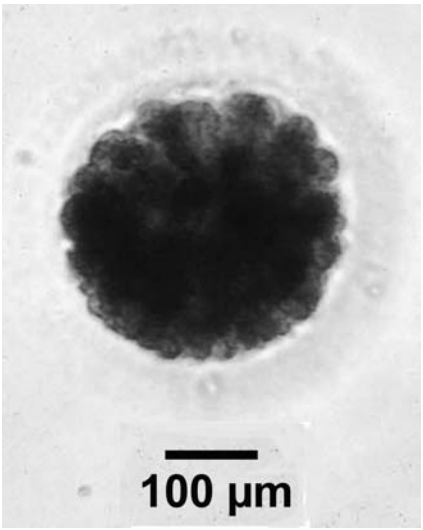

Fig. 7.6 A morula stage embryo derived from an adult *Balaenoptera bonaerensis* (Antarctic minke whale) after *in vitro* maturation and fertilization. The embryo developed for 8 days after *in vitro* insemination followed by co-culture with granulose cells. After Asada, M., Tetsuka, M., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2001. Theriogenology 56: 521-533, Fig. 3

COC cultured in media supplemented with either E_2 + FSH or PMSG + hCG (Fig.7.7). The proportion of matured oocytes cultured in the medium supplemented with FSH + E_2 (26.7%) was significantly ($P < 0.05$) higher than that supplemented with PMSG + hCG (6.9%), although the reason for this was not determined. Furthermore, the proportion of matured oocytes (26.7%) was not increased when compared to previous studies (27.3 and 31.8% for Fukui *et al*. 1977a and Asada *et al*. 2001, respectively).

Another study (Iwayama *et al*. 2004) classified 2,909 *Balaenoptera bonaerensis* COCs into 4 groups by morphology of cumulus cells and the appearance of the cytoplasm of the oocytes: grade A (compact with more than two layers of cumulus cells and homogeneous cytoplasm); grade B (denuded cumulus cells), grade C (expanded cumulus cells), and grade D (degenerated cumulus cells). The proportions of grade A COC that were used for IVM following vitrification and warming were 41.5 and 38.3% for adult and prepubertal *B. bonaerensis*, respectively. The mean numbers of COC collected per ovary were 14.0 and 21.0 for the adult and prepubertal *B. bonaerensis*, respectively, without a significant ($P < 0.05$) difference.

In a preliminary study measuring the osmolarity of whale follicular fluid (wFF), it was found that the osmolarity in wFF (387.9mOsM, n=26) and in fetal serum (363.7mOsM, n=23) of *Balaenoptera bonaerensis* (Fig. 7.8) were much higher than those in cattle and pigs (approximately 300mOsM). Lambertsen *et al*. (1986) described that, as for other cetaceans, serum osmolarity was

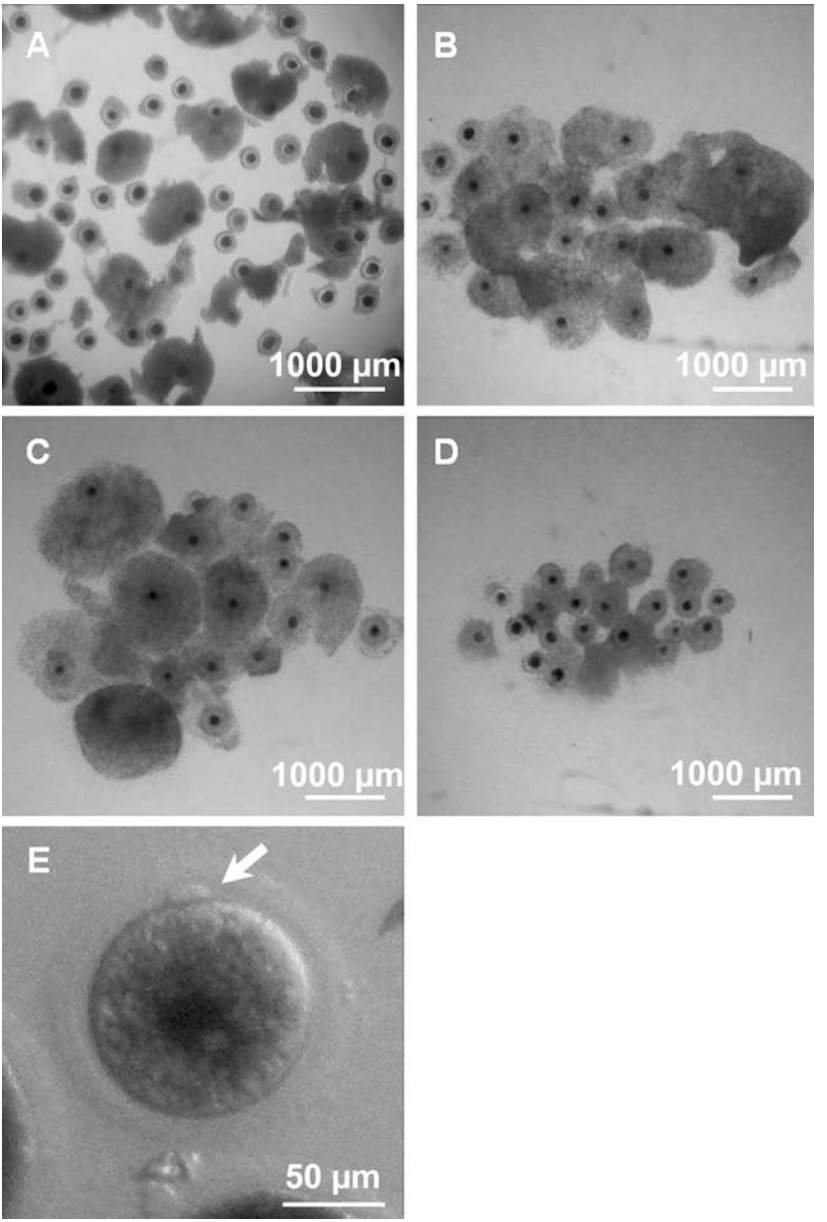

Fig. 7.7 Cumulus-oocyte complexes (COCs) and *in vitro* culture for oocyte maturation of *Balaenoptera bonaerensis* (Antarctic minke whale). **A**. COCs immediately after recovery from follicles. **B**. After *in vitro* maturation (IVM) culture in medium supplemented with FSH + E_2. **C**. After IVM culture in medium supplemented with PMSG + hCG. **D**. After IVM culture in medium with no hormones. **E**. An *in vitro* matured oocyte with the first polar body (arrow) in IVM medium supplemented with FSH + E_2. After Iwayama, H., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2005. Journal of Reproduction and Development 51: 69-75, Fig. 2.

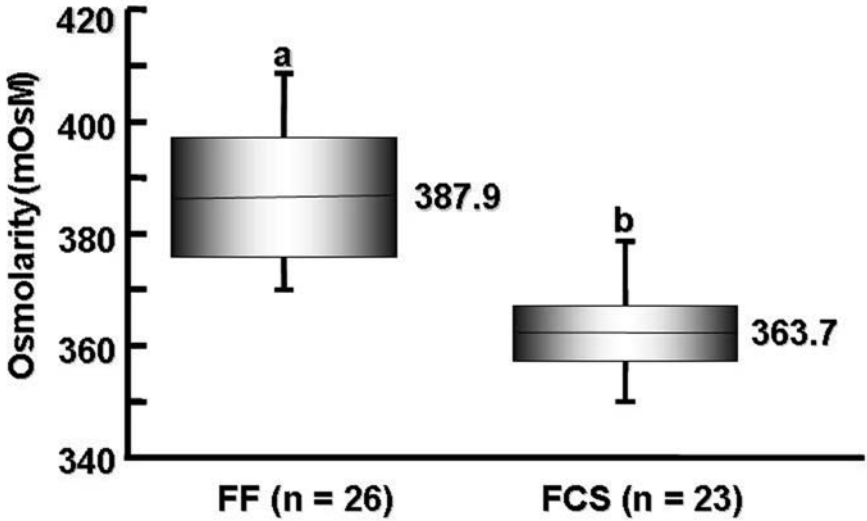

Fig. 7.8 Comparison of the osmolarity of follicular fluid (FF) and fetal cord serum (FCS) in *Balaenoptera bonaerensis* (Antarctic minke whale). Letters a, b indicate significant differences ($P < 0.01$). Original.

distinctly higher in two *B. physalus* (330mOsM and 359mOsM) than in terrestrial mammals (approximately 300mOsM). Interestingly, the osmolarity (470mOsM, n=3) in the ocular secretions (tears) of *T. truncatus* also is higher than that of human and terrestrial mammals (approximately 300mOsM) (Young and Dawson 1992). No information is available concerning the composition of follicular fluid of marine mammals, especially in Mysticeti.

The preliminary measurement of osmolarity of wFF led us to adjust osmolarity of the IVM medium containing 10% wFF to 390mOsM by changing the concentrations of NaCl, KCl, $MgSO_4$ (anhydrous) and $CaCl_2 \cdot 2H_2O$ at a constant ratio with Medium 199 (Iwayama *et al*. 2004). The modified IVM medium with the high osmolarity by the addition of wFF resulted in 29.2% of matured oocytes in adult *Balaenoptera bonaerensis* following vitrification and warming (Fig. 7.9), which was similar to that of fresh oocytes cultured for IVM (26.7%, Iwayama *et al*. 2005). The addition of wFF to an IVM culture medium tremendously shortened the culture interval to 28-40 h from the previously reported 84-120 h interval (Fukui *et al*., 1997a: Asada *et al*. 2001). This decrease may reflect an improved environment (medium) for *B. bonaerensis* oocytes to mature *in vitro* versus the medium without wFF. In bovine and porcine IVM culture, 10% FF usually is added to the medium to promote maturation and subsequent developmental capacity (Kikuchi *et al*. 2002; Ali *et al*. 2004). Future development of IVM or IVC culture media without FF or serum is suggested to avoid contamination of cultured oocytes or embryos and to further define the composition of the culture media.

7.2.2 Cryopreservation of Oocytes

Cryopreservation of sperm, eggs (oocyte), and embryos has great potential in basic research and animal husbandry. To date, various methods for embryo cryopreservation have been developed in laboratory and farm animals, and embryos of more than 20 mammalian species have been successfully cryopreserved (Mukaida and Kasai 2003). Recently, cryopreservation of oocytes and embryos in wildlife species, including cetaceans (Asada *et al*. 2000; Iwayama *et al*. 2005), has been attempted. In general, cells are sensitive to cryopreservation. During freezing and thawing, mammalian cells are at risk for damage by various factors, including toxicity of cryoprotectants, chilling injury, osmotic swelling, and shrinkage. Because oocytes and embryos contain a large amount of cytoplasm, ice formation in the cytoplasm is a major cause of cell injury during the freezing process.

Several freezing methods for mammalian oocytes have been developed. The first conventional method is a slow freezing method. Asada *et al*. (2000) used Dulbecco's physiological solution (D-PBS) containing 1.5 M ethylene glycol (EG), 0.1 M sucrose, and 10% heat-treated fetal calf serum as a cryopreservation medium to freeze immature oocytes collected from *Balaenoptera bonaerensis*. The morphologically viable proportion of post-thawing *B. bonaerensis* oocytes was 39.7%. The maturity of the animals (immature and mature whales) and the presence or absence of cumulus cells did not affect the proportion of viable oocytes. Although 20-30% of cryopreserved *B. bonaerensis* oocytes resumed meiosis in vitro, only 4 out of 194 (2.1%) post-thawed oocytes matured to M-II stage after IVM culture for 5.5 d.

Vitrification, characterized by an ultra-rapid cooling rate (16,700 to 23,000 °C/min) has been shown to be a promising method for oocyte cryopreservation. Vitrification procedures using a very high concentration of cryoprotectant (30-50%) are simple, with high survival. As it is a less toxic cryoprotectant, EG is widely used. For vitrification, several containers such as electron microscope grids (Martino *et al*. 1996), open-pulled straws (OPS, Vajita *et al*. 1998), Cryoloops (Lane *et al*. 1999), and Cryotops (Katayama *et al*. 2003) have been developed. Hochi *et al*. (2004) reported that Cryotop was superior to OPS and Cryoloop for vitrification of 1-cell rabbit zygotes. Fujihira *et al*. (2004b) used Cryotop to examine effects of pretreatment with cytochalasin B (CB) and two types of cryprotectant solutions (EG only or EG + dimethyl sulfoxide: DMSO) in porcine immature oocytes. They found that pretreatment of CB (7.5 mg/ml for 30 min) was beneficial for the vitrification of immature porcine oocytes, and that 30% EG solution resulted in significantly ($P < 0.05$) higher maturation (37.1%) than 15%EG + 15% DMSO solution (23.9%), although the development rate to blastocysts did not differ (13.5 and 14.3%, respectively) following intracytoplasmic sperm injection (ICSI). These results on porcine oocytes have encouraged the study of cryopreservation of whale immature oocytes. *In vitro* maturation rates of frozen-thawed porcine and *Balaenoptera bonaerensis* oocytes were markedly lower than those of other species (Didion *et al*. 1990; Asada *et al*. 2000). Perhaps one reason why porcine

and *B. bonaerensis* oocytes have low cryotolerance is the high amount of intracellular lipids. Fujihira *et al*. (2004a) compared the amounts of four types of lipids (triglycerol, total cholesterol, phospholipids, and non-esterified fatty acids) in immature oocytes from pigs and *B. bonaerensis.* They found that the amounts of the four lipids were significantly ($P < 0.05$) higher in vitrified-warmed oocytes from immature and adult *B. bonaerensis* than those from prepubertal pigs. From this study, it seems that *B. bonaerensis* oocytes, as well as porcine oocytes, are sensitive to freezing or vitrification.

Iwayama *et al*. (2004) compared OPS and Cryotop as the cryo-device for vitrification of GV stage oocytes recovered from prepubertal and adult *Balaenoptera bonaerensis* (Fig. 7.9). *B. bonaerensis* cumulus cell-oocyte complexes (COC) were vitrified in a solution containing 15% EG, 15% DMSO and 0.5 M sucrose. The post-warmed oocytes with normal morphology were cultured for 40 h in an IVM medium with the osmolarity adjusted to 390mOsM by adding 10% whale follicular fluid (wFF). The proportions of morphologically normal oocytes after vitrification and warming were significantly ($P < 0.05$) higher when the COC were cryopreserved by Cryotop (prepubertal, 80.8%; adult, 88.4%) rather than OPS (prepubertal, 64.2%; adult, 67.7%). The oocyte maturation rate also was significantly ($P < 0.05$) higher in the adult Cryotop group (29.1%) than those of the prepubertal Cryotop group (14.4%), the adult OPS group (14.3%), and the prepubertal OPS group (10.6%). These results indicate that Cryotop is a better cryodevice than OPS for vitrification of immature oocytes from adult *B. bonaerensis*. By adding wFF in an IVM culture medium following vitrification and warming, the proportion of *in vitro* matured oocytes (29.1%) has been greatly improved when compared with the highest proportion (31.8%, Asada *et al*. 2001) of matured oocytes freshly collected from *B. bonaerensis*. Improvements of cryopreservation methods and *in vitro* oocyte maturation systems would support the maturational and developmental potential of immature whale oocytes. A particularly relevant area in need of research involves the cryopreservation of immature oocytes, because these cells collected at GV or GVBD stage do not have a temperature-sensitive meiotic spindle as do matured (M-II stage) oocytes (Pukazhenthi and Wildt 2004).

Cryopreservation of ovarian tissue is an attractive and alternative option for gene banks because fetal, young and adult ovaries contain numerous female germ cells and ovarian tissue is much easier to collect and cryopreserve than are oocytes or embryos (Shaw *et al*. 2000). Candy *et al*. (1997) reported that 80% of primordial follicles and 50% of small growing follicles survived after cryopreservation. Recently, mouse ovaries containing several growing stages of oocytes in small follicles were frozen by a conventional slow freezing method and after thawing they were grafted under the kidney capsule of ovariectomized recipient mice for 2 wk (Cleary *et al*. 2001). The study showed that follicles other than primordial follicles survived within the ovary after both cryopreservation and grafting. Although freezing methods (e.g. cooling rate) must to be established for the individual follicle types (large or small

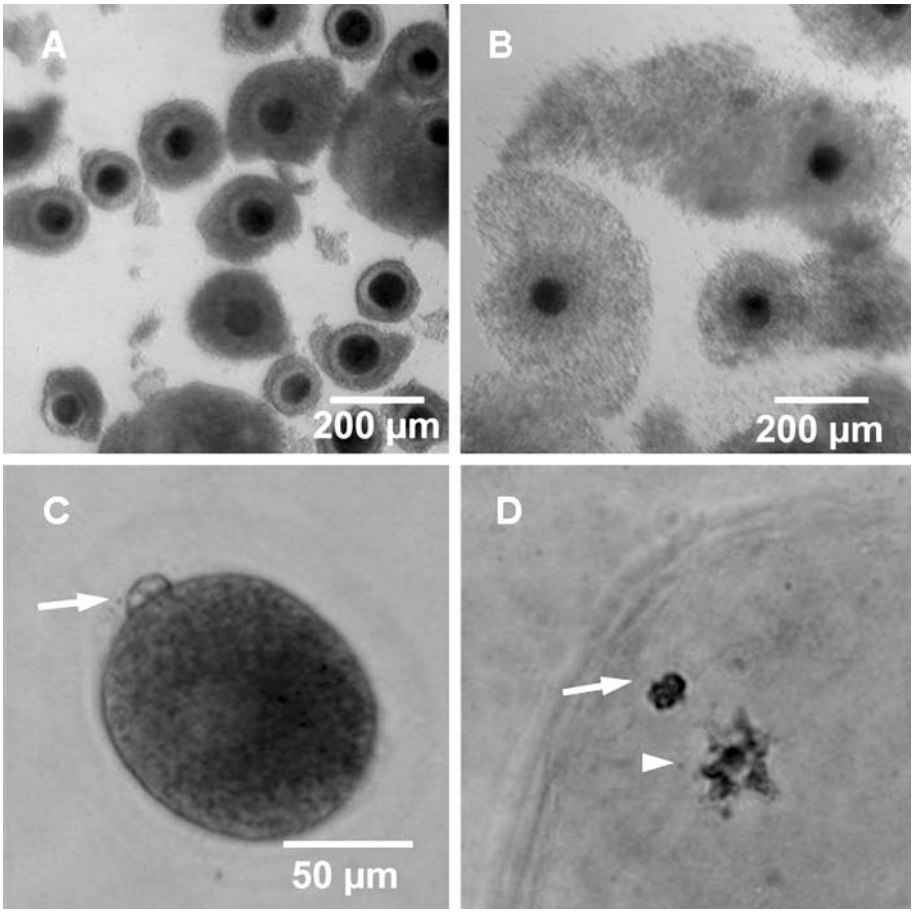

Fig. 7.9 **A**. Cumulus-oocyte complexes (COCs) of *Balaenoptera bonaerensis* (Antarctic minke whale) used for vitrification. **B**. The COCs after warming and *in vitro* maturation culture. The cumulus cell layers were expanded. **C**. An oocyte extruding the first polar body (arrow). **D**. Whole-mount preparation (× 400) of a polar body-extruding oocyte (arrow: the first polar body; arrowhead: the second metaphase plate). After Iwayama, H., Hochi, S., Kato, M., Hirabayashi, M., Kuwayama, M., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2004. Zygote 12:333-338, Fig. 1.

follicles), cryopreservation of ovarian tissue would be a promising means for long-term storage of dolphin and whale oocytes.

7.3 OVARIAN CYCLE

The ovarian cycle (estrous cycle or reproductive cycle) was not determined in cetaceans until the early 1980's. Development of hormonal assays to measure several hormones, such as estradiol-17β (E_2) and luteinizing hormone (LH), that regulate estrus and ovulation, made it possible to assess ovarian activity

throughout the year and, in captive facilities, allowed us to determine the estrous cycle for a particular species. For example, Schroeder and Keller (1990) determined that the length of the *Tursiops truncatus* estrous cycle ranges from 24 to 35 d, with an average of 27 d. The ovarian cycle of female dolphins and *Orcinus orca* is further classified into three general phases by progesterone (P_4) levels: 1) ovarian active phase, 2) pseudo-pregnancy phase, and 3) resting (anestrous) phase. The ovarian active phase is reflected by high levels (5-15 ng/ml) of P_4 and the pseudo-pregnancy phase is the period of maintaining a high P_4 level for several months. In the resting phase, distinctive P_4 levels usually are not observed, but secretion of E_2 (around 5 pg/ml) is continued. This continual secretion of E_2, indicates that folliculogenesis is not arrested, as is in seasonal breeders of domestic animals (e.g. sheep).

It is agreed that most cetaceans, including dolphins and Mysticeti, are spontaneous ovulators and seasonal breeders. It is further believed that cetaceans only ovulate once a year except for some dolphins and whales, such as *Tursiops truncatus*, *Pseudorca crassidens* (False killer whale) and *Megaptera novaeangliae* that may ovulate several times (poly-estrous cycles) during the breeding season if conception fails to occur. Male dolphins and whales also have a seasonal cycle, in that testis weight and sperm production of migrating Mysticeti increase during the late autumn or early winter, correlating closely with the female breeding pattern (Lockyer 1984). The gestation period for Mysticeti is about one year, resulting in a two year reproductive cycle; however, a three-year or longer reproductive interval is possible depending on circumstances (Lockyer 1984). *Balaenoptera physalus* is thought to mate in December and January and have a gestation period of 11 to 12 mo (Kjeld *et al*. 1992). All larger southern Mysticeti, except *Balaenoptera edeni* (Bryde's whale), are thought to undertake seasonal migrations between winter breeding areas in tropical or subtropical waters and summer feeding areas in the Southern Ocean (Mackintosh 1966). The breeding season of Antarctic Mysticeti is generally considered to be austral winter, May to August (Lockyer 1984). Kasamatsu *et al*. (1995), while surveying breeding areas and southbound migration of *Balaenoptera bonaerensis*, reported that *B. bonaerensis* moved southward from the breeding areas by October through November, and most of them had migrated into Antarctic waters by January. Among the large southern Mysticeti, *B. bonaerensis* are unique animals, suggesting that most mature female whales ovulate and conceive while still lactating (Kato and Miyashita 1991).

7.3.1 Regulating Hormones

In Mysticeti, little is known about circulating reproductive hormone levels, including sex steroids and correlation with reproductive activity. Progesterone (P_4) is known as one of the important sex steroids produced in the ovaries and placenta of many mammalian species. Elevation of P_4 is used for ovulation and pregnancy diagnosis in captive dolphin breeding programs (Sawyer-Steffan *et al*. 1983; Kirby 1990). Further, Yoshioka *et al*. (1989)

examined the correlation between serum P_4 levels and female reproductive status in 46 *Stenella coeruleoalba* (striped dolphins) and 11 *Globicephala macrorhynchus* taken during October in Taiji, Japan. Progesterone (P_4) levels in immature, resting and lactating individuals were as low as 1 ng/ml or less for both species. In *S. coeruleoalba*, the diameter of CL of ovulation showed significant positive correlation to serum P_4 levels. Additionally, Yoshioka and Fujise (1992) measured P_4 levels in 204 female *Balaenoptera bonaerensis* taken by Japanese researchers in the Antarctic during the non-breeding season and found that immature and resting females without CLs in the ovaries showed P_4 levels lower than 1 ng/ml, while ovulated and pregnancy females had much higher levels with averages of 17.0 and 17.6 ng/ml, respectively. These data indicate that P_4 concentrations below 1 ng/ml can be considered as basal circulating levels but not as ovulated or pregnancy levels. Tamura-Takahashi and Ui (1977) first characterized *B. borealis* LH and reported that the molecular weight determined by sedimentation equilibrium was 31,000, which was slightly larger than that (approximately 28,000) from other mammals, such as human, ovine, bovine and porcine. Yoshioka *et al*. (1986) examined annual changes in serum P_4, E_2 and LH levels in three female *Tursiops truncatus* and observed no cyclic elevation of P_4 levels during winter; however, they observed a markedly high LH level (over 10 ng/ml) that was assumed to be the LH-surge in one of the dolphins. This surge was similar to ovarian hormonal patterns seen in other spontaneously ovulating mammals. Their results also indicated that the calving interval in *T. truncatus* is about 3-4 yr and estrus and ovulation do not always occur annually.

Walker *et al*. (1988) analyzed hormone concentrations in the urine of six captive *Orcinus orca* for intervals up to 2 yr. The female reproductive pattern of *O. orca* is characterized by a gestation of 17 mo and an ovarian cycle of 6-7 wk. The hormone changes associated with the ovarian cycle of *O. orca* are similar to those of most other mammalian species. A bimodal pattern of bioactive FSH with a pronounced rise of estrogen predominates the pre-ovulatory hormone profile. After ovulation, increased P_4 production is observed for approximately 4 wk in the non-conceptive ovarian cycle. During the luteal phase and early pregnancy, when P_4 metabolites are elevated, estrogen metabolite excretion remains low. Atkinson *et al*. (1999) also examined P_4 profiles to study general reproductive patterns in three captive female *P. crassidens* and found that plasma P_4 concentrations reflected ovarian activity for most of the year with increased concentrations in the spring and summer, indicating that the adult female false killer whale has spontaneous ovulations and is seasonally polyestrus. During the study there were varying periods of no apparent ovarian activity from 3 to 10 consecutive months (see also Chapter **6**).

Recently, it has been possible to detect the ovulatory LH surge in *O. orca* urine by radio-immunoassay (RIA) or enzyme-immunoassay (EIA) techniques (Robeck *et al*. 2004). To predict the timing of AI, Robeck *et al*. (2004) determined more accurate timing of the LH surge in relation to urinary estrogens using twice-daily samples, and reported that the mean preovulatory follicle diameter

in *Orcinus orca* was 3.9 cm (n=6) and that ovulation occurred 38 h after the peak of the LH surge. Non-invasive hormonal monitoring throughout the season has been extensively studied since the early 1980's in wildlife, including dolphins and whales. However, in the case for *Balaenoptera bonaerensis* repeated blood or urine sampling would be impossible to obtain for determining the hormonal patterns during the breeding or non-breeding seasons. In early studies using *B. bonaerensis* (Iga *et al*. 1996), concentrations of P_4, E_2 and testosterone (T) in follicular fluid, serum and corpus luteum (CL) tissue were evaluated by EIA. The concentrations of steroid sex hormones varied with follicular diameter in immature and early and late gestation whales. Large follicles (> 8 mm) could be classified according to their E_2 levels into growing (≥ 0.2 ng/ml) and atretic (< 0.2 ng/ml) follicles. Suzuki *et al*. (2001) measured plasma and pituitary concentrations of FSH, LH and steroid hormones (P_4, E_2 and T) by EIA in 95 male and 67 female *B. bonaerensis*. Suzuki *et al*. (2001) reported that the pituitary concentrations of FSH and LH were higher in females than in males ($P < 0.01$) and in mature females than in immature females ($P < 0.05$). They further reported that pituitary FSH and LH levels were significantly (r=0.69; $P < 0.01$) correlated in both immature and mature whales, regardless of gender. Their results showed that gender and maturity influence gonadal and pituitary function of *B. bonaerensis* during the feeding season. These data for plasma and pituitary concentrations of gonadotropins and steroid hormones were obtained from captured and dead whales. Therefore, no information was available on the pulses of FSH and LH secretion in live Mysticeti or the secretion patterns of individual whales during the feeding season or the breeding period. This study was the first to provide important data of hormonal correlation of plasma and pituitary levels with morphological condition of *B. bonaerensis* in different genders and stages of maturation.

7.3.2 Estrus, Ovulation and Corpus Luteum

Sexual maturity in female whales is usually determined by the first ovulation (presence of CL in the ovary). However, it is not always easy to recognize whether a live whale has ovulated or not. Larsen and Kapel (1983) reported that the body length at which 50% of western Greenland *B. acutorostrata* are sexually mature can be estimated at 745 cm, although there is much variation (710 –770 cm).

It is important to know the time and duration of estrus for natural mating and artificial breeding but, in cetaceans, estrus is not always easy to determine. In *Tursiops truncatus*, ovulation may occur 2-3 times per year with a peak period of August to November and with great variation between individuals (Schroeder 1990). Similar to other seasonal breeders, estrous behavior is not always associated with ovulation. Therefore, monitoring the ovarian changes by ultransonography following hormonal treatment for induction of ovulation and measurement of serum or urine hormone patterns, such as E_2 and LH to determine the time of ovulation, would be important

tools for establishing controlled breeding technologies such as AI in dolphins and other species.

In cetaceans, the process of follicular development and transformation to CL after ovulation is similar to other mammals (Ivashin 1984). The usual cycle of ovulation is once (or occasionally twice) per season in both the Odontoceti and the Mysyiceti. The Graafian follicle is supplied with blood from the follicular artery, which is near the base of the follicle. Its branches cover the whole follicle on the surface, except at the top, *i.e.* the area of the future rupture (ovulation). The CL morphology is round and is at the periphery of the ovary. The diameter of CL varies from 10.9 cm, 8.3-18 cm, 3.2-8.8 cm, 6-17 cm, 5.8-16 cm, and 4-9 cm for *Balaenoptera musculus*, *B. physalus*, *Megaptera novaeangliae*, *Eschrichtius robustus* (Gray whale), *Physeter macrocephalus* (Sperm whale), and *B. acutorostrata* whales, respectively (Ivashin 1984; Lockyer 1984) (Fig. 7.1). Iga *et al*. (1996) found that the P_4 concentrations in CL tissues of early and late pregnancy Antarctic minke whales were 11.7 and 4.0 mg/wet g, respectively, and indicated that the CL appears to be a major source of P_4 for the maintenance of pregnancy. The developing CL becomes folded and blood vessels and connective tissue are observed in the folds and in the center of the corpus. The CL produces hormones, mainly P_4, during the period of pregnancy and then degenerates into a whitish mass of connective tissue known as the corpus albicans (CA). These CA usually persist throughout life in whales, although in land animals they usually disappear after a time, possibly to minimize ovarian size (Tinker 1988). If the CL of ovulation develops without pregnancy, it is soon formed into a CA of ovulation. Ivashin (1984) classified two types of scars in the CL of Mysticeti (specifically, *B. physalus*, *M. novaeangliae* and *E. robustus*), *Delphinus delphis* (common dolphin) and *P. macrocephalus*; *i.e.*, in *B. physalus*, one type is from pregnancy and are usually located over the surface of the ovary, ranging from 3-10 cm and the other type is from ovulation with the size rarely exceeding 1.5-3 cm.

7.4 ACKNOWLEDGMENTS

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CHAPTER 8

Testis, Spermatogenesis, and Testicular Cycles

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8.1 INTRODUCTION

An understanding of the reproductive biology of wild populations provides important insight for the interpretation of data from genetic, behavioral, and evolutionary studies and plays a key role in the establishment of management and conservation strategies. Past research on reproduction in cetaceans has mainly focused on females, while studies that exclusively examine male reproduction remain rare (Chittleborough 1954; Best 1969; Collet and Saint Girons 1984; Mitchell and Kozicki 1984; Hohn *et al.* 1985; Desportes *et al.* 1993; Desportes 1994; Desportes *et al.* 1994; Thayer *et al.* 2003). The focus on female research is probably due to the fact that female reproductive events are key to population modeling for use in management strategies (Honma *et al.* 2004). Nevertheless, information on male reproductive and breeding behavior can improve population models and management strategies as well as provide valuable information regarding population health (O'Hara *et al.* 2002). Therefore, the focus of research into the reproductive biology of cetaceans has recently shifted to detailed studies of both sexes (Hohn and Brownell 1990; Read and Gaskin 1990; Slooten 1991; Sørensen and Kinze 1994; Van Waerebeek and Read 1994; Read and Hohn 1995; Hohn *et al.* 1996).

The majority of studies of cetacean reproduction are based on material obtained from hunted (Best 1969; Miyazaki 1984; Desportes *et al.* 1993) or stranded animals (Ross 1979; 1984; Calzada *et al.* 1996) or from animals incidentally caught and killed in fishing nets (Perrin *et al.* 1976; Perrin *et al.* 1977; Read 1990a, 1990b; Slooten 1991; Van Waerebeek and Read 1994; Hohn *et al.* 1996) or anti-shark nets (Cockcroft and Ross 1990). Data on the

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reproduction of live wild animals are difficult to obtain and studies of reproduction in captive animals may not be representative of wild populations. Although animals may be approached by boat, most of the reproductive activities occur underwater and thus are inaccessible for boat-based researchers. Regardless of this difficulty, a number of studies have successfully observed reproductive and other life history events in wild populations of dolphins in the past two decades (Wells *et al.* 1987; Herzing 1997) and studies on live wild animals are becoming increasingly more common. However, the examination of material from dead specimens is vital in order to validate data from less invasive techniques, such as ultrasound and hormonal studies, on wild animals.

8.2 TESTES MASS AS INDICATOR OF MATING SYSTEM

The morphology of the male gonads of cetaceans has been studied extensively (Slijper 1966; Harrison *et al.* 1972; de Smet 1977). Cetaceans differ from most other mammals in that they are testicond, meaning the testes are located inside the abdominal cavity thus improving streamlining (Slijper 1962; de Smet 1977). There are minor differences in the morphology of the testes between species and the testis volume may change with age and sexual activity (de Smet 1977); see also section on testicular cycles below) and between species (see section on mating systems and testis size below).

Thermoregulation of the intra-abdominal testes is carried out by a counter-current heat exchange, as has been studied in *Tursiops truncatus* (Bottlenose dolphin) (Pabst *et al.* 1995; and Chapter **4** of this volume). Further, there are no differences in spermatogenesis between mammals with abdominal and scrotal testes (Setchell 1978a). Historically, a detailed examination of the testes has relied on dead specimens being available for dissection. Recent advances in technology, such as ultrasonography, now allow the examination of live captive animals (Robeck *et al.* 2004; Brook *et al*. 2000).

8.3 SPERMATOGENESIS AND ATTAINMENT OF SEXUAL MATURITY (ASM)

Spermatogenesis is an important indicator of the reproductive biology of a male mammal, both with respect to the onset of maturity and the onset of breeding in seasonally reproducing species. Estimates of the size and age at ASM (Barlow 1985; Chivers and Myrick 1993) and data on the reproductive cycle of a species are necessary to understand its reproductive strategy and ecology. Furthermore, such information is necessary for inter- and intraspecific comparisons (Hohn *et al.* 1985). Data for the length at sexual maturity for a given species are especially useful in the field or for animals for which no age estimates are available (Perrin and Reilly 1984). Furthermore, these data are needed for the management of species that are subject to mortality by man (Read and Gaskin 1990; Slooten 1991; Chivers and Myrick

1993; Desportes *et al.* 1993; Hohn *et al.* 1996). For example, an increase in length at ASM over time is reported for male *Stenella attenuata* (Spotted dolphin) incidentally caught in the tuna purse-seine fishery in the Eastern Tropical Pacific, with no coincidental increase in age at ASM (Hohn *et al.* 1985). Perrin and Henderson (1984) report that mature testis weight can vary greatly between populations, possibly as a function of the degree of exploitation. Therefore such data may play an important role in stock assessment studies (Perrin and Henderson 1984) and in determining the degree of exploitation of a stock or population (Read and Gaskin 1990; Slooten 1991; Hohn *et al.* 1996).

The definition of ASM in male cetaceans is complex (Perrin and Reilly 1984) as there is no single criterion for the onset of sexual maturity (Perrin and Henderson 1984). Testis histology (i.e., stage of spermatogenesis) and seminiferous tubule diameter, testis weight or length, sperm abundance, the presence of sperm in the epididymis, and serum testosterone levels all have been used to indicate ASM and are described in detail below.

8.3.1 Spermatogenesis

There is some discrepancy as to how many different stages of maturity can be defined in odontocetes. Whereas the most common practice is to distinguish between immature, pubertal (also called prepubescent or maturing), and mature animals (Best 1969; Hohn *et al.* 1985; Sørensen and Kinze 1994), a few studies define four different stages of maturity, namely immature, early maturing, late maturing, and mature (Kasuya and Marsh 1984; Desportes *et al.* 1993; Kasuya and Tai 1993). For ease of comparison with other mammalian studies we will use only three stages of maturity here, namely immature, early spermatogenesis and late spermatogenesis.

Little research has been done on the gonadal development of immature cetaceans, which is probably largely due to the difficulties in obtaining representative samples throughout the various ontogenetic stages preceding maturity. Such baseline studies are becoming increasingly important in view of the potential effects that a number of environmental pollutants have on the endocrine system of marine mammals, as many of these compounds act as hormone mimics (Atkinson 1997; Karakosta *et al.* 1999). Karakosta *et al.* (1999) describe the histological development of immature testes in *Phocoena phocoena* (Harbor porpoise) and classify them into three developmentally distinct classes. These were based on the degree of testicular maturity as indicated by the relative amounts of interstitial and seminiferous tubule tissue present and the frequency of prospermatogonia. Generally immature animals are characterized by tightly packed, narrow seminiferous tubules with no lumen, surrounded by abundant interstitial tissue (Best 1969; Hohn *et al.* 1985; Desportes *et al.* 1993; Plön 2004). In *Megaptera novaeangliae* (Humpback whale), Chittleborough (1954) reported the presence of some seminiferous tubules with open lumens, but no dividing spermatocytes.

The tubules are lined by an epithelium comprising a single layer of prospermatogonia along the basement membrane and Sertoli cells situated closer to the center of the tubule (O'Hara *et al.* 2002; Plön 2004) (Fig. 8.1A). Sertoli cells are sustentacular or nurse cells for the developing gametes that are positioned basally within mammalian seminiferous tubules and provide the physical support within which the spermatogonia are embedded (Setchell 1978b; Miller *et al.* 2002). Early spermatogenesis is characterized by two to four cell layers of spermatogonia and spermatocytes in the seminiferous epithelium (Setchell 1978b; Plön 2004). No spermatids are present and very little interstitial tissue is present between the seminiferous tubules (Hohn *et al.* 1985; Plön 2004) (Fig. 8.1B). Late spermatogenesis is characterised by large seminiferous tubules with an open lumen (Best 1969; O'Hara *et al.* 2002; Plön 2004). A complex seminiferous epithelium is present that is comprised three or more cell layers of spermatogonia, spermatocytes and spermatids (Plön 2004). Little interstitial tissue is present (Fig. 8.1C, D). In *Phocoena phocoena*, spermatogenesis proceeds in helical waves along the seminiferous tubules and thus more than one stage of spermatogenesis is observed at the same time (Neimanis *et al.* 2000). Each segment of the wave represents a different stage of germ cell maturation and this wave-like nature of spermatogenesis stops during the process of regression (Neimanis *et al.* 2000). This arrest of spermatogenesis may be caused either directly by the apoptosis of germ cells and/or hormonal cues that prevent spermatogonia from dividing or indirectly if Sertoli cells cease to maintain their supportive role for germ cells (Neimanis *et al.* 2000). Furthermore, Karakosta *et al.* (1999) report that during the seasonal regression of the testes when active spermatogenesis ceases the testes still retain characteristically larger seminiferous tubules than immature testes, in addition to having spermatocytes present in the lumen. Thus mature and immature testes are easily distinguished throughout the year. The physiological mechanisms driving these seasonal changes in testes activity have not been studied in detail in cetaceans, but are probably similar to those found in other mammals (Bronson and Heidemann 1994; Sørensen and Kinze 1994; Neimanis *et al.* 2000).

Both Sertoli and Leydig cells are commonly used as indicators of reproductive status in mammals (Setchell 1978b). Leydig cells are the interstitial cells of the testes located within the septal connective tissue and play a role in secreting testosterone (Setchell 1978b; Miller *et al.* 2002). In *Physeter macrocephalus* (Sperm whale), there is considerable variation of histological appearance of the Leydig cells in mature males (Best 1969). As the degree of vacuolation of the cells indicates the androgen content of the testis, the diameter (size) of the cells represents an indication of the hormone content (Best 1969). Using this measurement, a cycle of Leydig cell activity can be determined and this seems to be correlated with the female breeding cycle (Best 1969). In addition, Leydig cell size varies with stage of maturity and pubertal whales have cells that range from immature to mature depending on the location within the testis (Clarke *et al.* 1994). In contrast, examination of

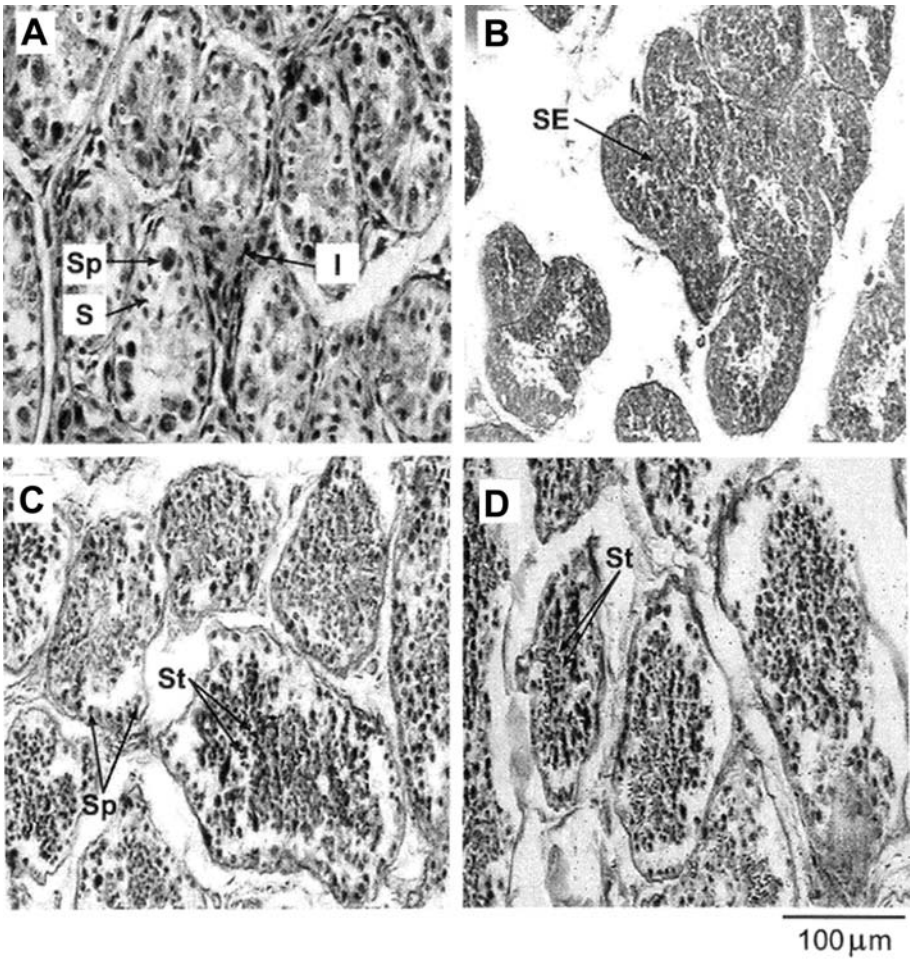

Fig. 8.1 Histological preparations of different maturity stages of the testes in *Kogia.* **A**. Immature testis, characterized by tightly packed, narrow seminiferous tubules with no lumen and abundant surrounding interstitial tissue (I). Sp: Spermatogonia, S: Sertoli cells. **B**. Early spermatogenesis is characterized by two to four cell layers of spermatogonia and spermatocytes in the seminiferous epithelium (SE). No spermatids are present and only little interstitial tissue is found between the seminiferous tubules. **C, D**. Late spermatogenesis as indicated by large seminiferous tubules with an open lumen and a complex seminiferous epithelium with spermatogonia, spermatocytes and spermatids (St) present. The tissue shows some damage due to post-partum decay, which is often observed in studies on stranded cetaceans. Original.

Balaena mysticetus (Bowhead whale) testes has shown Leydig cells to be either unidentifiable or in low numbers in animals expected to be mature and active (O'Hara *et al.* 2002). Consequently, by means of immunohistochemistry these

cells can be distinguished by using calretinin immunohistochemical staining (Miller *et al.* 2002). Differential staining intensity observed between individuals may indicate a seasonal cycle in *B. mysticetus*, but these data need to be verified by a corresponding serum hormone analysis (Miller *et al.* 2002). Further discussion on male reproductive endocrinology is provided in Chapter **6** of this volume.

Histological examination of the testes is the most accurate way to determine ASM in male cetaceans (Kasuya and Marsh 1984; Perrin and Donovan 1984; Hohn *et al.* 1985; Desportes *et al.* 1993). Unfortunately, it is a lengthy process and must be carried out in a laboratory. Regardless, many studies have been initiated to explore cetacean spermatogenesis (Best 1969; Kasuya and Marsh 1984; Desportes *et al.* 1993; Kasuya and Tai 1993; Plön 2004) and seminiferous tubule diameter (Hohn *et al.* 1985; Cockcroft and Ross 1990; Desportes *et al.* 1993; Plön 2004).

In the majority of species, both testes mature at the same rate (Chittleborough 1954; Collet and Saint Girons 1984; Miyazaki 1984; Van Waerebeek and Read 1994; O'Hara *et al.* 2002; Plön 2004) and therefore usually only one testis is used for examination (Kasuya and Marsh 1984; Cockcroft and Ross 1990; Honma *et al.* 2004)). On occasion sperm is only present in detectable amounts in one testis (Clarke *et al.* 1994), and attempts to evaluate both gonads should therefore be encouraged (O'Hara *et al.* 2002). In some species such as *Physeter macrocephalus* (Best 1969), *Globicephala melas* (Long-finned pilot whale) (Desportes 1994) and *Berardius bairdii* (Baird's beaked whale) (Kasuya *et al.* 1997), a zonal maturation of the testes occurs. The testes of *P. macrocephalus* appear to mature from the center outwards (Best 1969) and this has subsequently been used as a guideline for taking samples for reproductive studies. Some *B. mysticetus* appear to show a similar maturation from the center to the periphery and thus sampling in this species was recommended from the center of the testis (O'Hara *et al.* 2002).

Most contemporary studies on the reproduction of cetaceans use samples from stranded animals or animals incidentally caught in fishing gear, thus utilizing tissue which could not be collected immediately after death and properly fixed and stored for histological examination (Karakosta *et al.* 1999; Neimanis *et al.* 2000; Plön 2004). Consequently much of the tissue is damaged and should be interpreted with care. Nevertheless, even with tissue in poor condition, it is possible to separate the three stages of maturity discussed above.

8.3.2 Other Indicators of Maturity

A rapid increase in testis weight is used as an indicator of ASM (Sergeant 1962; Gambell 1968; Perrin *et al.* 1976; Perrin *et al.* 1977; Kasuya and Marsh 1984; Hohn *et al.* 1985; Kasuya and Tai 1993); however, large individual variation of testis weight among mature males is reported for both mysticetes (Chittleborough 1954; Gambell 1968) and odontocetes (Collet and Saint Girons 1984; Desportes *et al.* 1993). Furthermore, differences in testis weight

are observed between two different stocks of *Stenella longirostris* (Spinner dolphin) with different degrees of exploitation (Perrin and Henderson 1984). Thus changes in testis mass should be interpreted with care.

Some studies on the stage of sexual maturity use the abundance of sperm from testicular, epididymal (Kasuya and Marsh 1984; Desportes *et al.* 1993) and vas deferens smears (Chittleborough 1954; Desportes *et al.* 1993), along with histological examination of testicular tissues (Chittleborough 1954; Best 1969; Mitchell and Kozicki 1984; O'Hara *et al.* 2002). In the field, the easiest method to determine sexual maturity is by determining if seminal fluid is present in the epididymis (Fisher and Harrison 1970; Harrison and Brownell 1971; Perrin *et al.* 1977; Cockcroft and Ross 1990; Van Waerebeek and Read 1994; O'Hara *et al.* 2002). Unfortunately, this method may underestimate sexual maturity because seminal fluid is generally not present until a slightly later stage in ontogeny than can be noted histologically in the testes (Sergeant 1962; Kasuya and Marsh 1984). For studies relying only on the field method, it has been assumed that the presence of seminal fluid in the epididymis means that sperm are produced continuously (Perrin and Reilly 1984; Desportes *et al.* 1993). This assumption is not valid because males of some species may have a resting phase during which testis size decreases and no sperm are produced (Collet and Saint Girons 1984; Perrin and Reilly 1984; see also section on testicular cycles below). The value of this tool is questionable unless the presence or absence of sperm in the seminal fluid is confirmed histologically.

Another indicator of sexual maturity is the level of testosterone in the blood (Harrison and Ridgway 1971; Wells 1984; Schroeder and Keller 1989; Desportes *et al.* 1993; Desportes 1994; Kita *et al.* 1999; Kjeld *et al.* 2003, 2004). Although an increase of serum testosterone levels can be determined in mature animals (Kjeld *et al.* 2003, 2004), Kita *et al*. (1999) found a lot of variation in levels between mature individuals of *Globicephala macrorhynchus* (Short-finned pilot whale) irrespective of testis size and seminiferous tubule diameter. In addition, the ranges of serum testosterone vary between different species of cetaceans (Desportes *et al.* 1994; Kjeld *et al.* 2003, 2004), which makes comparisons between species difficult. Therefore, plasma testosterone cannot be used as single indicator of sexual maturity, but does provide a good means of monitoring sexual activity over time within a species (Desportes *et al.* 1994).

Most studies use a combination of criteria to determine the onset of sexual maturity in males (Best 1969; Cockcroft and Ross 1990; Desportes *et al.* 1993; O'Hara *et al.* 2002). This appears to be the best solution in view of the constraints encountered with each factor mentioned above. In addition, some overlap may be encountered with some parameters between the different categories of maturity, as has been reported for testis size (both length and weight) in *Stenella attenuata* (Hohn *et al.* 1985), *Kogia* spp. (Pygmy and Dwarf sperm whales) (Plön 2004), and in *Globicephala melas* (Desportes *et al.* 1993), with the exception of testis length in the latter. Brook *et al.* (2000) report that

testis length alone is not a reliable measure of testis volume as the longest testis measured in a *Tursiops truncatus* was only half the volume of the largest testis measured. Ultrasonography may lend great insight into testicular maturation as characteristic echo patterns can identify the maturity status of individual animals (Brook *et al.* 2000).

Perrin and Donovan (1984) suggest that in order to assess the different stages of maturity in male cetaceans, testes weights should be recorded, the epididymis should be examined for sperm, and smears from the periphery as well as from the center of the testis should be examined histologically. Gonadal characteristics like testis weight (Chittleborough 1954; Hohn *et al.* 1996), seminiferous tubule diameter (Mitchell and Kozicki 1984; Hohn *et al.* 1985), and testis length (Desportes *et al.* 1993; Hohn *et al.* 1996) are reliable indicators of maturity, whereas other factors such as age (Hohn *et al.* 1985; Desportes *et al.* 1993), body length (Chittleborough 1954; Hohn *et al.* 1985; Desportes *et al.* 1993), body weight (Desportes *et al.* 1993), and color phase of the animal (Hohn *et al.* 1985; Kasuya *et al.* 1988) are less reliable. Which characteristic presents the most reliable indicator of maturity may vary between species and even between populations of the same species (Perrin and Henderson 1984; Hohn *et al.* 1985). The variety of methods used in determining sexual maturity and age and length at ASM in male cetaceans must be considered and interspecies comparisons cautiously explored (DeMaster 1984; Perrin and Reilly 1984; Hohn *et al.* 1985). It has been suggested that an index of testis development, which defines maturity in terms of unit testis weight (g) per unit of testis length (mm), may remove some of the variability in testis weight among species of different sizes and thus allow comparison between different stocks or species (Hohn *et al.* 1985). Although such an index was calculated by Collet and Saint Girons (1984), Hohn *et al.* (1985), and Desportes *et al.* (1993), the above authors have all calculated the testis index in different ways, rendering a comparison impossible.

8.4 TESTICULAR CYCLES

Whether a mammal reproduces seasonally or continuously depends largely on its environment (Bronson 1989; Bronson and Heidemann 1994). Seasonal differences in food availability, rainfall, temperature, photoperiod, predation and female body condition are all important determinants of the timing and duration of a seasonal cycle in mammals (Bronson 1989; Bronson and Heidemann 1994; Urian *et al.* 1996). Most habitats have at least some seasonal variation in climate and food availability and this is especially pronounced in higher latitudes, where annual variations in temperature can be extreme (Bronson 1989; Bronson and Heidemann 1994; Urian *et al.* 1996). Although reproductive seasonality has been widely researched in terrestrial mammals (Bronson 1989; Bronson and Heidemann 1994), the factors influencing seasonality in marine mammals are little understood. This can primarily be attributed to the complex marine environment with its largely unpredictable

spatial and temporal variation in biotic and abiotic factors (Sørensen and Kinze 1994). Seasonality of reproduction has been widely monitored in marine invertebrates and fish, but it is somewhat more difficult to find cues for seasonality in marine mammals. As males have to shape their annual reproductive pattern around the pattern that is most advantageous for the females (Bronson 1989), reproductive seasonality has largely been examined in view of the timing and duration of the calving period.

The study of male seasonality in cetaceans is problematic as it requires samples to be obtained throughout the year, but as most samples are obtained opportunistically from either stranded or bycaught animals, a representative sample is not always possible. In addition, samples obtained from baleen whales often were restricted to the hunting season (Mackintosh and Wheeler 1929; Chittleborough 1954; Laws 1961). Keeping these restraints in mind, changes in testis weight (Hohn *et al.* 1985; Read 1990b; Slooten 1991; Van Waerebeek and Read 1994; Hohn *et al.* 1996; Neimanis *et al.* 2000), testis volume (Gaskin *et al.* 1984), seminiferous tubule diameters (Fisher and Harrison 1970; Collet and Saint Girons 1984; Hohn *et al.* 1985; Neimanis *et al.* 2000), sperm abundance (Gaskin *et al.* 1984; Hohn *et al.* 1985; Desportes *et al.* 1993), spermatogenic activity (Sergeant 1962; Fisher and Harrison 1970; Desportes *et al.* 1993; Hohn *et al.* 1996), Leydig cell diameter (Clarke *et al.* 1994), and serum testosterone levels (Harrison and Ridgway 1971; Wells 1984; Desportes *et al.* 1993; Fukui *et al.* 1996; Mogoe *et al.* 2000; Kjeld *et al.* 2003, 2004) have all been used as indicators of a male seasonal cycle in cetaceans. Furthermore, testis length differs significantly between periods of high and low testicular activity in *Globicephala melas* (Desportes *et al.* 1993) and *Phocoena phocoena* (Neimanis *et al.* 2000). In contrast, no difference was found between mature and active versus mature and inactive males in either the range of mean testis length or of mean testis mass in *Balaena mysticetus* (O'Hara *et al.* 2002). Based on the above criteria, seasonality in testicular activity was reported for a number of wild populations of mysticetes (Chittleborough 1954; for a review see Lockyer 1984) and odontocetes (Sergeant 1962; Hohn *et al.* 1985; Read 1990b; Van Waerebeek and Read 1994; Read and Hohn 1995) (see Table 8.1). Studies of captive *Tursiops truncatus* (Harrison and Ridgway 1971; Schroeder and Keller 1989) and *Stenella longirostris* (Long-snouted spinner dolphin) (Wells 1984) and more recently wild caught *Balaenoptera borealis* (Sei whale), *B. physalus* (Fin whales) and *B. acutorostrata* (North Atlantic minke whale) (Fukui *et al.* 1996; Mogoe *et al.* 2000; Kjeld *et al.* 2003, 2004) indicate that serum testosterone levels change seasonally, reflecting seasonal testicular activity. Combined studies of serum hormone levels and morphological gonadal characteristics in recent years indicate that very low serum testosterone levels in Antarctic *B. acutorostrata* coincided with a decrease in testes weight (Fukui *et al.* 1996; Mogoe *et al.* 2000). In male *B. borealis* serum testosterone levels were found to be a more sensitive index of male seasonal testicular activity than testis weight (Kjeld *et al.* 2003, 2004). Such results show that measurements of sex hormone concentrations not only corroborate

Table 8.1 Data on male seasonal cycles. Although an effort was made to include only data from studies that had samples available year-round, a number of studies (*) relied on seasonal sampling and may thus be biased.

Species	*Location*	*Seasonality (indicated by annual peak levels, season of elevated levels in parentheses)*	*Indicator*	*Source*
Blue whale *Balaenoptera musculus*	Southern Ocean	April-May	testis histology	Mackintosh and Wheeler 1929
Fin whale *Balaenoptera physalus*	Southern Ocean	April-July	testis histology	Mackintosh and Wheeler 1929*
Fin whale *Balaenoptera physalus*	Southern Ocean	May-June (April-August)	testis histology	Laws 1961*
Sei whale *Balaenoptera borealis*	Southern Hemisphere	None	testis weight, testis volume, testis histology	Gambell 1968
Sei whale *Balaenoptera borealis*	Northwest Atlantic	June-October	testis weight, testis histology	Mitchell and Kozicki 1974
Gray whale *Eschrichtius robustus*	Eastern North Pacific	late Nov-early December	testis weight, testis histology	Wolman 1985
Humpback whale *Megaptera novaeangliae*	Western Australia	July-August	testis weight, testis histology	Chittleborough 1954*
Sperm whale *Physeter macrocephalus*	Eastern South Atlantic (off South Africa)	year-round	testis weight, testis histology	Best 1969
Dwarf sperm whale *Kogia sima*	Eastern South Atlantic and Western Indian Ocean (off South Africa)	year-round	testis weight, testis histology	Plön 2004
Long-finned pilot whale *Globicephala melas*	North Atlantic (off Faroe Islands)	June (March-September)	testis weight, epididymal smears	Desportes *et al.* 1993
Long-finned pilot whale *Globicephala melas*	North Atlantic (off Faroe Islands)	April, August-September	testosterone level	Desportes *et al.* 1993*
Long-finned pilot whale *Globicephala melas*	North Atlantic (off Faroe Islands)	March, May-June	testis histology	Desportes *et al.* 1993
Short-finned pilot whale *Globicephala macrorhynchus*	North-western Pacific	year-round	testis weight, testis histology, epididymal smear, testicular smear	Kasuya and Marsh 1984; Kasuya *et al.* 1993; Kasuya and Tai 1993

Table 8.1 Contd. ...

Table 8.1 Contd. ...

Common dolphin *Delphinus delphis*	Eastern North Atlantic, Bay of Biscay, France	April-August	testis histology	Collet and Saint Girons 1984
Common dolphin *Delphinus delphis*	Eastern North Atlantic, Channel coast, France	December-July	testis histology	Collet and Saint Girons 1984
Bottlenose dolphin *Tursiops truncatus*	California (captive)	September-October and April-May	serum testosterone levels	Harrison and Ridgway 1971
Northern white-belly spinner dolphin *Stenella longirostris*	Eastern tropical Pacific	February, July-August	testis weight	Perrin and Henderson 1984
Eastern white-belly spinner dolphin *Stenella longirostris*	Eastern tropical Pacific	March-June	testis weight	Perrin and Henderson 1984
Spinner dolphin *Stenella longirostris*	Hawaii (captive)	June-July (March-September)	serum testosterone levels	Wells 1984
Spotted dolphin *Stenella attenuata*	Eastern tropical Pacific	July-August, April (May)	testis and epididymis weight, testis histology, index of testis development	Hohn *et al.* 1985
Dusky dolphin *Lagenorhynchus obscurus*	Peru-western South Atlantic	September-October (August-November)	testis weight	Van Waerebeek and Read 1994
Harbour porpoise *Phocoena phocoena*	North Sea (Danish waters)	July (May-August)	testis weight, testis histology	Sørensen and Kinze 1994
Harbour porpoise *Phocoena phocoena*	Gulf of Maine, USA	late June-early July	testis weight, testis histology	Read and Hohn 1995
Harbour porpoise *Phocoena phocoena*	Bay of Fundy, Canada, and Gulf of Maine, USA	mid-June-end of July (mid-June-mid- September)	testis histology	Neimanis *et al.* 2000
Vaquita *Phocoena sinus*	Gulf of California	late February-late April	testis weight, testis and epididymis histology	Hohn *et al.* 1996

anatomical/histological data, but also surpass them in sensitivity of detecting cyclical changes in the male reproductive cycle.

While testes weights vary seasonally in a number of cetaceans, there is little histological evidence for a seasonal complete cessation of spermatogenesis (also termed aspermatogenesis) in either mysticetes or odontocetes (Perrin and Donovan 1984). The most comprehensive study on seasonal regression in testes and its associated histology has been carried out in *Phocoena phocoena* (Neimanis *et al.* 2000). During the phase of testicular regression, spermatocytes and round spermatids first disappeared from the lumina of randomly scattered seminiferous tubules, and degenerated spermatogenic cells were present (Neimanis *et al.* 2000). This was followed by a decrease in numbers and gradual disappearance of the spermatozoa in the tubular lumina (Neimanis *et al.* 2000). Ultimately, all signs of spermatogenesis were absent, but the seminiferous tubules retained an alternating lining of Sertoli cells and spermatogonia (Neimanis *et al.* 2000). Regressive changes were accompanied by an increase in the interstitial tissue area by up to two times, a decrease in the diameter of the seminiferous tubules, and basement membranes which were almost twice as thick as those in fully active testes (Neimanis *et al.* 2000). The changes in testes histology were accompanied by changes in the epididymides. While the lumina of the epididymides were packed with spermatozoa and the epithelial lining was thick and well-developed during full activity of the testes, the spermatozoal density decreased and degenerating spermatogenic cells were observed during seasonal regression of the testes (Neimanis *et al.* 2000). Additional parameters of gonadal size, including mean testicular length, mean testicular and epididymal weight, and mean seminiferous tubule diameter, also showed a significant change during the time of regression of the testes and decreased approximately 1.5, 3.5 and 1.5 times, respectively (Neimanis *et al.* 2000). Complete cessation of spermatogenesis in other cetaceans has only been reported for *Megaptera novaeangliae* (Chittleborough 1954) and the *Delphinus delphis* (Common dolphin) (Collet and Saint Girons 1984). In *Lagenorhynchus obscurus* (Dusky dolphin) and *Phocoena sinus* (Vaquita) complete cessation is also reported to occur, but is rare (Van Waerebeek and Read 1994; Hohn *et al.* 1996), while in *Globicephalus melas* (Desportes *et al.* 1993), *S. attenuata* (Hohn *et al.* 1985) and *S. coeruleoalba* (Striped dolphins) (Miyazaki 1977) the testes regress, but not to the degree seen in *P. phocoena* (Neimanis *et al.* 2000). In contrast, a number of species, like *Physeter macrocephalus* (Best 1969; Mitchell and Kozicki 1984), *Kogia sima* (Dwarf sperm whale) (Plön 2004), and *Tursiops truncatus* (Cockcroft and Ross 1990), show continuous spermatogenesis throughout the year.

One would expect a seasonal peak in male testicular activity to occur shortly prior to or coinciding with the time of female estrus (Bronson and Heidemann 1994). Historically, few cetacean researchers have concentrated on this aspect and evidence based on the examination of the male reproductive organs has only been gathered for few cetacean species, such as *Megaptera novaeangliae* (Chittleborough 1954), *L. obscurus* (Van Waerebeek and

Read 1994), *Globicephalus melas* (Sergeant 1962; Desportes *et al.* 1993; Martin and Rothery 1993), *Stenella attenuata* (Hohn *et al.* 1985), and captive *Tursiops truncatus* (Harrison and Ridgway 1971). Again the most detailed studies on wild cetaceans have been carried out in *Phocoena phocoena*, where seasonal changes in testicular size and activity have been used to infer and corroborate the mating season (Gaskin *et al.* 1984; Read 1990b; Sørensen and Kinze 1994; Read and Hohn 1995; Neimanis *et al.* 2000). Generally in mammals, matings are expected to occur when the epididymides are filled with spermatozoa because spermatozoa mature in the epididymis and are stored there before copulation (Amann *et al.* 1993). However, selection should favor maximum testicular activity for the entire duration of the conception period in case some females ovulate early or late in the breeding season and others fail to conceive during the first estrus cycle (Neimanis *et al.* 2000). The data for *P. phocoena* support this as males are active for about one month longer than the data for female conception indicate (Neimanis *et al.* 2000) (Fig. 8.2). In *S. attenuata* times of elevated testes weights coincide with the mating season, but although the seasonal peak in both the northern and southern offshore stock is similar, calving seasons differ between the two stocks and the testes never fully regress (Hohn *et al.* 1985). Some calves are born year-round, indicating that a number of females may ovulate at any time throughout the year and thus year-round spermatogenic activity in males is expected (Hohn *et al.* 1985; Neimanis *et al.* 2000). Recent advances in monitoring serum sex hormones may help elucidate the seasonal reproductive activities of cetaceans in more detail as peaks of male and female sex hormones coincide with the mating season in the North Atlantic stock of *Balaenoptera acutorostrata* (Kjeld *et al.* 2004). These data indicate that the male seasonal reproductive cycle is complex and different parameters can peak at different times of the year (Desportes *et al.* 1993). In addition, a number of seasonal patterns may be found in the reproductive cycles of cetaceans, which are perhaps better referred to as differing degrees of seasonality (see Table 8.1). Differing patterns of male seasonal cycles have even been reported for different populations of the same species (Perrin and Henderson 1984). More research is needed to elucidate male cycles of most cetacean species.

8.5 MALE MATING STRATEGY

This chapter focuses on the testes of male cetaceans. Therefore, it would be an oversight to exclude the numerous theories on male mating strategies developed for this group. These theories are to a large extent based on testis size.

8.5.1 Indicators of Mating Strategy

Copulation and other sexual behavior of cetaceans is frequently observed in captivity (Saayman and Tayler 1977), but less often in natural environments (Herzing 1997). This is especially true for large or rare species. Given that a

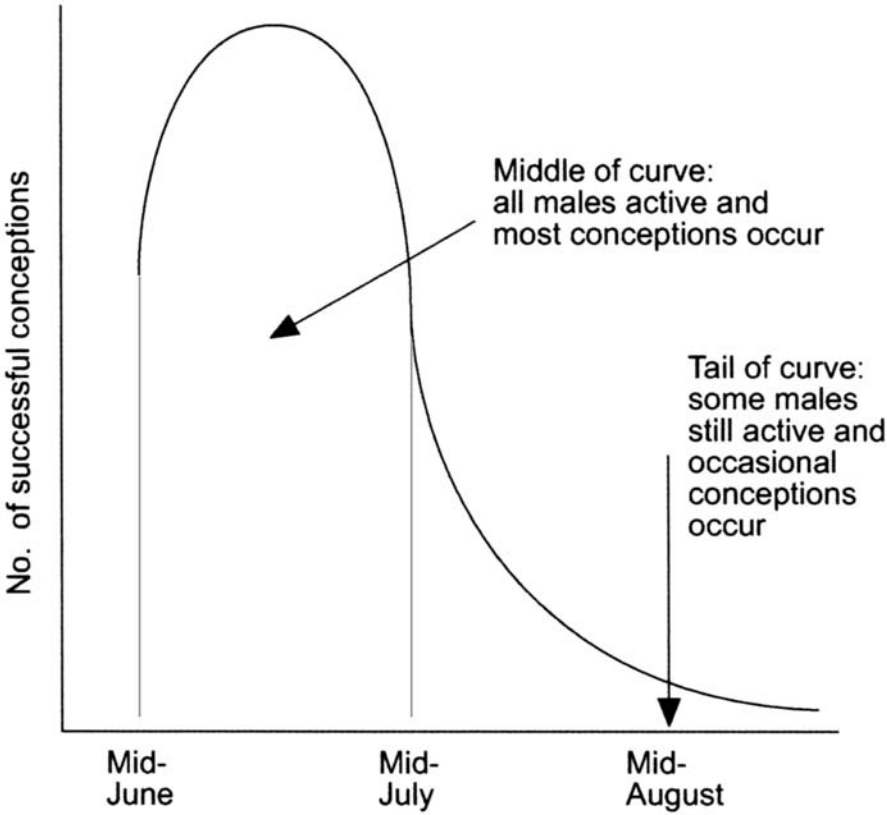

Fig. 8.2 Schematic representation of the number of successful conceptions in relation to male testicular activity in harbor porpoises from the Bay of Fundy. Reproduced from Neimanis, A. S., Read, A. J., Foster, R. A. and Gaskin, D. E. 2000. Journal of Zoology 250: 221-229, Fig. 8.

number of odontocetes appear to use sexual contact (both homo- and heterosexual) to strengthen the social bonds of a group or school (Kasuya *et al.* 1993), observations in the wild may not give unequivocal evidence about a species' reproductive strategy.

Numerous factors are involved in shaping the mating system of a species and thus multifactorial models are needed to predict mating systems (Sandell and Liberg 1992). Indicators like testis mass to body mass ratio, sexual dimorphism, and group size are used to provide information about the mating system of terrestrial mammals (Harcourt *et al.* 1981; Kenagy and Trombulak 1986; Rose *et al.* 1997). In cetaceans, these parameters together with the degree of scarring resulting from intrasexual fights (McCann 1974; Heyning 1984; MacLeod 1998) are used to provide a starting point for the development of hypotheses about the mating system of a species (Brownell and Ralls 1986; Slooten 1991; Aguilar and Monzon 1992; Cockcroft 1993; Van Waerebeek and Read 1994). This is especially useful for species for which data from behavioral observations in the wild are either difficult or costly to obtain.

8.5.2 Testis Mass to Body Mass Ratio

In mammals, testis size usually increases as body size increases, regardless of the breeding system (Harcourt *et al.* 1981; Kenagy and Trombulak 1986), but variation in relative testis size within and between species generally reflects variations in the requirements for sperm production (Setchell 1978a; Kenagy and Trombulak 1986). Therefore testis weight as a percentage of body weight is often used as an indicator of the mating system of a species (Harcourt *et al.* 1981; Kenagy and Trombulak 1986; Rose *et al.* 1997). Relatively large testes are a result of the selective pressures of multiple inseminations, sperm competition within the female reproductive tract, spontaneous ovulations, and seasonal reproduction (Harcourt *et al.* 1981; Kenagy and Trombulak 1986). Therefore large testes in relation to body weight are usually associated with a multimale breeding system (or polyandry), in which the males compete with each other in the form of sperm competition (Harcourt *et al.* 1981; Kenagy and Trombulak 1986). Sexual dimorphism is usually absent in these species (Aguilar and Monzon 1992).

Small testes in relation to body weight are indicative of low copulatory frequency and thus of monogamy or extreme polygynous single-male mating systems, the latter involving one male mating with a number of females (i.e., harem) (Harcourt *et al.* 1981; Kenagy and Trombulak 1986). Sexual dimorphism is usually great in species where males have to fight over access to a number of females. In cetaceans, intraspecific fighting is thought to be reflected in the amount of scarring (MacLeod 1998). Generally very little sexual dimorphism is found in monogamy species (Harcourt *et al.* 1981). Intermediate levels of sexual dimorphism, large testes (and thus assumed high copulatory frequency), and low degrees of scarring indicate a multimale breeding system, for example promiscuity or multimale polygyny (Harcourt *et al.* 1981; Van Waerebeek and Read 1994) (Table 8.2).

Combined testis weights comprise less than one percent of body mass in most terrestrial mammals and cetaceans have slightly, but significantly larger testes relative to body weight (Kenagy and Trombulak 1986). In addition, there are large differences in testis size between the mysticetes and the odontocetes (Kenagy and Trombulak 1986; Aguilar and Monzon 1992). There are a number of possible explanations for cetaceans possessing larger testes than terrestrial mammals. An aquatic mode of life may facilitate larger testes due to the support of body weight in the aquatic medium or it may necessitate larger testes due to a possibly different reproductive physiology involved in internal fertilization in an aquatic medium (Kenagy and Trombulak 1986). Furthermore, cetaceans as a group may show more frequent copulations than other mammals and thus exhibit larger testes (Kenagy and Trombulak 1986; Connor *et al.* 2000). However, marsupials have significantly smaller testes than eutherian mammals, but within their range the testis sizes of the marsupials are still indicative of the different mating systems mentioned above (Rose *et al.* 1997). Thus relatively small testes in cetaceans probably still indicate a monogamy or extreme polygynous mating system, whereas

Table 8.2 Proposed male mating strategies for different species of odontocetes based on testis size, sexual dimorphism, degree of scarring and group size. Testis size is expressed as a percentage of the total body weight.

	Criterion	*Dusky dolphin* [1]	*Vaquita* [2]	*Harbor porpoise* [3]	*Common dolphin* [4]	*Hector's dolphin* [5]	*Bottlenose dolphin* [6]	*Humpback dolphin* [7]	*Dwarf sperm whale* [8]	*Pygmy sperm whale* [9]	*Sperm whale* [10]	*Short-finned pilot whale* [11]	*Ziphiidae* [12]
Testis size (% of body size)	Small						1	0.7	2	1.7	0.01-0.05		√
	Medium		5	3-4	4.2	2.9							
	Large	8.5											
Sexual dimorphism	Males larger than females							√			√	√	√
	Little or no sexual dimorphism				√		√		√	√			
	Females larger than males		√	√									
Group size	Solitary or small groups		√	√		√		√	√	√			
	Medium sized schools	√					√					√	√
	Large schools				√								
Scarring	Little or no scarring	√	√	√	√	√	√	√	√	√		√	
	Extensive scarring										√		√
Proposed mating strategy	SC	SC	SC	SC	R	R	?	R	R	R	JH	H	

Table 8.2 Contd. ...

Table 8.2 Contd. ...

	Criterion	*Franciscana*[13]	*Estuarine dolphin*[14]	*Tucuxi*[15]			*Right whale*[16]	*Gray whale*[16]	*Humpback whale*[16]
Testis size (% of body size)	Small	0.12					1.31	0.27	0.1
	Medium		3.3	2.5-5					
	Large								
Sexual dimorphism	Males larger than females								
	Little or no sexual dimorphism	√	√	√					
	Females larger than males						√	√	√
Group size	Solitary or small groups	√	√	√			√	√	√
	Medium sized schools								
	Large schools								
Scarring	Little or no scarring	√	√	√	Aggressive interactions	Little or none	√		
	Extensive scarring					Some	√		√
Proposed mating strategy		SM	MM	P			SC	SC	SC

SC=sperm competition; R=roving males; JH=joint harem; H=harem; ?=unknown.
SM=serial monogamy; MM=multimale mating system; P=polyandrous; SC=sperm competition.
[1]van Waerebeek and Read 1994; Carwardine 1995; [2]Hohn *et al*. 1996; [3]Gaskin *et al*. 1984; Carwardine 1995; Read and Hohn 1995; Hohn *et al.* 1996; Fontaine and Barrette 1997; [4]Cockcroft 1993; [5]Slooten 1991; Carwardine 1995; Slooten and Dawson 1994; Dawson, pers.com.; [6]Wells *et al.* 1987; Cockcroft 1993; [7]Cockcroft 1993; [8]Plön 2004; [9]John Heyning, unpubl. data; [10]Best *et al.* 1984; Gaskin *et al*. 1984; Kato 1984; MacLeod 1998; [11]Kasuya and Tai 1993; Magnusson and Kasuya 1997; MacLeod 1998; Kasuya, pers. com.; [12]Aguilar and Monzon 1992; MacLeod 1998.[13]Rosas and Monteiro-Filho 2001; [14]Weber Rosas and Monteiro-Filho 2002; [15]Best and da Silva 1984; [16] Brownell and Ralls 1986

relatively large testes are assumed to indicate frequent copulations and sperm competition.

In most mysticetes the combined testis weight makes up less than one percent of the total body mass, except in *Eubalaena* spp. (Right whale), where it comprises up to 1.31 percent of the body weight (Brownell and Ralls 1986). Although a similar percentage has been reported for *Sousa chinensis* (Humpback dolphin) (0.7 percent) and *Tursiops truncatus* (one percent) (Cockcroft 1993), most odontocetes have a somewhat larger testis weight to body weight ratio (Table 8.2). In *Cephalorhynchus hectori* (Hector's dolphin) the testes make up 2.9 percent of the total body weight (Slooten 1991) (Table 8.2). Furthermore, values of 3.5 percent and three to four percent have been reported for *Phocoena phocoena* (Gaskin *et al.* 1984; Read 1990b), 4.2 percent for *Delphinus delphis* (Cockcroft 1993), almost five percent in *P. sinus* (Hohn *et al.* 1996), and five percent for *Sotalia fluviatilis* (Tucuxi) (Best and da Silva 1984) (Table 8.2). *Lagenorhynchus obscurus* (Dusky dolphin) has testes weighing up to 8.5 percent of the total body weight, amongst the highest recorded for mammals (Van Waerebeek and Read 1994) (Fig. 8.3; Table 8.2).

There is a difference between sexual and social maturity in a number of cetacean species. Social maturity has been defined as the stage when males may gain access to receptive females and successfully fertilise them (*sensu* Best 1969; Kasuya and Marsh 1984; Desportes *et al.* 1993; Kasuya *et al.* 1997). In some species exemplified by *Globicephala melas*, sexually mature males may be capable of producing sperm, but may not reach social maturity until a later stage (Desportes *et al.* 1993). In *Tursiops truncatus*, only males older than 21 years appear to sire calves (Duffield and Wells 2002). An extreme example is *Berardius bairdii*, in which testis weight continues to increase for almost 20 years after ASM (Kasuya *et al.* 1997). In other species, such as *Globicephala macrorhynchus*, full sexual maturity (based on histology) and social maturity occur at the same time (Kasuya and Marsh 1984). A substantial increase of combined testis weight after ASM of 7.62 fold is reported for *G. melas* (Desportes *et al.* 1993) and an increase of 4.3 and 11.7 fold has been reported for *Kogia breviceps* and *K. sima*, respectively (Plön 2004) .

Fig. 8.3 Testes of different cetacean species. **A**. Bowhead whale (*Balaena mysticetus*) (photo courtesy of J. C. George, North Slope Borough, Department of Wildlife Management, Barrow, Alaska, USA); **B**. Dusky dolphin (*Lagenorhynchus obscurus*) (photo courtesy of Andy Read, Duke University, NC, USA); **C**. Whole male uro-genital apparatus of a Striped dolphin (*Stenella coeruleoalba*) (photo courtesy of Bruno Cozzi, University of Padova, Italy). **D**. Cross section through Harbour porpoise (*Phocoena phocoena*) testes (left and right testis, respectively). The autopsy of the 1.4 m long animal indicated that it was killed as a result of fatal interactions with bottlenose dolphins; one of the many hits was found in the right testis (photo courtesy of Rod Penrose, Collaborative UK Marine Mammal and Marine Turtle Strandings Project, Cardigan, West Wales, UK).

Colour

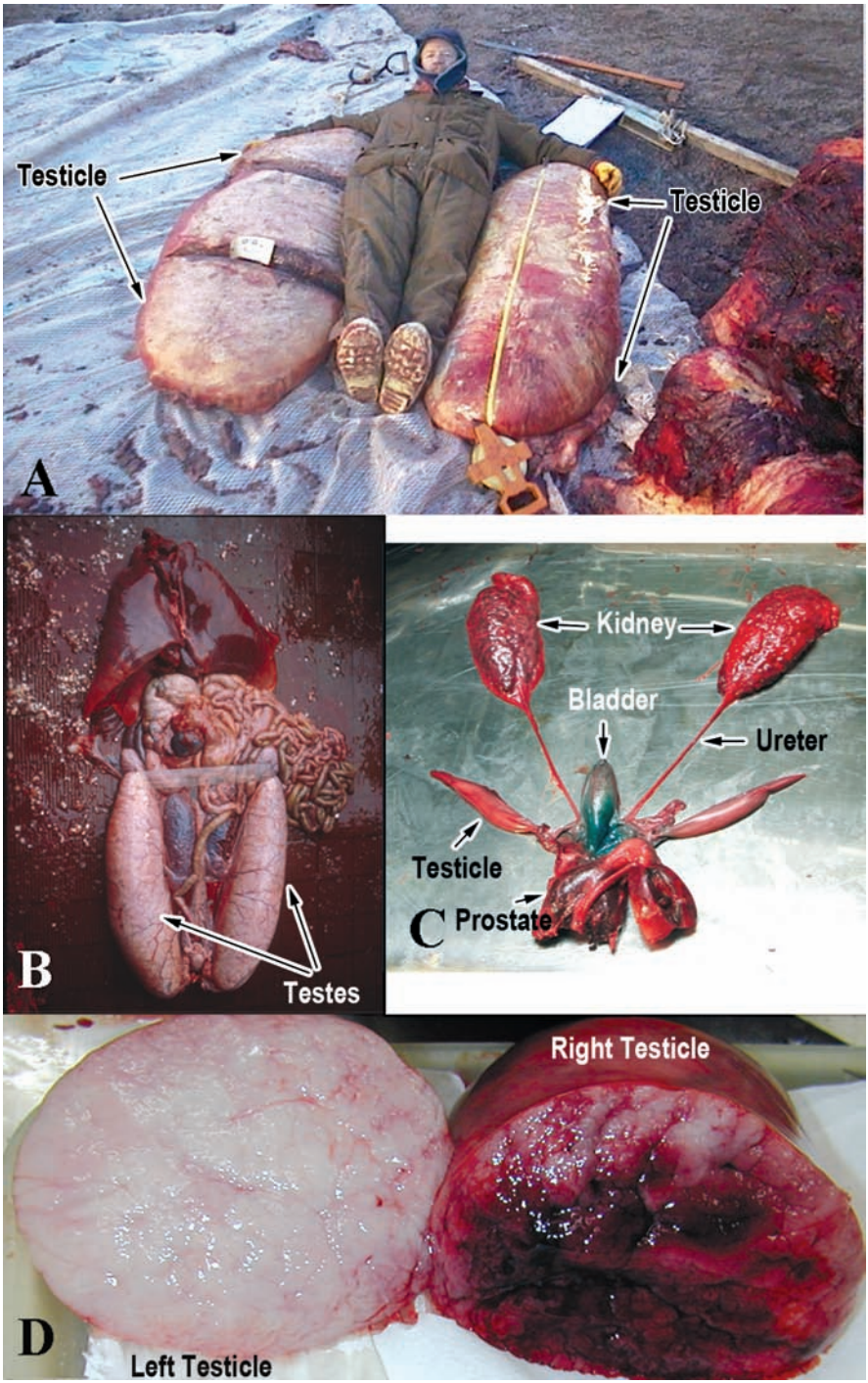

Fig. 8.3

8.5.3 Sexual Dimorphism

Sexual dimorphism is another important indicator of the mating system of a species (Table 8.2). Cetaceans generally lack secondary sexual characters, with the exception of *Monodon monoceros* (Narwhal), where only males possess an up to 2.6 m long tusk (Gerson and Hickie 1985). Sexual dimorphism is most pronounced in the largest odontocete, the Sperm whale (Best *et al.* 1984), with males being up to five metres longer than females (Leatherwood and Reeves 1983). Sexual dimorphism in the shape and color of the melon is reported for *Hyperoodon ampullatus* (Northern bottlenose whale) (Bloch *et al.* 1996) and in the coloration of the patch around the genital area for *Cephalorhynchus hectori* and *C. commersonii* (Commerson's dolphin) (Slooten and Dawson 1994). In most medium-sized odontocetes sexual dimorphism may be expressed as differences in girth and weight rather than length (Hohn and Brownell 1990; Cockcroft and Ross 1990; Cockcroft 1993; Tolley *et al.* 1995). Although only slight or no differences in asymptotic length are found between the sexes in *Tursiops truncatus* (Hohn and Brownell 1990; Cockcroft and Ross 1990), males are about 30% heavier (Cockcroft and Ross 1990; Cockcroft 1993), more robust and possess larger appendages than females of the same length (Tolley *et al.* 1995). Thus it appears that robustness rather than length plays a role in male-female (Cockcroft and Ross 1990) as well as male-male intraspecific interactions (Tolley *et al.* 1995). This may well be the case for a number of other cetacean species, for example *Delphinus delphis* males are about 10% heavier than females (Cockcroft 1993). In the smallest odontocetes, namely the phocoenids and the delphinid genus *Cephalorhynchus*, and in the large baleen whales sexual dimorphism is reversed, with the females being larger than the males (Brownell and Ralls 1986; Read and Gaskin 1990; Slooten 1991; Connor *et al.* 2000). There is some indication of reversed sexual dimorphism in the two *Kogia* species (Plön 2004) (Table 8.2). Mammals in which females are larger than males have a variety of social systems ranging from monogamy to harems (Ralls 1976; Brownell and Ralls 1986; Clapham 1996) and in this respect sexual dimorphism gives no indication as to the mating system of the species. However, sexual dimorphism in cetaceans has not been very well researched in the past and should be the subject of further investigation to shed more light on the mating system.

8.5.4 Group Size

Group size influences the mating system of cetaceans (Evans 1987; Cockcroft 1993) as do social structure and association patterns (Evans 1987; Wells *et al.* 1987). Large testes and little sexual dimorphism in conjunction with large schools, as found in *Delphinus delphis*, are attributed to sperm competition, whereas small testes, great sexual dimorphism and small group sizes, as found in *Sousa chinensis*, are thought to indicate that larger males dominate smaller males and deny access to females (Cockcroft 1993) (Table 8.2).

8.5.5 Intra-specific Scarring

As already established, knowledge about the degree of sexual dimorphism represents a starting point for the exploration of cetacean mating systems because it indicates the degree of male-male competition and the role it plays in determining male reproductive success (Connor *et al.* 2000). However, the extent of body scarring resulting from intrasexual fights is thought to present a further clue (Table 8.2). Depending on the type of scarring, they are indicators of intraspecific fighting in a number of odontocetes (Best *et al.* 1984; Heyning 1984; Kato 1984; Gerson and Hickie 1985; MacLeod 1998) (Table 8.2). In some instances body scars are an indicator of the male maturity status (Kato 1984) and "quality" (Gerson and Hickie 1985; MacLeod 1998) and thus indirectly add information about the breeding system of a species (Kato 1984; MacLeod 1998). A number of odontocete species show a clear reduction in the number of teeth (Gaskin 1982; Heyning 1984; MacLeod 1998) and this is especially obvious in teuthophagous species, such as *Physeter* (Kato 1984) and both *Kogia* species, and reaches an extreme in the ziphiids (Mead 1984; Heyning and Mead 1996; MacLeod 1998). Recent studies suggest that the ziphiids employ suction-feeding in which prey are sucked into the mouth in a vacuum-like fashion without the need for teeth to grasp or chew the prey (Heyning and Mead 1996). A similar mechanism is considered likely in both genera of the sperm whales (Heyning and Mead 1996; Bloodworth and Marshall, 2005) and a number of other odontocete species (Norris and Møhl 1983). Thus in species that use suction-feeding, the retained teeth might play a role in social interaction (Heyning 1984; Kato 1984; MacLeod 1998; Connor *et al.* 2000), since in some species the remaining teeth show an adaptation for use as weapons (Best *et al.* 1984). Body scarring is reported for a number of odontocete species (McCann 1974; MacLeod 1998) and even for one mysticete (Chu and Nieukirk 1988). However, Connor *et al.* (2000) point out that it is likely that most serious fighting in cetaceans may occur by means of strikes with the peduncle, flukes or other body parts. This would not result in any obvious wounds and thus, in the absence of any direct behavioral observations, the examination of body scars may lead to an underestimation of the frequency and severity of intra- and interspecific aggression (Connor *et al.* 2000).

8.5.6 Other Factors Influencing Mating Strategy

The factors discussed above all influence mating strategy, but do so somewhat from the male's "point of view." A range of factors such as the length (Whitehead 1990; Sandell and Liberg 1992; Magnusson and Kasuya 1997) and synchrony (Best and Butterworth 1980) of female estrus, as well as the group density and dispersion of the females (Best and Butterworth 1980; Krebs and Davies 1981; Whitehead 1990; Sandell and Liberg 1992; Magnusson and Kasuya 1997; Connor *et al.* 2000) have a profound effect on the mating strategy of a species and are determined by the biology of the

female. For example, the density and dispersion of females largely determines the difference between a harem strategy and a roving male strategy (Best and Butterworth 1980; Krebs and Davies 1981; Whitehead 1990; Sandell and Liberg 1992; Magnusson and Kasuya 1997). See also Chapter **13**, this volume.

8.5.7 Proposed Mating Systems

Combining the aforementioned factors that influence male mating strategies, researchers have proposed a number of different mating strategies for cetaceans, ranging from floating leks in *Megaptera novaeaenglia* (Clapham 1996), multimale polygynous breeding systems (or joint harems) in both the southern and northern *Globicephala macrorhynchus* off Japan (Kasuya and Marsh 1984; Kasuya *et al.* 1993; Kasuya and Tai 1993; Magnusson and Kasuya 1997), to a roving male strategy proposed for *Physeter macrocephalus* (Best and Butterworth 1980; Whitehead 1990; Magnusson and Kasuya 1997) and *Tursiops truncatus* (Wells *et al.* 1987; for a review see Connor *et al.* 2000). Based on the comparatively small testis size of around two percent of the total body weight, indicating moderate copulation frequency (as opposed to high copulation frequency in sperm competition), potentially reversed or small sexual size dimorphism, little scarring and small group size, a promiscuous or polygynous mating system with more than one male gaining access to females is suggested for either *Kogia* species (Plön 2004). Where females range widely and are solitary or occur in small groups, as is the case in *Kogia,* males may employ a roving strategy in search of receptive females in order to maximize their reproductive opportunities rather than monopolizing and fighting over a number of females (Connor *et al.* 2000). The roving male strategy is also suggested for *P. macrocephalus* (Whitehead 1990; Magnusson and Kasuya 1997) (Table 8.2), although there is evidence for male-male aggressive interaction in this species (Kato 1984). *Tursiops truncatus* also shows little sexual dimorphism (Wells *et al.* 1987; Tolley *et al.* 1995) and has relatively small testes, which make up one percent of the total body weight (Cockcroft 1993). Extensive studies on *T. truncatus* in Sarasota, Florida, show that male pairs may adopt a roving strategy in which one male may dominate the other, but both mate with receptive females without showing any aggressive interaction (Wells *et al.* 1987) (Table 8.2). Alliance formation between males is only observed in habitats where males encounter each other frequently (Connor *et al.* 2000). Thus if a male has a low probability of encountering a rival male while with a female, he would be better off without an ally (Connor *et al.* 2000).

One exception to the phenomenon that larger testes are found in cetaceans compared to terrestrial mammals (Aguilar and Monzon 1992) appears to be the ziphiids, which have some of the smallest testes sizes recorded for odontocetes and also show some of the highest degree of intraspecific scarring (MacLeod 1998). This suggests a mating system where males fight over access to females, like a harem (Connor *et al.* 2000) (Table 8.2).

Sperm competition may occur in some mysticetes (Brownell and Ralls 1986) as well as in some odontocetes like *Phocaena sinus* (Hohn *et al.* 1996), and *Delphinus delphis* (Cockcroft 1993). Based mainly on large testis size, it is suggested that *Lagenorhynchus obscurus* may have a promiscuous mating system with sperm competition, and this is supported by the fact that little intraspecific scarring is observed on the males (Van Waerebeek and Read 1994) (Table 8.2). Similarly, the large testis size in *P. sinus* together with the small group size and reversed sexual dimorphism also suggest sperm competition for this species (Hohn *et al.* 1996) (Table 8.2).

Although it may be assumed that mating systems do not vary within a species, the mating systems of *Tursiops truncatus* may vary slightly between populations (Tolley *et al.* 1995; Connor *et al.* 2000) and geographical variation in the mating system is reported for *Stenella longirostris* (Perrin and Mesnick 2003). Although these data indicate that cetaceans, like most mammals, generally have some form of polygynous or promiscuous mating system (see Evans (1987) for a summary) (Krebs and Davies 1981), the use of techniques like molecular analysis have, in recent years, provided hard evidence to support these observations (Amos *et al.* 1993; Clapham and Palsbøll 1997; Duffield and Wells 2002). The analysis of testes size, group size and sexual dimorphism may give a good first indication as to the mating system of a species, but in some instances it remains speculative and only behavioral observations in the wild (Slooten *et al.* 1993) and genetic analysis will eventually give unequivocal evidence as to which males dominate the matings (Amos *et al.* 1993; Duffield and Wells 2002). In these cases, genetic analysis of paternity may lead to interesting results.

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CHAPTER 9

The Mature Cetacean Spermatozoon

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9.1 INTRODUCTION

The morphological features (i.e. descriptors beyond total length) of cetacean spermatozoa have been reported (albeit often minimally) for only 14 of the nearly 80 extant species. These 14 species represent six families, one from the suborder Mysticeti and five from the suborder Odontoceti (Matano *et al.* 1976; Fleming *et al.* 1981; Mogoe *et al.* 1998; Beilis *et al.* 2000; Kita *et al.* 2001; Miller *et al.* 2002; Fukui *et al.* 2004; Meisner *et al.* 2005). Two investigations have been comparative studies of cetacean spermatozoa, one employing light microscopy (LM) and scanning electron microscopy (SEM) (Kita *et al.* 2001) and the other using LM, SEM and transmission electron microscopy (TEM) (Miller *et al.* 2002). In addition, Meisner *et al.* (2005) included five cetaceans in their SEM examination of sperm head morphology of 36 mammalian species. Although most accounts have been of epididymal spermatozoa, Mogoe *et al.* (1998) described sperm from the vasa deferens. Others examined spermatozoa collected using electroejaculation (Fleming *et al.* 1981) or trained medical behaviors (Miller *et al.* 2002). Fukui *et al.* (2004) noted the occurrence of significant morphological changes secondary to cryopreservation. This chapter reviews the morphological and ultrastructural characteristics reported for cetacean spermatozoa and provides new information on spermatozoa from some species investigated previously, as well as from five other species, including representatives of two additional families. Lastly, we offer observations on the ultrastructural effects of cryopreservation.

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9.2 STRUCTURE OF MAMMALIAN SPERMATOZOA

Terrestrial mammalian spermatozoa are composed of a head and a tail; the head consists of acrosomal and postacrosomal regions and the tail includes a neck, midpiece, principal piece and terminal piece (Fawcett 1975; Barth and Oko, 1989; Eddy and O'Brien 1994). Across species, spermatozoal size generally is not proportional to body size of adult males, and sperm head morphology is variable (Forman 1968; Martin *et al.* 1975; Gould 1980; Meisner, 2005). The same can be said for the cetacean spermatozoa examined thus far (Matano *et al.* 1976; Fleming *et al.* 1981; Mogoe *et al.* 1998; Beilis *et al.* 2000; Kita *et al.* 2001; Miller *et al.* 2002; Fukui *et al.* 2004; Meisner, 2005). Terminology for sperm structure has been standardized with respect to the various observational techniques (LM, SEM, and TEM), although individual researchers occasionally modify the standard nomenclature (Fawcett, 1975; Matano *et al.*, 1976; Barth and Oko, 1989; Meisner *et al.* 2005). Eddy and O'Brien (1994) have presented a comprehensive review of mammalian spermatozoal structure, including terminology, plasma membrane domains, cytoskeletal details, chemical composition, and speculations on the functions of the various components.

9.2.1 Light Microscopy

In light microscopy, head length includes the portion of the sperm occupied by the nucleus, plus any apical extension of the acrosome, and is measured from the top of the acrosome to the base of the nucleus. Head width usually is measured across the broadest point. Depending on the condition of the sperm (i.e., presence or absence of the plasma membrane and/or the acrosome) and the stains used, the upper (acrosomal) region and the lower (postacrosomal) region of the head may be distinguished and measured, and an acrosome/postacrosome ratio calculated. Measurement of the neck is frequently omitted, as it is very short and often inconsistently discernible by light microscopy. Midpiece is usually understood to refer to everything from the base of the nucleus to the start of the principal piece. The midpiece, principal piece and terminal piece (end piece) may be reported as a single unit (the tail) simply because of the difficulty in discerning these regions. The majority of sperm in LM preparations are present in *en face* orientation.

9.2.2 Electron Microscopy

Transmission and scanning electron microscopy reveal a wealth of information on mammalian sperm structure and incorporate a plethora of terminology. Fawcett (1975) and Eddy and O'Brien (1994) describe in depth the morphology and ultrastructure of mammalian sperm. Barth and Oko (1989) review the LM and TEM structure of normal and abnormal *Bos taurus* sperm. Matano *et al.* (1976) and Meisner *et al.* (2005) detail SEM morphology and terminology, especially of the head. Some of this terminology is defined below and illustrated in Figures 9.1, 9.2, and 9.3.

9.2.2.1 Head

In TEM, the head is defined as extending from the apex of the acrosome to the posterior ring, where the plasma membrane and the nuclear envelope fuse to form a narrow groove between the head and the neck (Figs. 9.1 and 9.2) (Fawcett 1975; Barth and Oko 1989; Eddy and O'Brien 1994). Because the posterior ring may or may not be visible by SEM, the length of the sperm head in SEM studies may be measured from the apex to the posterior ring (Meisner *et al.* 2005) or more likely, to the more easily distinguished base of the nucleus. Head width is generally measured at the broadest level, although Meisner *et al.* (2005) define head width as the breadth of the head at the midpoint of a line drawn from the tip of the head to the posterior ring. In many terrestrial mammals, the sperm head is differentiated into dorsal and ventral surfaces (Matano *et al.* 1976). The dorsal surface is convex, whereas the ventral surface is concave and distinguished by a raised, U-shaped acrosomal ridge (marginal segment, apical segment, anterior band, peripheral rim) that runs along the apex and the sides of the head above the acrosomal equator (Figs. 9.1 and 9.4). The head is composed of two main regions, the anterior acrosomal region (also known as acrosomal cap or head cap) and the posterior postacrosomal region. The acrosomal and postacrosomal regions are sharply delineated by the postacrosomal sheath border (subacrosomal ring, serrated band) which lies below the posterior margin of the equatorial region of the acrosome (Fig. 9.1) and which is visible by SEM (Matano *et al.* 1976) and TEM.

The nucleoplasm throughout the head and into the neck is highly condensed and contains small, scattered vacuoles (Figs. 9.1, 9.2, and 9.3). Below the posterior ring, the peripheral nucleoplasm expands and becomes diffuse, forming the lateral diverticula or posterior nuclear space (Fig. 9.2). Within the neck, the condensed nucleoplasm from above the posterior ring extends to the implantation fossa or implantation socket (fossa) at the base of the nucleus (Fig. 9.2). This implantation fossa and the cytoskeletal structures associated with it (the basal plate or rim and the capitulum) create a strong physical link between the sperm head and the tail (Fawcett 1975; Barth and Oko 1989; Eddy and O'Brien 1994).

In the acrosomal region, the membrane-bound acrosome with its moderately electron-dense matrix lies close to the nucleus and immediately beneath the plasma membrane, although the plasma membrane may balloon away from the acrosome (Fig. 9.1). The acrosome itself is divided into a number of specialized regions or structures (Figs. 9.1, 9.3, and 9.6). The thickened acrosomal ridge – also known as the apical ridge – may be elaborated into a more-or-less extensive hood-like flap (Figs. 9.1 and 9.3; Matano *et al.*, 1976), sometimes referred to as the apical segment (Meisner *et al.*, 2005). When the apical ridge is enlarged, the acrosomal matrix is often differentiated into areas of greater and lesser electron density (Figs. 9.1 and 9.3) (Barth and Oko 1989; Matano *et al.* 1976). The apical body or perforatorium (Fig. 9.3) is a narrow, usually moderately electron-dense,

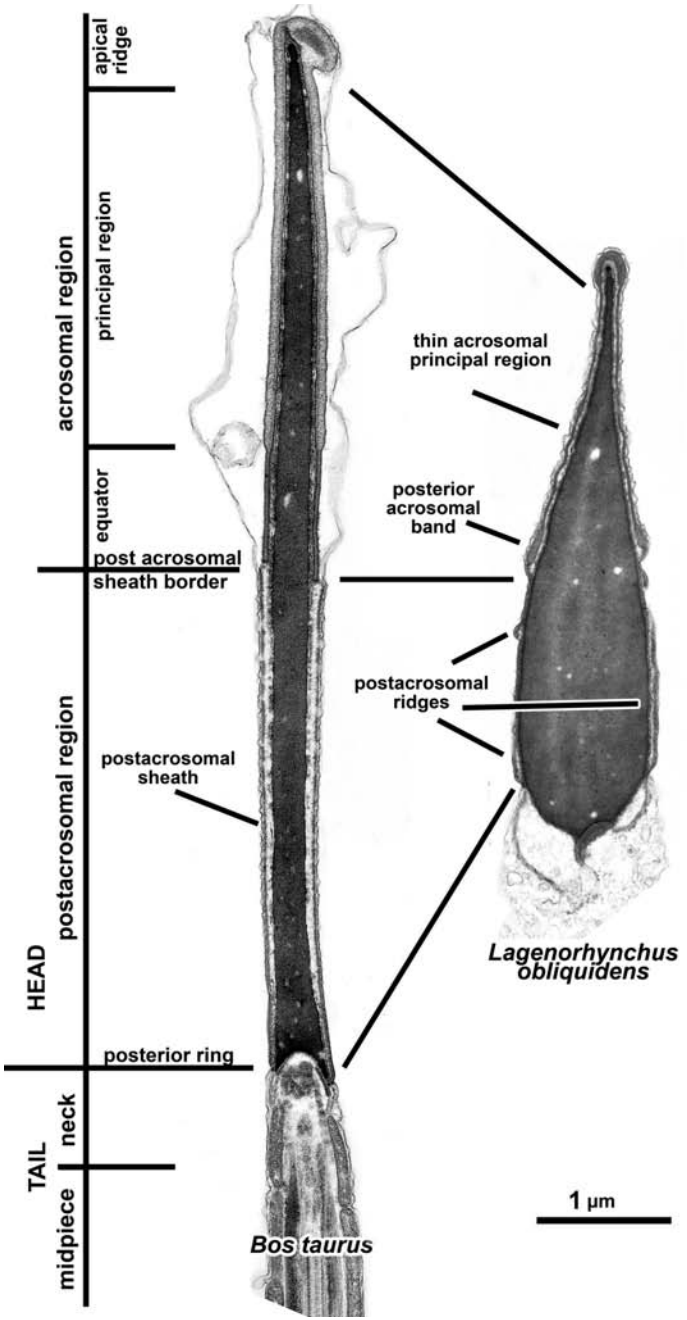

Fig. 9.1 *Bos taurus* and dolphin sperm. Electron micrographs of sagittal longitudinal sections illustrating the general head morphology of mammalian sperm.

Bos taurus sperm received from ABS Global, Inc., DeForest, WI and prepared for TEM using standard, microwave-assisted procedures. Original.

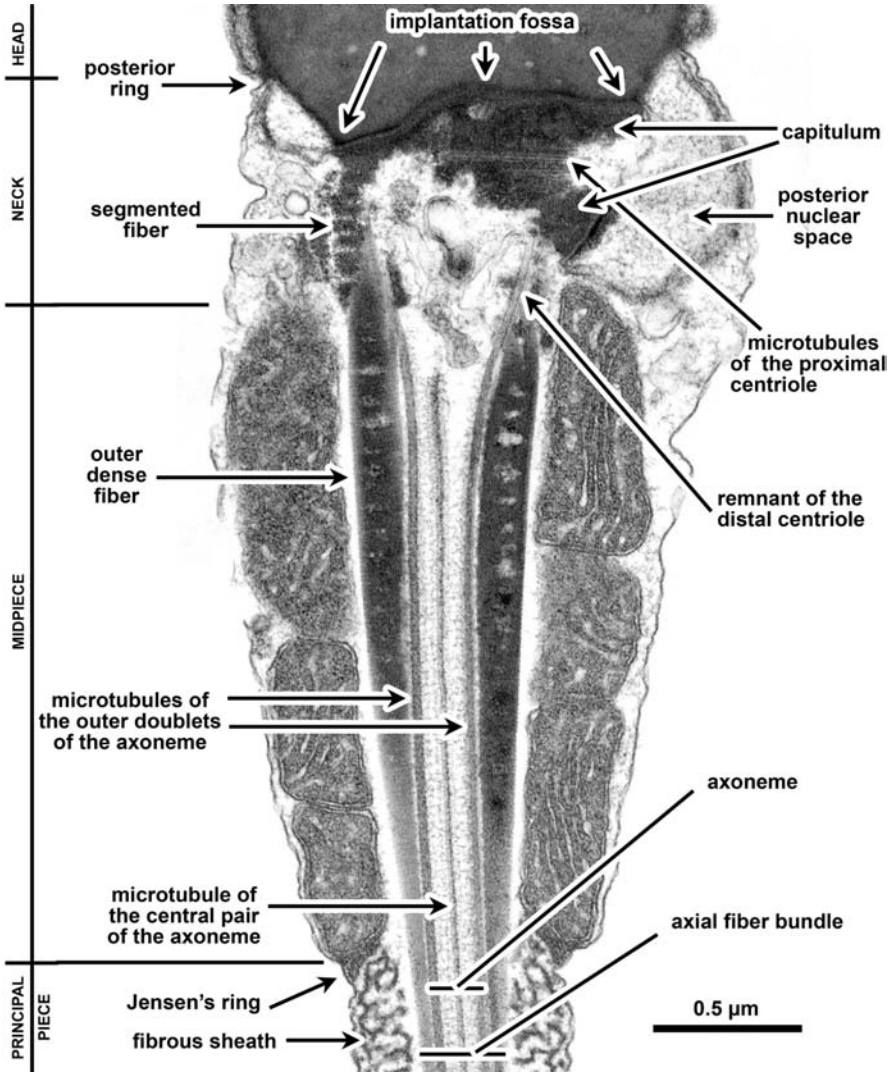

Fig. 9.2 Transmission electron micrograph of the neck and midpiece of the spermatozoa of *Lagenorhynchus obliquidens* (Pacific white-sided dolphin). The posterior ring is a narrow groove between the head and the neck. The posterior nuclear space is formed by expansion of the peripheral nucleoplasm just below the posterior ring. A complex series of nuclear and cytoplasmic densities and filaments anchor the base of the nucleus to the centrioles and striated fibers at the implantation fossa and capitulum. Nine prominent segmented fibers (segmented or striated columns, laminated fibers and plates) extend from the capitulum below the implantation fossa to the top of the midpiece where they overlap the outer dense fibers associated with each of the nine pairs of peripheral microtubules of the axoneme. Jensen's ring is a band of granular, electron-dense material that surrounds the base of the midpiece below the mitochondrial sheath. A single column of mitochondria are stacked to either side of the axial fiber bundle.

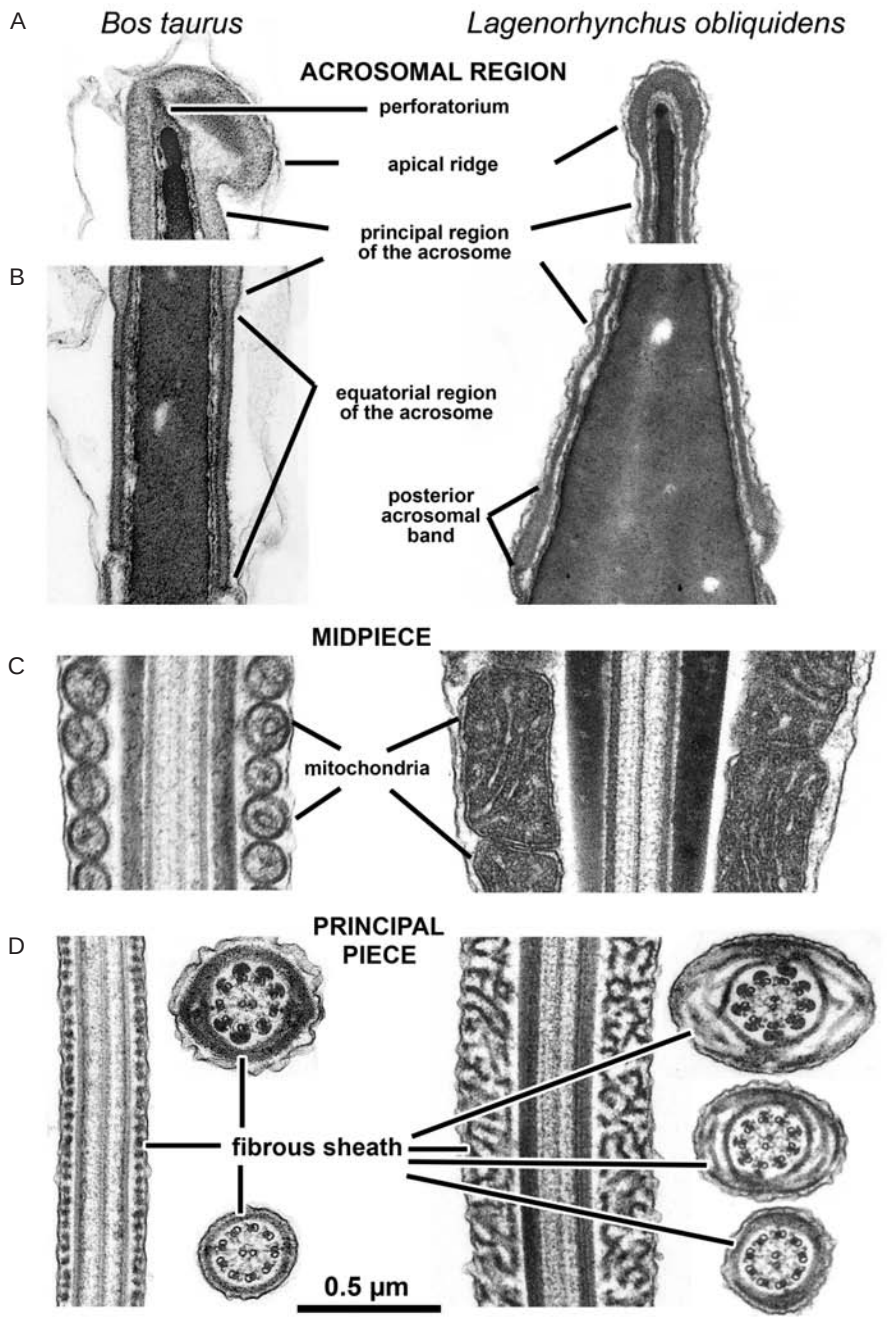

Fig. 9.3 Transmission electron micrographs of sperm in sagittal longitudinal and cross-sectional views, illustrating some differences between *Bos taurus* and a

Fig. 9.3 Contd. ...

cone-shaped, cytoskeletal structure between the acrosome and the apex of the nucleus (Fawcett 1975; Barth and Oko 1989; Eddy and O'Brien 1994). The perforatorium is a major feature of falciform rodent sperm but a minor structure in spatulate sperm (Eddy and O'Brien 1994). The principal region or segment constitutes the largest portion of the acrosome, extending from the acrosomal ridge across the *en face* surface of the acrosomal region to the top of the equatorial segment. The principal region is usually smooth when well-fixed sperm are examined by SEM, although it may appear "delicately wavy" (Matano *et al.* 1976). This region is uniformly thick for *Bos taurus* sperm (Fig. 9.1). The equatorial region or segment is the thinner posterior region of the acrosome that lies above the boundary with the postacrosomal region and forms a variably-shaped band around the base of the acrosomal region (Fig. 9.1) (Matano *et al.* 1976; Barth and Oko 1989; Eddy and O'Brien 1994; Meisner *et al.* 2005). The equatorial region of well-preserved sperm is visible by SEM approximately midway down the length of the sperm head, as its name suggests. SEM preparations may show the presence of a specialized, semi-circular region of the equator known as the equatorial subsegment (Meisner *et al.* 2005). The portion of the acrosome apical to the equator participates in the acrosomal reaction, while the equatorial region is thought to be the site of sperm-oocyte fusion (Manandhar and Toshimori 2001).

The postacrosomal region extends from the base of the acrosome to the posterior ring (Fig. 9.1). By SEM, the postacrosomal region appears flat and smooth, with small, vertical furrows immediately below the equator (Matano *et al.* 1976; Meisner *et al.* 2005). The plasma membrane of this region is associated closely with an electron-dense layer called the dense lamina (also known as the lamina densa, the outer dense lamina, the postacrosomal dense lamina, postnuclear cap, postnuclear dense body, or the postacrosomal sheath). The dense lamina often has regular periodic densities about 12 nm apart and is separated from the nucleus by a narrow, relatively uniform, electron-lucent space (Fawcett 1975; Eddy and O'Brien 1994). It is slightly more prominent in spatulate sperm than in falciform sperm (Eddy and O'Brien 1994).

Fig. 9.3 Contd. ...

representative cetacean (*Lagenorhynchus obliquidens*). **A**. Apex of the acrosomal region showing the perforatorium of *Bos taurus* sperm and the thick acrosome of the apical crest; **B**. the lower acrosomal region showing the thin equatorial region typical of *Bos taurus* and the thick posterior acrosomal band of *L. obliquidens*. **C**. The mitochondria of the spermatozoal midpiece, which are small in *Bos taurus* and large in *L. obliquidens*. **D**. Longitudinal and cross-sectional views of the principal piece; the cross sections show the principal piece at different points along its length (larger diameter cross sections are at the proximal end, progressively smaller diameters and less prominent fibrous sheaths are in progressively more distal regions). *Bos taurus* sperm received from ABS Global, Inc., DeForest, WI and prepared for TEM via standard, microwave-assisted procedures. Original.

9.2.2.2 Tail

The neck (connecting piece) extends from the posterior ring to the top of the midpiece, although mitochondria from the apex of the midpiece may extend into the neck (Figs. 9.1, 9.2, and 9.6). Within the neck at the implantation fossa, there is a complex series of nuclear and cytoplasmic densities and filaments that anchors the base of the nucleus to the centrioles and striated fibers (Fig. 9.2) (Fawcett 1975; Barth and Oko 1989; Eddy and O'Brien 1994). During spermiogenesis, the distal (longitudinal) centriole at the base of the nucleus is continuous with the axoneme of the midpiece and the tail; in many mammalian sperm, the distal centriole eventually disintegrates, although the proximal (horizontal) centriole remains intact (Eddy and O'Brien 1994). Nine prominent segmented fibers (segmented or striated columns, laminated fibers and plates) extend from the capitulum below the implantation fossa to the top of the midpiece where they overlap the dense fibers (outer dense fibers) associated with each of the nine pairs of peripheral microtubules of the axoneme (the axial filament complex) (Fig. 9.2). The segmented fibers are a prominent feature in negatively-stained TEM (NS-TEM) preparations where the plasma membrane of the neck is absent or interrupted (Fig. 9.5).

The midpiece is defined by the mitochondrial column (mitochondrial sheath), which is visible by both SEM and TEM (Figs. 9.2, 9.3, 9.4, 9.5, and 9.6). The midpiece extends from the apex of the mitochondrial column to the constriction at Jensen's ring (Fawcett 1975; Barth and Oko 1989; Eddy and O'Brien 1994). Jensen's ring (or annulus) is a band of granular, electron-dense material that surrounds the base of the midpiece below the mitochondrial sheath (Fig. 9.2). It is attached to the plasma membrane and separates the mitochondrial sheath of the midpiece from the fibrous sheath of the principal piece. In longitudinal sections, a single column of mitochondria is stacked on either side of the axial fiber bundle or core complex (the axoneme and its associated dense fibers). In general, the mitochondria are arranged in loose spirals (Figs. 9.3 and 9.5). SEM and TEM studies reveal that the number and size of the mitochondria, the number of mitochondrial helices, and the arrangement of mitochondria relative to one another may be very complex and species specific (Eddy and O'Brien 1994).

The principal piece extends from below Jensen's ring to the discontinuity formed by the loss of the fibrous sheath just above the proximal end of the narrow terminal piece. The gradually tapering principal piece of the tail consists of the axial fiber bundle and the fibrous sheath (Fawcett 1975; Barth and Oko 1989; Eddy and O'Brien 1994). The fibrous sheath is a synapomorphy of the Amniota (Jamieson 1999). It lies between the plasma membrane and the axial fiber bundle and comprises a zone of horizontal (circumferential), rib-like, electron-dense fibers (the circumferential ribs) that fuse with two thicker longitudinal columns composed of vertically oriented filaments; in Cetacea the dense fibers usually disappear some distance above Jensen's ring (Fig. 9.3).

The terminal piece (end piece) extends from its junction with the principal piece to the end of the tail. It is approximately circular in cross section

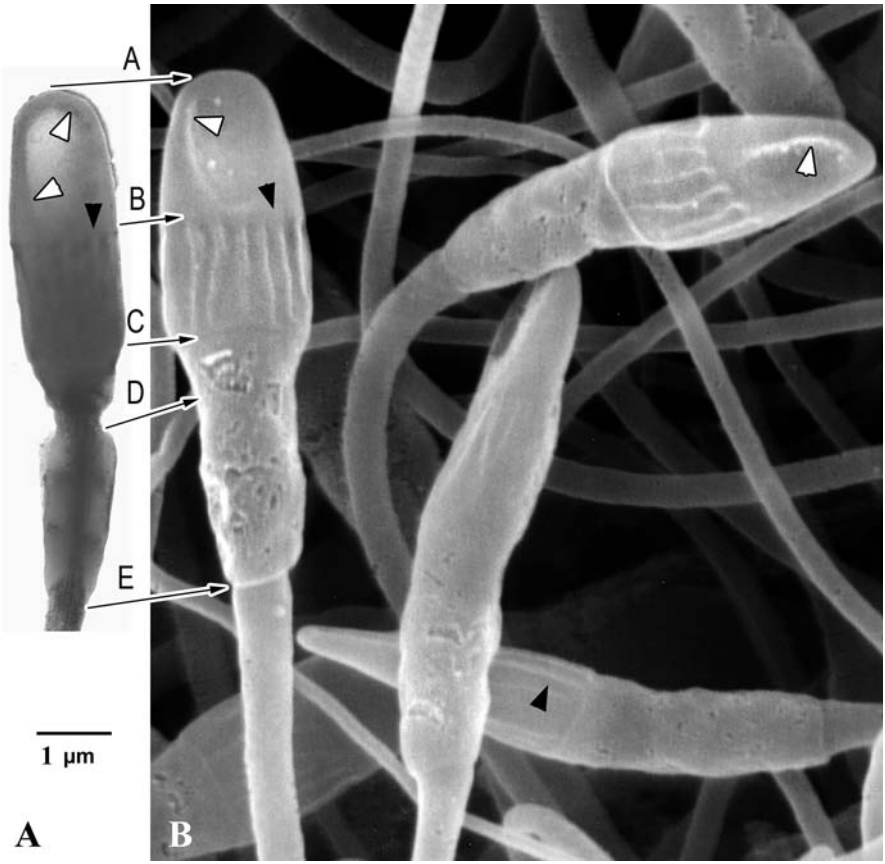

Fig. 9.4 Electron micrographs of *Lagenorhynchus obliquidens* (Pacific white-sided dolphin) spermatozoa. **A**. Negatively-stained spermatozoon. **B**. Scanning electron micrograph. Arrows indicate corresponding regions on the adjacent negatively stained and scanned sperm: (A-C) head; (A-B) acrosomal region; (B-C) postacrosomal region; (C) posterior ring; (C-D) neck; (D-E) midpiece; (E) Jensen's ring; below (C) tail; below (E) principal piece. White arrowheads, raised acrosomal band; black arrowheads, postacrosomal ridges. From Miller, D.L., Styer, E.L., Decker, S.J. and Robeck, T. 2002. Anatomia Histologia Embryologia 31: 158-168, Fig. 1.

throughout its length, has no noticeable taper, and consists solely of the plasma membrane and the axoneme. The axoneme gradually becomes disorganized, ending with a diminished number of unpaired microtubules toward the distal end of the terminal piece.

9.2.2.3 Distinguishing features of cetacean sperm

Several features distinguish cetacean sperm from sperm of terrestrial mammals (Figs. 9.1, 9.3, and 9.6). In Cetacea, there is no extension of the apical ridge into an apical crest with density differences in the acrosomal matrix, nor is there a prominent apical body (Figs. 9.1 and 9.3) (Fleming *et al.* 1981; Miller *et al.* 2002; D.L.M. and E.L.S., unpublished data). Rather, the apical ridge seems

to have evolved into a protuberance that caps the apex and sides of the acrosomal region and encircles the base of the acrosome in an area corresponding to the thin equatorial region of the acrosome in terrestrial mammalian sperm (Fig. 9.1, 9.3, and 9.6) (Fleming *et al.* 1981; Miller *et al.* 2002; D.L.M. and E.L.S., unpublished data). We will refer to this continuation of the apical ridge that encircles the base of acrosome in the area usually occupied by the thin equatorial region as the "posterior acrosomal band" and use "acrosomal band" to refer to the apical ridge plus the posterior acrosomal band. In Cetacea, instead of being uniformly thickened as is illustrated for *Bos Taurus* sperm in Figures 9.1 and 9.3, the principal region of the acrosome is uniformly thin [e.g., *Tursiops truncatus* (Atlantic bottlenose dolphin) and *Lagenorhynchus obliquidens* (Pacific white-sided dolphin)] or it is rough [*Orcinus orca* (Killer whale)] (Miller *et al.* 2002; D.L.M. and E.L.S., unpublished data). The area occupied by the equator in terrestrial mammalian sperm is instead occupied by the posterior acrosomal band (Figs. 9.1 and 9.3). Toshimori *et al.* (1998) found that the protein equatorin (christened for its location) is found in (and limited to) the equatorial region in rats and mice and is involved in sperm-oocyte fusion. While it is unknown if equatorin exists in species other than rats and mice, immunolabeling for this protein might help to establish the nature of the equatorial region in cetacean sperm.

The postacrosomal region of the cetacean sperm examined thus far is smooth-surfaced like that of *Bos taurus*, except for Delphinidae. Thus far, the sperm from Delphinidae, excluding *Orcinus orca*, have a postacrosomal region characterized by a series of relatively evenly-spaced longitudinal postacrosomal ridges (Fig. 9.1 and 9.4) (Flemming *et al.* 1981; Kita *et al.* 2001; Miller *et al.* 2002; Meisner *et al.* 2005; D.L.M. and E.L.S., unpublished data). These ridges contain pads of electron-dense material intimately associated with the plasma membrane and separated from the nucleus by a fairly uniform electron-lucent space (Fig. 9.6). Occasionally, the subplasmalemmal pads of the ridges are seen to consist of the regular densities present in the dense lamina of *Bos taurus* sperm (Flemming *et al.* 1981; Miller *et al.* 2002; D.L.M. and E.L.S., unpublished data). Between the ridges, the plasma membrane is appressed to the nuclear envelope (Fig. 9.6).

Variations in mitochondrial arrangement occur across mammalian and cetacean species (Fawcett 1970; Eddy and O'Brien 1994; Kita *et al.* 2001; Miller *et al.* 2002; Meisner *et al.* 2005). The mitochondrial sheath of *Balaena mysticetus* (Bowhead whale) sperm is reminiscent of that of *Bos taurus*: in longitudinal sections there are numerous (ca 18) mitochondria on either side of the axial fiber bundle (Fig. 9.5). These mitochondria appear to spiral around the midpiece. In other cetaceans examined by SEM and TEM [*T. truncatus*, *L. obliquidens*, *O. orca*, *Delphinapterus leucas* (Beluga whale)], there are only 3-5 relatively large mitochondria bracketing the axial fiber bundle in longitudinal sections (Fig. 9.2), and it is unclear whether these mitochondria are arranged in a spiral or in tiers (Flemming *et al.* 1981; Miller *et al.* 2002; D.L.M. and E.L.S., unpublished data).

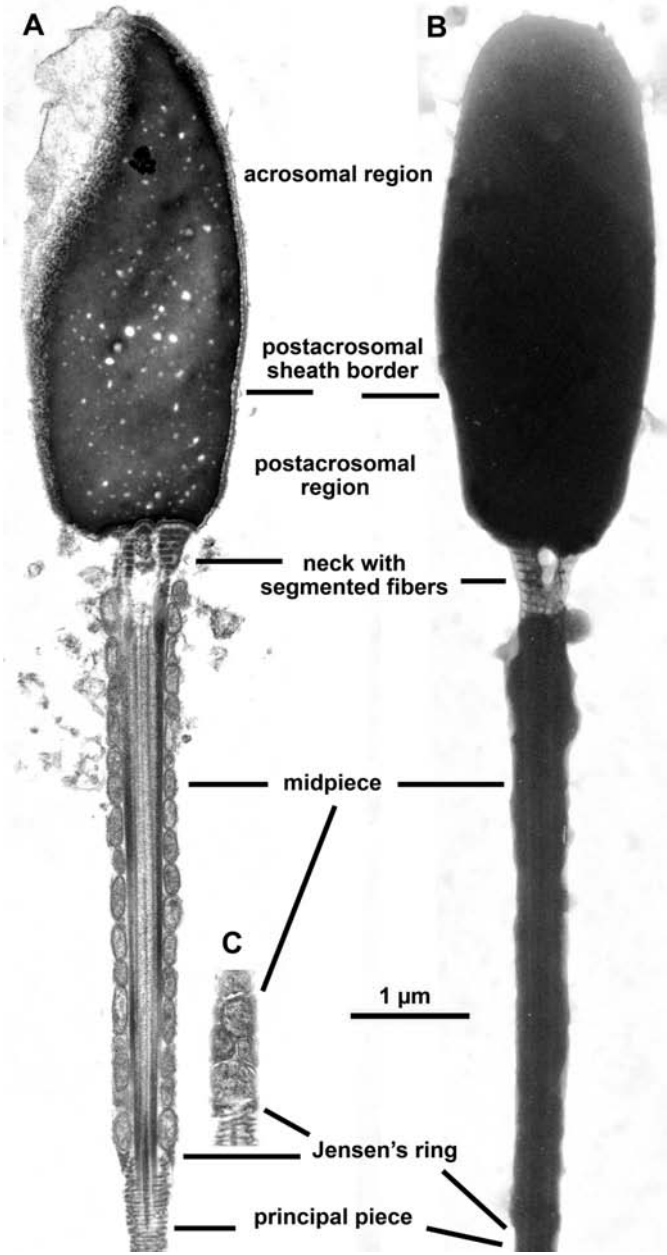

Fig. 9.5 *Balaena mysticetus* (Bowhead whale) sperm. **A**. Transmission electron micrograph (TEM) showing a *en face* longitudinal section of the sperm and major divisions of the head and midpiece. **B**. These divisions are less discernible when intact sperm are viewed by negatively-stained-TEM. **C**. TEM of a tangential longitudinal section of the distal end of the midpiece, showing the spiraling of mitochondria about the midpiece. Original.

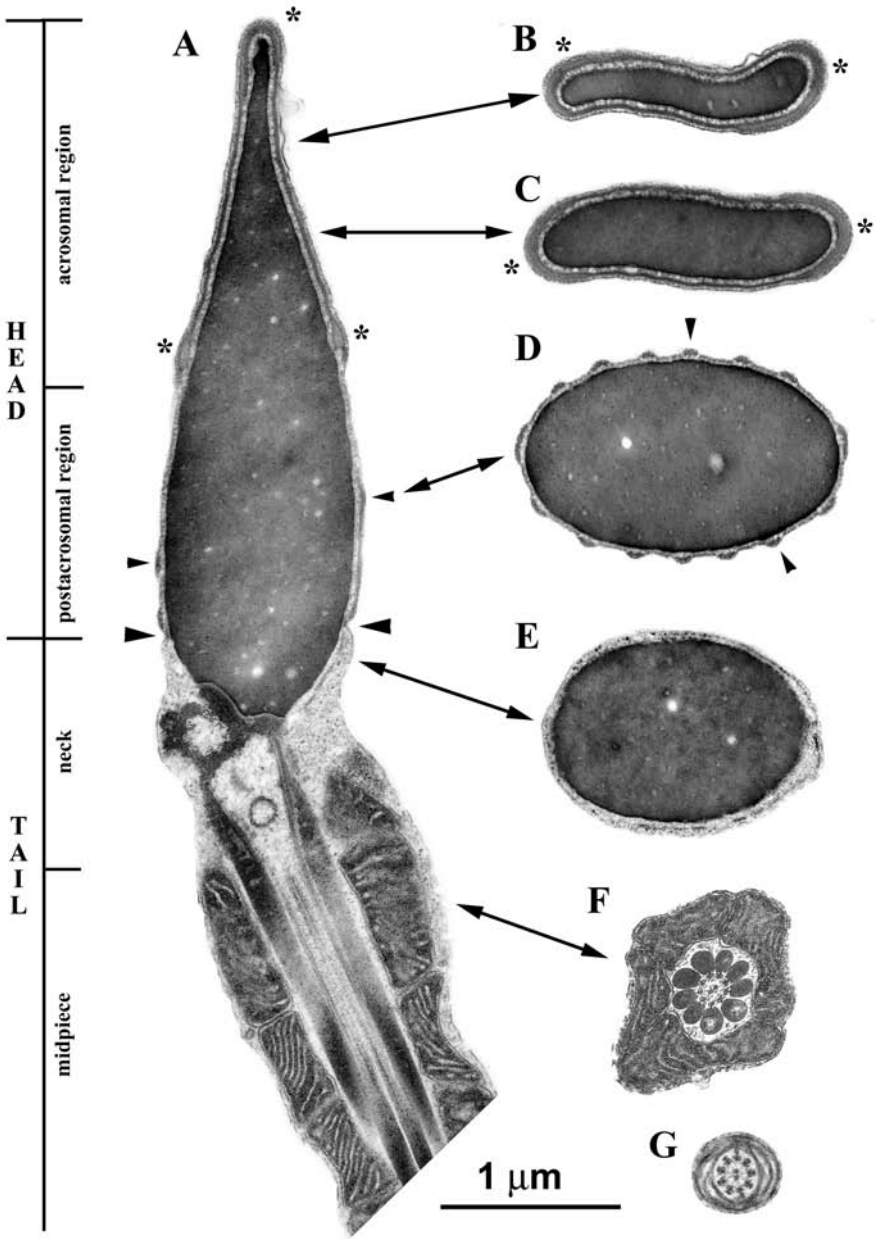

Fig. 9.6 *Tursiops truncatus* (Atlantic bottlenose dolphin). Ultrathin sagittal longitudinal section (A) and cross sections (B-F) of the head, neck and midpiece and a cross section of the proximal region of the tail. The diagram at left indicates corresponding anatomical/morphological regions of the longitudinal section. The

Fig. 9.6 Contd. ...

The fibrous sheath of the principal piece in cetacean sperm is an elaborate reticulum of thin, electron-dense strands (Flemming *et al.* 1981; Miller *et al.* 2002; D.L.M. and E.L.S., unpublished data) in contrast to the regular circumferential ribs and longitudinal columns of *Bos taurus* sperm (Fig. 9.3). In cross sections of the distal end of the principal piece, this fibrous reticulum appears as a single ring of electron-dense material. In cross sections of more proximal regions, the fibrous sheath appears as a reticulum or as several concentric rings that fuse into a single thick strand opposite the two central microtubules of the axoneme.

9.3 CETACEAN SPERMATOZOAL DIMENSIONS

A variety of preparative and observational techniques have been used to determine spermatozoal dimensions, including brightfield and phase light microscopy, SEM, TS-TEM, and NS-TEM. For example, sperm of *Lagenorhynchus obliquidens* have been examined by phase microscopy (Kita *et al.* 2001), as well as by brightfield microscopy, SEM, TS-TEM and NS-TEM (Miller *et al.* 2002; D.L.M. and E.L.S., unpublished data). With the exception of total sperm length and the length of the tail, longitudinal dimensions of the various structures are fairly consistent, regardless of the preparative technique. Total sperm length and tail length measured using NS-TEM were approximately 11-16 μm greater than when measured using phase contrast or brightfield light microscopy. This discrepancy may be related to unreliable visualization of the thin, 11 μm-long terminal piece, or to difficulties in accurately measuring the sinuous tail. Other measurement discrepancies are of lesser magnitude, e.g., 0.1-0.4 μm. Similar incongruities (0.1-0.7 μm) exist between dimensions of the head and neck of *Orcinus orca* sperm determined by brightfield light microscopy as compared to SEM or NS-TEM (Miller *et al.* 2002).

Spermatozoal dimensions for 21 cetacean species representing eight families are presented in Table 9.1 and spermatozoa exemplifying six families are illustrated in Figure 9.7. Sperm of *Physeter catodon* (Sperm whale) have the shortest average total length while those of *Balaena mysticetus* are slightly

Fig. 9.6 Contd. ...

double-headed arrows indicate the levels of the corresponding cross sections. A, longitudinal section of the head, neck and midpiece. B-G. Cross sections: B, acrosomal region showing the characteristic sigmoid shape of the thin upper portion; C, acrosomal region showing reduced curvature as the thickness increases distally; D, postacrosomal region showing 15 of 16 possible longitudinal ridges (small arrowheads); E, neck immediately below the posterior ring (large arrowhead) with areas of both condensed and diffuse nucleoplasm; F, midpiece with large dense fibers associated with the microtubule doublets of the axoneme; G, proximal region of the tail showing the fibrous sheath and the greatly reduced dense fibers associated with the microtubule doublets of the axoneme. Expanded regions of the acrosome are noted (*). Original.

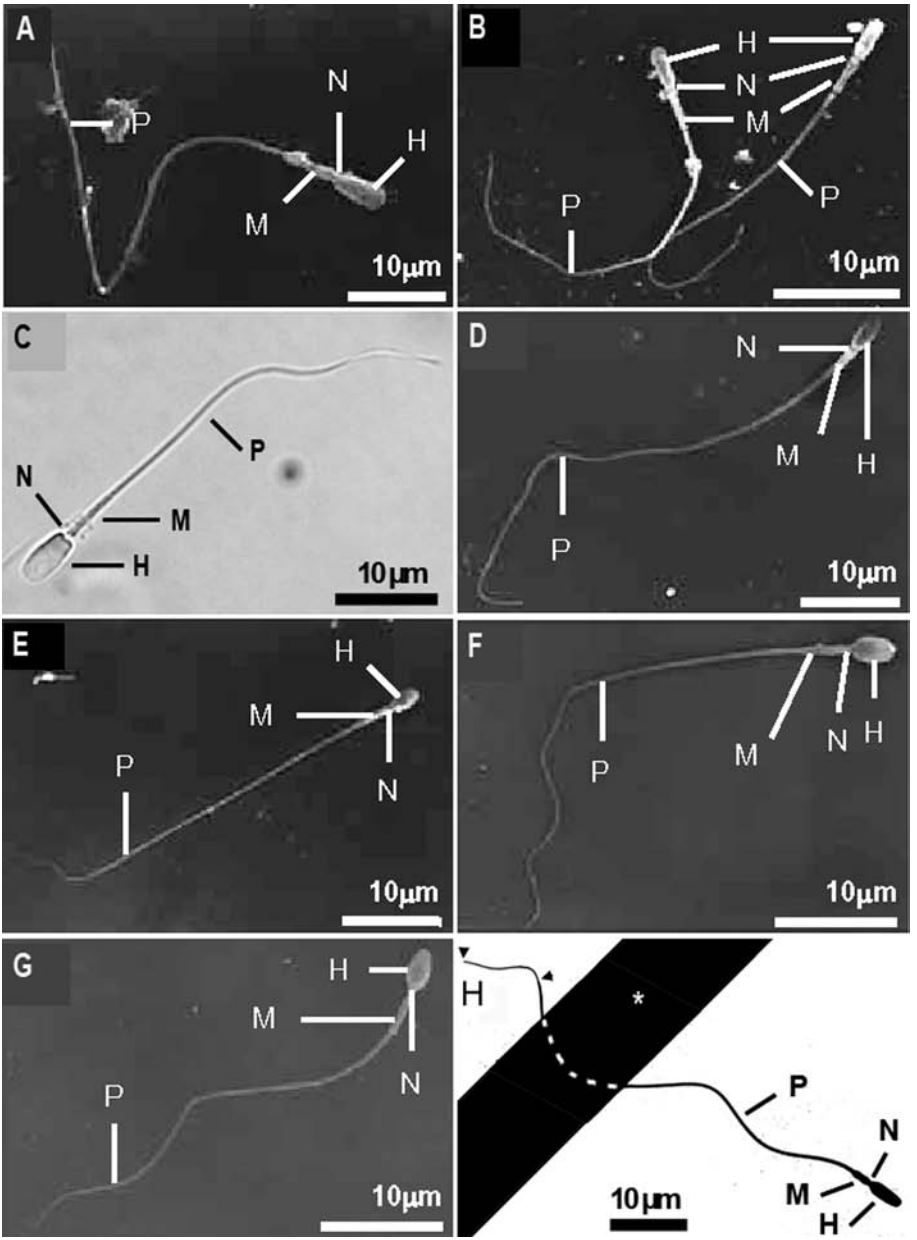

Fig. 9.7 Cetacean spermatozoa representing six families and eight species with the head (H), neck (N), midpiece (M), principal piece (P), and a specimen grid wire (*) indicated. **A**, **B**, **D**-**G** views, scanning electron microscopy; **C**, unstained brightfield light microscopy; and **H**, negatively-stained transmission electron microscopy. **A**. Balaenopteridae: *Balaenoptera brydei* (Bryde's whale), **B**. Ziphiidae:

Fig. 9.7 Contd. ...

longer (Table 9.1). In contrast, *Orcinus orca*, *Globicephala macrorhynchus* (Short-finned pilot whales) and *Grampus griseus* (Risso's dolphin) have the greatest total sperm length, followed closely by *Phocoena phocoena* (Common porpoise) (Table 9.1).

Sperm head length is quite similar among the 21 species (Table 9.1). The shortest heads are those of *Neophocaena phocaenoides* (Finless porpoises), *Balaenoptera brydei* (Bryde's whale) and *Delphinapterus leucas*, whereas the longest heads are characteristic of *P. phocoena*, *Peponocephala electra* (Melon-headed whales), *Balaena mysticetus*, *Phocoena spinipinnis* (Burmeister's porpoise), and *Balaenoptera acutorostrata* (Minke whale). The sperm heads of *Orcinus orca* and *D. leucas* are extremely wide *en face*, whereas sperm heads of *Berardius bairdii* (Baird's beaked whale) are very narrow (Fig. 9.11).

Spermatic tails range from ca 35.7-70 µm, comprising 90-95 percent of total sperm length. Tail length is directly proportional to total sperm length, corroborating previous observations on cetaceans (Kita *et al.* 2001). Cummins and Woodall (1985) report comparable findings in their survey of 284 species representing 49 percent of mammalian families and all but four mammalian orders. They also note that total length of mammalian sperm correlates inversely with maximal adult male body mass (Fig. 9.8) among all orders except Chiroptera (Cummins and Woodall 1985). Although no clear relationship is evident between total sperm length and adult male body mass within Cetacea (Fig. 9.9), to some extent larger cetaceans tend to have shorter spermatozoa. While data are fragmentary and characterized by appreciable within-family variation, three species of Balaenopteridae exhibit an unambiguous inverse relationship between estimated body mass and total sperm length. Further, the cetacean with the largest body mass, *Physeter catodon*, has the shortest sperm; whereas sperm from the smallest cetacean, *Neophocaena phocaenoides* (Finless porpoise), measure only 12 µm less than the longest sperm (*Orcinus orca* and *Grampus griseus*).

No correlation is apparent between estimated body mass and total sperm length among the seven species of Delphinidae. Whereas average total sperm length is similar across members of Delphinidae, adult male body size ranges from a maximum length and weight of 9 m and 5568 kg, respectively, for *O. orca* to only slightly more than 2 m and 110 kg for *Delphinus delphus* (Common dolphin). In contrast, there is a direct relationship between body mass and

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Fig. 9.7 Contd. ...

Berardius bairdii (Baird's beaked whale), **C**. Balaenidae: *Balaena mysticetus* (Bowhead whale), **D**. Delphinidae: *Globicephala macrorhynchus* (Short-finned pilot whale), **E**. Phocoenidae: *Neophocaena phocaenoides* (Finless porpoise), **F**. Kogiidae: *Kogia breviceps* (Pygmy sperm whale), **G**. Kogiidae: *Kogia sima* (Dwarf sperm whale), and **H**. Delphinidae: *Lagenorhynchus obliquidens* (Pacific white-sided dolphin). Figures **A**, **B**, **D**, and **E** are from Kita, S., Yoshioka, M., Kashiwagi, M., Ogawa, S. and Tobayama, T., 2001. Fisheries Science 67(3): 482-492, Fig. 2. Figures **C**, **F**, **G** and **H** are original.

Table 9.1 Reported and unpublished dimensions of cetacean spermatozoa and characteristic head shape (ND = no data).

Taxon	*Head shape (*en face*)*	*Total length (μm)*	*Head length (μm)*	*Head width maximum in* en face *view (μm)*	*Tail length (μm)*	*Source*
Mysticeti						
Balaenopteridae						
Bryde's whale *Balaenoptera brydei* (syn. *B. edeni*)	Obovate	56	3.8	2.1	52.2	Kita *et al.* 2001
Minke whale *Balaenoptera acutorostrata*	"elliptical" & "conical"	56.7	5.2	3.0	51.7	Mogoe *et al.* 1998
Humpback whale *Megaptera novaeangliae*	"flattened oval"	52.5 (mean) 32.2-61.4	4 (mean) 3-5	ND	ND	Chittleborough 1955
Balaenidae						
Bowhead whale *Balaena mysticetus*	Oblong to elongated ellipse	46.8	5.3	2.4	41.5	D.L.M. and E.L.S, unpublished data
Odontoceti						
Ziphiidae						
Baird's beaked whale *Berardius bairdii*	Lyrate "bowling pin"	51.6	4.3	1.5	47.3	Kita *et al.* 2001
Delphinidae						
Killer whale *Orcinus orca*	Almost square with reniform acrosome "Japanese fan-shaped"	74.4	4.4	3.9	70.0	Kita *et al.* 2001
	Almost square with a reniform acrosome	78	4.0	3.3	75.8	Miller *et al.* 2002
Short-finned pilot whale *Globicephala macrorhynchus*	Oblong elongated ellipse	74.1	4.6	2.0	69.5	Kita *et al.* 2001

Table 9.1 Contd. ...

Table 9.1 Contd. ...

Long-finned pilot whale *Globicephala melas*		67	ND	ND	ND	Cummins and Woodall 1985
Risso's dolphin *Grampus griseus*	Oblong elongated ellipse	74.4	4.5	2.0	69.9	Kita *et al.* 2001
Common dolphin *Delphinus delphus*	Oblong elongated ellipse	70.6	4.3	2.1	66.3	Kita *et al.* 2001
Bottlenose dolphin *Tursiops truncatus*	Oblong elongated ellipse	70.2	4.4	2.1	65.8	Kita *et al.* 2001
	Oblong to elongated ellipse	ND	3.9	2.0	ND	D.L.M. and E.L.S, unpublished data
	Elongated ellipsoid	65	4.5	2.0	60	Fleming *et al.* 1981
Pacific white-sided dolphin *Lagenorhynchus obliquidens*	Oblong elongated ellipse	69.3	4.2	2.0	65	Kita *et al.* 1981
	Oblong to elongated ellipse	62-68	4.6	1.9	60	Miller *et al.* 2002
Melon-headed whale *Peponocephala electra*	Oblong elongated ellipse	71.9	5.5	2.3	66.4	Y.E. and S.K., unpublished data
Phocoenidae						
Finless porpoise *Neophocaena phocaenoides*	"ellipsoid"	62.7	3.6	2.1	59.1	Kita *et al.* 2001
Dall's porpoise *Phocoenoides dalli*	"ellipsoid"	60.5	4.0	2.2	56.5	Kita *et al.* 2001
Burmeister's porpoise *Phocoena spinipinnis*	ellipsoid	66.0	5.3	2.6	59.7	Beilis *et al.* 2000
Common porpoise *Phocoena phocoena* (syn. *Phocoena communis)*	"4-sided to oval with rounding off of the front and rear corners" (Ballowitz, 1907)	73.8	5.8 (mean) 5.4-6.3	1.8	ca 68	Cummins and Woodall 1985; Ballowitz 1907; Retzius 1909

Table 9.1 Contd. ...

Table 9.1 Contd. ...

Kogiidae						
Pygmy sperm whale *Kogia breviceps*	Broadly elliptical "racket-shaped"	53.8	4.4	2.7	50.0	Y.E. and S.K., unpublished data
Dwarf sperm whale *Kogia sima* (syn. *K. simus*)	Lanceolate or ovate	49.3	4.3	2.4	45.0	Y.E. and S.K., unpublished data
Physeteridae						
Sperm whale *Physeter catodon* (syn. *P. macrocephalus)*	Oblong to elongated ellipse	ND	5.2	1.9	ND	Matano *et al.* 1976 (measured from photos in the paper)
	Elliptical with a blunt front end	40.6	4.9	2.7	35.7	Yamane 1936
Monodontidae						
Beluga, White whale *Delphinapterus leucas*	Almost square w/reniform acrosome	ND	3.8	3.4	ND	Miller *et al.* 2002

Ballowitz, E. 1907. Archiv für Mikroskopische Anatomie und Entwicklungsgeschichte 70: 227-237.
Beilis, A, Cetica, P., Merani, M.S. 2000. Marine Mammal Science 16: 636-639.
Chittleborough, R.G. 1955. Australian Journal of Marine and Freshwater Research 6: 1-30.
Cummins, J.M. and Woodall, P.F. 1985. Journal of Reproduction and Fertility 75: 153-175.
Fleming, A. D., Yanagimachi, R. and Yanagimachi, H. 1981. Journal of Reproduction and Fertility 63: 509-514.
Kita, S., Yoshioka, M., Kashiwagi, M., Ogawa, S. and Tobayama, T. 2001. Fisheries Science 67: 482-492.
Matano, Y., Matsubayashi, K., Omichi, A. and Ohtomo, K. 1976. Gunma Symposia on Endocrinology. 13: 27-48.
Miller, D.L., Styer, E.L., Decker, S.J. and Robeck, T. 2002. Anatomia Histologia Embryologia 31: 1-11.
Mogoe, T., Fukui, Y., Ishikawa, H. and Ohsumi, S. 1998. Marine Mammal Science 14: 854-860.
Retzius, G. 1909. Biologische Untersuchungen NF 14: 163-178.
Yamani, J. 1936. Zeitschrift für Züchtung 34B: 105-109.

sperm length in the two species of Kogiidae, *Kogia breviceps* (Pygmy sperm whale) and *K. sima* (Dwarf sperm whale). Morphological information on sperm from additional species is needed to clarify the relationship between body mass and sperm length within Cetacea.

9.4 CETACEAN SPERMATOZOAL MORPHOLOGY BY SPECIES

In general, familial variation in cetacean sperm head morphology is akin to that observed for terrestrial mammals (Forman 1968; Gould 1980). Most cetacean sperm heads are characterized as elliptical, oblong, ellipsoid, or elongated ellipses (Table 9.1 and Figs. 9.3, 9.7, and 9.11). The outstanding exceptions are the almost square heads of *Orcinus orca* and *Delphinapterus leucas*, the lyrate head of *Berardius bairdii*, and the lanceolate or ovate heads of *Kogia sima*. With the exception of *O. orca*, sperm heads among Delphindae are remarkably similar in shape and size. Many theories have been proposed to account for differences in head morphology. For example, Smith and Yanagimachi (1990) and Roldan *et al.* (1992) suggested that the origin of variability in terrestrial mammalian sperm head morphology may be traced to morphological traits of the uterus and oviduct, along with secretions from these organs. Likewise, Cetica *et al.* (1998) related differences in sperm head morphology among Dasypodidae to reproductive strategy.

This latter theory may help to explain variation in cetacean sperm head morphology. For example, sperm head morphology of *Berardius bairdii* and *Orcinus orca* differs markedly from other cetaceans. In accordance with Cetica *et al.* (1998), such sperm head morphological traits may derive from male reproductive strategy and/or the mechanism of capacitation. Likewise, in Kogiidae, sperm head shape differs between *Kogia breviceps* and *K. sima*, which tend to be sympatric species. Baskevich and Lavrenchenko (1995) report similar observations for geographically overlapping species of Muridae and surmise that variation in sperm head form likely serves as a mechanical barrier to crossbreeding between sympatric species within a family. Matano *et al.* (1976) provide supporting evidence for this theory by demonstrating that successfully crossbreeding primate species have similar sperm morphology. Thus, differences in sperm head morphology between *K. breviceps* and *K. sima* may constitute a mechanism preventing crossbreeding.

Besides diversity in head conformation, spermatozoa also display species-associated variation in midpiece size and mitochondrial arrangement. For example, midpieces of *Balaenoptera brydei*, *B. bairdii*, and *B. mysticetus* spermatozoa are longer than those of the other species. Similarly, the arrangement of mitochondria in *B. mysticetus* sperm is more like that in *Bos taurus* than in Delphinidae. Possible reasons for this include a relationship between body size and spermatic midpiece length, or between mitochondrial array and spermatozoal energy needs. Thus far, no such associations have been noted; nevertheless as hypothesized for head shape, variation in the cetacean spermatozoal midpiece may correspond to a range of reproductive

strategies. The exploration of spermatozoal energy requirements also might help to elucidate potential connections between these factors.

Hereafter, surface features of spermatozoa are described for selected species from eight cetacean families. Unless otherwise noted, when viewed *en face*, the acrosomal regions of the heads are thin, flat (i.e., smooth), and slightly concave (Figs. 9.4 and 9.11). Also unless otherwise noted, when viewed from the side, the sperm heads are roughly bottle-shaped, the postacrosomal region being relatively broad and thick and the acrosomal region tapering more or less quickly to a narrow tip (Figs. 9.4 and 9.10). Various combinations of phase contrast and brightfield light microscopy, SEM, NS-TEM, and TS-TEM have been used to observe surface features, with the most common techniques being phase contrast light microscopy and SEM (Matano *et al.* 1976; Fleming *et al.* 1981; Mogoe *et al.* 1998; Beilis *et al.* 2000; Kita *et al.* 2001; Miller *et al.* 2002).

Sperm have been characterized by TS-TEM from only five cetacean species: *Tursiops truncatus* (Fleming *et al.* 1981; D.L.M. and E.L.S., unpublished data); *Lagenorhynchus obliquidens*, *Orcinus orca* and *Delphinapterus leucas* (Miller *et al.* 2002); and *Balaena mysticetus* (D.L.M. and E.L.S., unpublished data). Poor preservation of postmortem samples hampered ultrastructural observations on *D. leucas* and *B. mysticetus* sperm. The spermatozoal ultrastructure is described and compared among the aforementioned five species (Figs. 9.4, 9.5, 9.6, 9.7, 9.10, and 9.11), as well as related to morphological observations (LM, SEM) on sperm of the same species.

9.4.1 Balaenopteridae: *Balaenoptera brydei* (Bryde's whale)

Kita *et al.* (2001) examined epididymal sperm of captured whales using phase contrast light microscopy and SEM. Sperm heads viewed *en face* are obovate or "paddle shaped" (Fig. 9.7A). The midpiece is longer than the head (also seen in *Balaena mysticetus*) as well as longer than the midpiece of either Delphinidae or Phocoenidae. The neck is thick and long compared with that of *Berardius bairdii*. The acrosome:postacrosome ratio is ca 1:1.

9.4.2 Balaenopteridae: *Balaenoptera acutorostrata* (Minke whale)

Mogoe *et al.* (1998) examined cryopreserved spermatozoa from the *vasa deferens* of captured *Balaenoptera acutorostrata* with SEM. Viewed *en face*, the heads of most sperm are "elliptical" (29 percent) or "conical" (36 percent), with both forms considered normal. Other shapes (microcephalic, round, thin, macrocephalic, and fan-shaped) are believed to be abnormal. The relatively large number of abnormal sperm heads may have been due to time (season) of collection, as Mogoe *et al.* (1998) acquired sperm during the feeding season instead of the breeding season. The acrosome:postacrosome ratio is ca 2:1, and the midpiece measures ca 3.4 μm long by 1.0 μm wide.

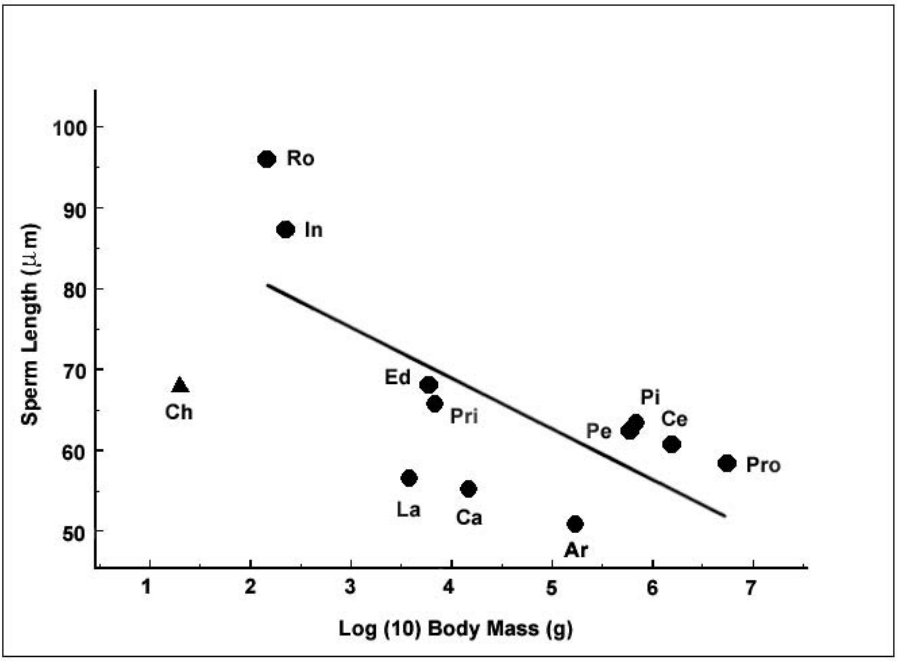

Fig. 9.8 Scatter plot of $\log_{10}$ of the mean maximum body mass (g) of adult males representing various mammalian orders vs mean sperm length; from Table 3 of Cummins and Woodall (1985. Journal of Reproduction and Fertility 75: 153-175). Trend line from 2D Linear Curvefit Plot, Axum 5.0 for Windows © 1988-96, MathSoft, Inc., Revision date 22 Aug. 1996. The twelve orders represented are: Artiodactyla (Ar), Carnivora (Ca), Cetacea (Ce), Chiroptera (Ch), Edentata (Ed), Insectivora (In), Lagomorpha (La), Perissodactyla (Pe), Pinnipedia (Pi), Primates (Pri), Proboscidea (Pro), Rodentia (Ro). Original.

9.4.3 Balaenidae: *Balaena mysticetus* (Bowhead whale)

Using brightfield LM (Fig. 9.7C), TS-TEM (Figs. 9.5 and 9.10a) and NS-TEM (Fig. 9.5), Miller and Styer (unpublished) examined epididymal sperm from a subsistence-harvested whale. Most of the heads of *B. mysticetus* sperm were shaped like oblong or elongated ellipsoids (Fig. 9.5). Occasional misshapen heads with narrow bases and ballooned apices were present in stained smears, but no double heads were observed. Heads averaged 5.3 μm long (range, 4.4-6.0) by 2.4 μm wide (range, 2.0-2.7). While poor preservation often rendered the ends of midpieces indiscernible, thereby hindering accurate measurement, midpiece lengths were comparable to or exceeded that of the heads. Occasional proximal droplets were noted. The length of the tail was approximately 41.5 μm. Many sperm had obviously short, blunt tails, presumed to be the equivalent of the folded tails observed in NS-TEM preparations. Scattered tails had small coils at the tip or were reflexed or loosely coiled from the base of the midpiece. In NS-TEM preparations, acrosome:postacrosome ratios were ca 2.4:1.

Transmission electron microscopy emphasized the poor spermatozoal preservation, which presumably resulted from degenerative changes associated with a prolonged period between death and sample collection. Plasma membranes were generally missing or fragmented but when present, were most often seen in the acrosomal equatorial region, in the postacrosomal region and in cross sections of terminal pieces. Sperm heads measured 4.3 µm long and 1.9 µm wide *en face* and 4.2 µm long by 1.0 µm wide in sagittal section. The acrosomal region was straight in cross section, rather than sigmoid as in *Tursiops truncatus* and *Lagenorhynchus obliquidens* (Figs. 9.4 and 9.6) (Miller *et al.* 2002). In sagittal and parasagittal sections, the acrosome appeared thick across the apex and along the sides of the acrosomal region, but narrow in the equatorial region above the boundary with the postacrosome. This parallels the situation in *Bos taurus* sperm, in which the acrosome narrows to a long, thin equatorial zone (Figs. 9.1 and 9.3), but differs from other cetaceans in which the acrosomal ridge continues as the thickened posterior acrosomal band above the junction of the acrosome with the postacrosome (Fleming *et al.* 1981; Miller *et al.* 2002; D.L.M. and E.L.S., unpublished data). The surface of the postacrosome was smooth and the plasma membrane was associated with a uniform dense lamina. Acrosome:postacrosome ratios were 2.9:3.3. The neck was ca 0.6-0.7 µm in length and the midpiece was slightly longer than the head (Fig. 9.5). Mitochondria were arranged in a shallow spiral of approximately 15-18 turns around the axoneme, again analogous to *Bos taurus* sperm (Figs. 9.1, 9.3, and 9.5). In longitudinal sections of the midpiece, mitochondria of *B. mysticetus* sperm were more elongated than those of *Bos taurus* sperm, which are approximately circular in outline, and were much smaller than the mitochondria of *T. truncatus* (Fig. 9.6), *L. obliquidens* (Fig. 9.2) and *Orcinus orca* (Miller *et al.* 2002; D.L.M and E.L.S., unpublished data). The proximal region of the principal piece appeared similar to that of *T. truncatus* and *L. obliquidens*, with a reticular fibrous sheath surrounding the axial fiber bundle (Miller *et al.* 2002). The nine pairs of peripheral microtubules (doublets) of the axoneme often could be discerned in cross sections of the midpiece, proximal piece and terminal piece, but the two central microtubules (central singlets) were usually unclear or absent.

9.4.4 Ziphiidae: *Berardius bairdii* (Baird's beaked whale)

Kita *et al.* (2001) examined epididymal sperm of captured animals using phase contrast light microscopy and SEM. In *en face* view, the sperm head is shallowly lyrate (i.e., somewhat resembling a peanut shell in shape) (Fig. 9.7B). When viewed from the side, the postacrosomal region is very broad in comparison to the acrosomal region (Fig. 9.10B). The acrosome extends beyond the narrow "waist" of the head, and the acrosomal:postacrosomal ratio is 3:2. The midpiece is long, like that of *Balaenoptera brydei* and the *Balaena mysticetus*. The maximum *en face* head width is the smallest among cetacean sperm

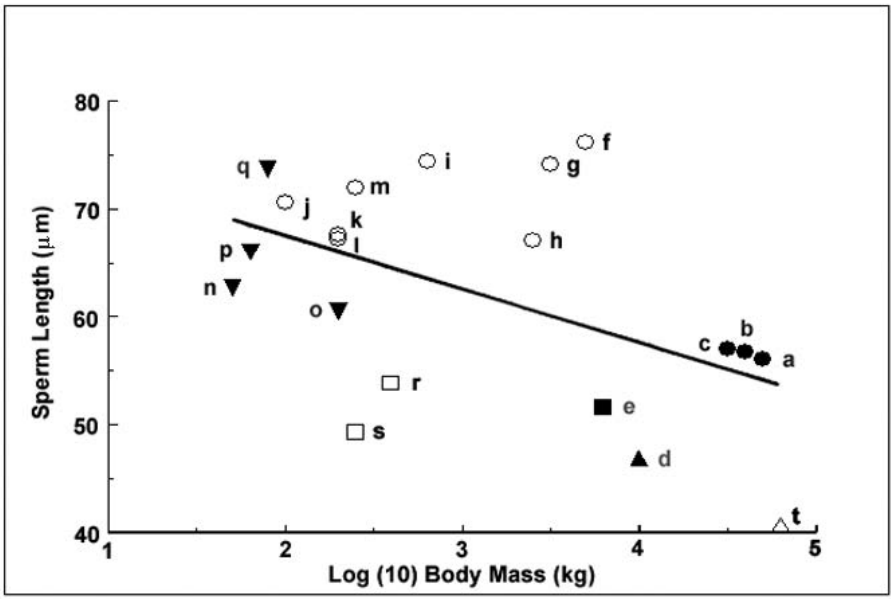

Fig. 9.9 Scatter plot of the $\log_{10}$ of the maximum body mass (kg) of adult males of selected cetacean species vs the total sperm length. Total sperm lengths from Table 9.1; maximum adult male body mass from Wilson and Ruff (1999, Smithsonian Institution Press, Washington, DC, U.S.A.); values were averaged when more than one was listed. Trend line from 2D Linear Curvefit Plot, Axum 5.0 for Windows © 1988-96, MathSoft, Inc., Revision date 22 Aug. 1996. Seven families are represented: Balaenopteridae (●), Balaenidae (▲), Ziphiidae (■), Delphinidae (○), Phocoenidae (▼), Kogiidae (□), Physeteridae (△). Sixteen species are shown: A. *Balaenoptera brydei* (Bryde's whale). B. *Balaenoptera acutorostrata* (Minke whale). C. *Megaptera novaeangliae* (Humpback whale). D. *Balaena mysticetus* (Bowhead whale). E. *Berardius bairdii* (Baird's beaked whale). F. *Orcinus orca* (Killer whale). G. *Globicephala macrorhynchus* (Short-finned pilot whale). H. *Globicephala melas* (Long-finned pilot whale). I. *Grampus griseus* (Risso's dolphin). J. *Delphinus delphus* (Common dolphin). K. *Tursiops truncatus* (Bottlenose dolphin). L. *Lagenorhynchus obliquidens* (Pacific white-sided dolphin). M, *Peponocephala electra* (Melon-headed whale). N. *Neophocaena phocaenoides* (Finless porpoise). O. *Phocoenoides dalli* (Dall's porpoise). P. *Phocoena spinipinnis* (Burmeister's porpoise). Q. *Phocoena phocoena* (Common porpoise). R. *Kogia breviceps* (Pygmy sperm whale). S. *Kogia sima* (Dwarf sperm whale). T. *Physeter catodon* (Sperm whale). Original.

examined to date, and the neck is short when compared with sperm of other species (Fig. 9.11B).

9.4.5 Delphinidae: *Orcinus orca* (Killer whale)

Kita *et al.* (2001) examined fresh and frozen semen from a captured *Orcinus orca* via phase contrast light microscopy and SEM; whereas Miller *et al.* (2002)

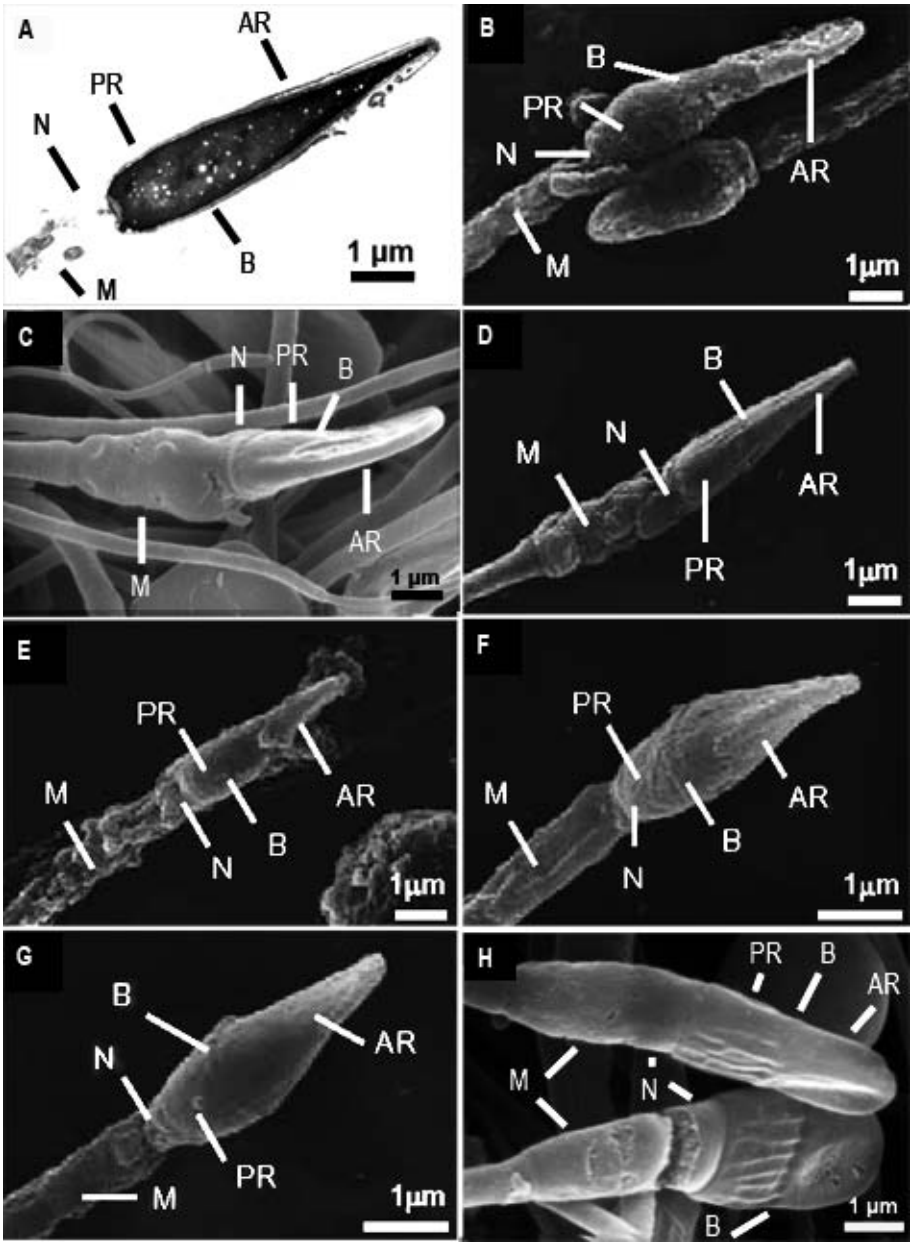

Fig. 9.10 Lateral aspects of cetacean spermatozoa representing five families and eight species and showing the acrosomal region (AR), the postacrosomal region (PR), postacrosomal sheath border (B) of the head. Also shown are the neck (N) and midpiece (M). **A**. Transmission electron micrograph of Balaenidae: *Balaena mysticetus* (Bowhead whale). **B**-**H**. Scanning electron micrographs, **B**. Ziphiidae:

Fig. 9.10 Contd. ...

used light microscopy, SEM, and TEM to examine sperm collected from a captive *O. orca* by trained behavior. The *en face* aspect of the spermatozoal head is square with rounded corners (Fig. 9.11C). As noted earlier, the head is the broadest among cetacean sperm studied thus far. The acrosome is reniform with raised edges (the acrosomal band), which surround a broad principal region that has a rough or uneven surface (Figs. 9.10C and 9.11C). The acrosome:postacrosome ratio varies from 1:2 (Kita *et al.* 2001) to 1.5:1 (Miller *et al.* 2002). The neck is thick and long (0.9-1.4 µm wide × 0.7-1.1 µm long) in comparison to *B. bairdii* sperm. The midpiece measures 0.9-1.7 µm wide by 3.0-3.1 µm long. SEM and TEM reveal that the surface of the midpiece has indentations that mirror the mitochondrial junctions visible by TEM.

With TEM (Miller *et al.* 2002) sperm heads measure ca 4.4 µm long and 4.0 µm wide *en face*. In sagittal sections, the heads are ca 3.7 µm long by 0.4 µm wide at the tip and 1.0 µm wide at the posterior ring. The acrosome is characterized by a uniformly thick (0.6-0.7 µm) acrosomal band and an unevenly thickened (0.25-0.7 µm) principal region. The variable thickness of the principal region is responsible for the rough surface seen in this region by SEM. The acrosomal matrix is moderately and uniformly electron dense. Cross sections of the upper portion of the acrosomal region are straight, with slight variations in breadth, and become progressively broader and more elliptical toward the boundary with the postacrosome. The smooth plasma membrane of the postacrosome is underlaid by a uniform dense lamina. In longitudinal sections of the midpiece, there are 4-5 mitochondria stacked to either side of the axial fiber bundle, and in cross sections, there are usually four encircling mitochondria. Throughout the principal piece, the axial fiber bundle is surrounded by a filamentous fibrous sheath. Cross sections of the principal piece are circular immediately below Jensen's ring, elliptical throughout most of the length, and circular at the distal end. The terminal piece is circular in cross section and less than 0.2 µm in diameter at its proximal end.

9.4.6 Delphinidae: *Globicephala macrorhynchus* (Short-finned pilot whale), *Peponocephala electra* (Melon-headed whale), *Grampus griseus* (Risso's dolphin), *Delphinus delphus* (Common dolphin), *Sousa plumbea* (Humpback dolphin), *Delphinus capensis* (Long-

Fig. 9.10 Contd. ...

Berardius bairdii (Baird's beaked whale), **C**. Delphinidae: *Orcinus orca* (Killer whale), **D**. Delphinidae: *Globicephala macrorhynchus* (Short-finned pilot whale), **E**. Phocoenidae: *Neophocaena phocaenoides* (Finless porpoise), **F**. Kogiidae: *Kogia breviceps* (Pygmy sperm whale), **G**. Kogiidae: *Kogia sima* (Dwarf sperm whale), and **H**. Delphinidae: *Lagenorhynchus obliquidens* (Pacific white-sided dolphin). Figures **B**, **D**, and **E** are from Kita, S., Yoshioka, M., Kashiwagi, M., Ogawa, S. and Tobayama, T., 2001. Fisheries Science 67(3): 482-492, Fig. 5. Figure **C** is from Miller, D.L., Styer, E.L., Decker, S.J. and Robeck, T. 2002. Anatomia Histologia Embryologia 31:158-168, Fig. 5. Figures **A**, **F**, **G**, and **H** are original.

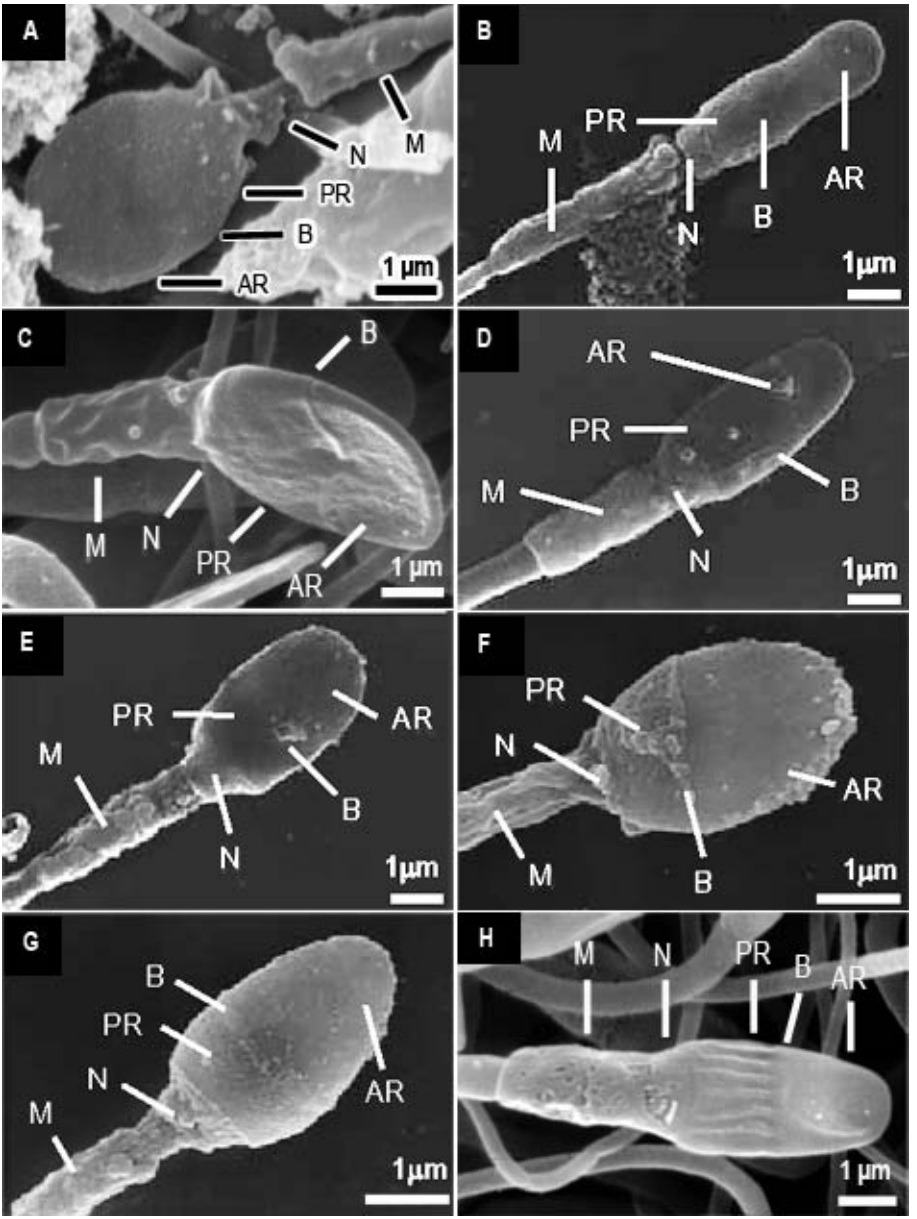

Fig. 9.11 *En face* view of cetacean spermatozoa representing five families and eight species and showing the acrosomal region (AR), the postacrosomal region (PR), postacrosomal sheath border (B) of the head. Also shown are the neck (N) and midpiece (M). Scanning electron micrographs. **A**. Monodontidae: *Delphinapterus leucas* (Beluga whale). **B**. Ziphiidae: *Berardius bairdii* (Baird's beaked whale), **C**. Delphinidae: *Orcinus orca* (Killer whale), **D**. Delphinidae:

Fig. 9.11 Contd. ...

beaked common dolphin), *Steno bredanensis* (Rough-toothed dolphin), *Stenella* spp. (Spotted dolphin), *Tursiops truncatus* (Atlantic bottlenose dolphin), *Lagenorhyncus obliquidens* (Pacific white-sided dolphin)

Kita *et al.* (2001) examined epididymal sperm from captured *Globicephala macrorhynchus*, *Grampus griseus*, and *Delphinus delphus*, and semen from a captive *Tursiops truncatus* and captive *Lagenorhynchus obliquidens*. Endo and Kita (unpublished) examined epididymal sperm acquired postmortem from *Peponocephala electra*. Miller *et al.* (2002) and Miller and Styer (unpublished data) examined semen from a captive *T. truncatus* and a captive *L. obliquidens* obtained using trained behaviors; Fleming *et al.* (1981) examined semen collected by electroejaculation from a captive *T. truncatus*; and Meisner *et al.* (2005) examined sperm from *Megaptera novaeangliae*, *Delphinus capensis* (Long-beaked common dolphin), and *Steno bredanensis* (Rough-toothed dolphin) and *Stenella* spp. that died after stranding. Techniques employed included: LM and SEM (Kita *et al.* 2001; Y.E. and S.K., unpublished data); SEM and TS-TEM (Fleming *et al.* 1981); brightfield light microscopy, SEM, TS-TEM and NS-TEM (Miller *et al.* 2002; D.L.M. and E.L.S., unpublished data); and SEM (Meisner *et al.* 2005). Whole sperm and higher magnification views of the heads, necks and midpieces of a number of these Delphinidae are shown in Figures 9.7, 9.10, and 9.11.

When viewed *en face* by SEM, the sperm heads of these Delphinidae are oblong or elongated ellipsoids, with almost parallel sides that taper slightly cranially to a relatively blunt apex (Figs. 9. 4 and 9.11). The acrosomal regions have a thickened acrosomal band that completely surrounds a smooth-surfaced principal region (Flemming *et al.* 1981; Kita *et al.* 2001; Miller *et al.* 2002; D.L.M. and E.L.S., unpublished data). Unfortunately, the sperm examined by Meisner *et al.* (2005) suffered so much postmortem damage that the thickened acrosomal band was absent or only partially present. The postacrosomal regions are characterized by a series of longitudinal ridges, which vary from six to eight per side in *en face* views and fuse into horizontal bands at the top and bottom of the postacrosome (Fig. 9.4). There are a maximum of 16 ridges when the midregion of the postacrosome is viewed in cross sections (Fig. 9.6). The purpose for these ridges remains unclear but a functional role in fertilization is possible. Head lengths range from 4.3 μm for

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Fig. 9.11 Contd. ...

Globicephala macrorhynchus (Short-finned pilot whale), **E**. Phocoenidae: *Neophocaena phocaenoides* (Finless porpoise), **F**. Kogiidae: *Kogia breviceps* (pygmy sperm whale), **G**. Kogiidae: *Kogia sima* (Dwarf sperm whale), and **H**. Delphinidae: *Lagenorhynchus obliquidens* (Pacific white-sided dolphin). Figures **B**, **D**, and **E** are from Kita, S., Yoshioka, M., Kashiwagi, M., Ogawa, S. and Tobayama, T., 2001. Fisheries Science 67(3): 482-492, Fig. 4. Figures **A**, **C**, and **H** are from Miller, D.L., Styer, E.L., Decker, S.J. and Robeck, T. 2002. Anatomia Histologia Embryologia 31:158-168, Figs. 1, 5 and 9. Figures **F** and **G** are original.

Delphinus delphus to 5.5 μm for *Peponocephala electra* (Table 9.1). By comparison, Meisner *et al.* (2005) measured head lengths from 3.6 μm to 4.2 μm; in this context, it should be noted that Meisner *et al.* (2005) found the head of *K. sima* sperm to be only 3.5 μm in length in contrast to 4.4 μm that Endo and Kita (unpublished data) observed (Table 9.1). This discrepancy in length is probably due to poor preservation of the sperm that Meisner *et al.* (2005) examined. The shorter lengths of other Delphinidae sperm may be due to variation among individual animals, differences in preparative techniques, or varying degrees of postmortem change. The acrosome:postacrosome ratios are 1:1 or greater. The necks are thick and long, similar to that of *O. orca* sperm. The structure of *Tursiops truncatus* and *Lagenorhynchus obliquidens* sperm are presented in greater detail below and in Table 9.2.

9.4.6.1 Pacific white-sided dolphin (*Lagenorhynchus obliquidens*)

The ultrastructure of *Lagenorhynchus obliquidens* sperm has been described in detail (Miller *et al.* 2002) and is summarized here. Dimensions and other features of the various spermatozoal regions are observable by NS-TEM, SEM, and TEM; some of this information is presented in Tables 9.1 and 9.2 and in Figures 9.1, 9.2, 9.3, 9.4, 9.7H, 9.10H, and 9.11H. The acrosomal band surrounds the smooth, slightly curved, fingernail-shaped principal region (Figs. 9.4 and 9.11H). The acrosomal matrix is moderately and homogeneously electron dense. In parasagittal longitudinal sections that pass through the very edge of the side of the head, the acrosome is uniformly thickened from its base to its apex. In sagittal longitudinal sections, the acrosome is thick at its apex and base and thin throughout the principal region. Cross sections of the apex of the acrosome are straight, short, and uniformly broad. Cross sections below the apex through the upper half of the acrosome are sigmoid with enlarged ends and a narrow principal region. Cross sections through the base of the acrosome are elliptical. Cross sections at about the midpoint of the postacrosome indicate there is a maximum of 16 approximately evenly spaced ridges (Miller *et al.* 2002). The plasma membrane over the ridges is underlaid by a pad of electron-dense material, which occasionally can be seen to have the regular densities expected of the postacrosomal dense lamina. These regular densities are frequently most apparent in cross sections toward the top and bottom of the postacrosomal region where two or more of the longitudinal ridges fuse to produce broader, single ridges. Cross sections near the middle of the postacrosome are ellipsoid. The neck and the midpiece are roughly circular in cross sections. The fibrous sheath of the principal piece is visible in longitudinal sections as an elaborate reticulum to either side of the axial fiber bundle. In cross sections, the fibrous sheath appears as a scant reticulum or a series of concentric electron-dense bands that fuse into a single broader band across the short diameter of the principal piece (Fig. 9.3). At the distal end of the principal piece, these concentric bands are reduced to a single band, which disappears at the junction with the terminal piece. The dense fibers also gradually diminish in size and terminate before the junction of the principal piece with the terminal piece (Fig. 9.3). The gradually tapering

Table 9.2 Comparison of sperm of *Lagenorhynchus obliquidens* (Pacific white-sided dolphin) and *Tursiops truncatus* (Atlantic bottlenose dolphin) examined by TEM. Dimensions are given in μm.

Sperm part	*L. obliquidens* *Miller et al. (2002)*	*T. truncatus* *D.L.M. and E.L.S. (unpublished data)*	*T. truncatus* *Flemming et al.* (1981)[1]
Head, L × W from sagittal longitudinal sections	3.8.0 × 1.1-1.2	3.4-3.9 × 1.0-1.4	3.8 × 1.0-1.2
Acrosomal Band, thickness	0.06-0.07	0.06-0.07	0.06-0.07
Acrosomal Principal Region, thickness	0.02-0.03	0.02-0.03	0.02-0.03
Acrosome:Postacrosome Ratio	1.5	1.3-1.5	1.2
Neck, L × W	1.1 × 1.1	1.1-1.3 × 1.0-1.1	0.7-1.1 × 1.0
Midpiece, $L \times W_{distal\ end} - W_{proximal\ end}$	2.3 × 1.0-1.4	2.3 × 1.0-1.5	$2.6 \times ND^2 - 1.7$
Midpiece, number of mitochondria to either side of the axial filament bundle; longitudinal sections	3-5, usually 4	3-5, usually 4	3-4
Midpiece, number of mitochondria in cross sections	3-5, usually 4	3-5, usually 4	4
Principal Piece, $L \times W_{proximal\ end}$	60 × 0.6	ND × 0.7	ND
Terminal Piece, $L \times W_{proximal\ end}$	11 × 0.2	ND × 0.2	ND

[1] Some measurements were made from the illustrations.
[2] ND, not done

Fleming, A.D., Yanagimachi, R. and Yanagimachi, H. 1981. Journal of Reproduction and Fertility 63: 509-514.
Miller, D.L., Styer, E.L., Decker, S.J. and Robeck, T. 2002. Anatomia Histologia Embryologia 31: 1-11.

principal piece is circular in cross section just distal to Jensen's ring, elliptical throughout most of its length, and finally circular again at its distal end. The narrow terminal piece is approximately circular in cross section throughout its length.

9.4.6.2 Atlantic bottlenose dolphin (*Tursiops truncatus*)

Interestingly, *Tursiops truncatus* sperm are virtually indistinguishable from *Lagenorhynchus obliquidens* sperm by morphological parameters, including: dimensions; the number and arrangement of mitochondria in the midpiece; the presence of a maximum of 16 longitudinal ridges in the postacrosomal region; the sigmoid nature of the upper portion of the acrosomal region; the presence of an acrosomal band; and the L:W ratios of cross sections at various levels of the head (Fig. 9.6; Tables 9.1 and 9.2) (D.L.M. and E.L.S., unpublished

data). Subjectively, cross sections of the acrosomal region of *T. truncatus* look somewhat broader than those of *L. obliquidens*, reflected by L:W ratios of 1.5:1 and 2:1, respectively (D.L.M. and E.L.S., unpublished data). Conversely, the cross section of the postacrosomal region of *T. truncatus* shown in Flemming *et al.* (1981) has a L:W of 2:1, suggesting that differences in L:W ratios noted for *L. obliquidens* and *T. truncatus* (D.L.M. and E.L.S., unpublished data) may be due to variation in sperm from individual animals. Flemming *et al.* (1981) describe midpiece mitochondria with different affinities for the heavy metal stains used in TEM. Miller and Styer (unpublished data) did not observe this phenomenon except in cryopreserved sperm, which displayed other freeze-related damage.

9.4.7 Phocoenidae: *Neophocaena phocaenoides*, *Phocoenoides dalli* (Finless and Dall's porpoises)

Kita *et al.* (2001) examined epididymal sperm collected from a stranded *Neophocaena phocaenoides* (Finless porpoise) (Figs. 9.7E, 9.10E and 9.11E) and from captured *Phocoenoides dalli* (Dall's porpoise) using phase contrast light microscopy and SEM. When viewed *en face,* the sperm heads are "ellipsoids" (Fig. 9.11E). The lateral aspect of the sperm heads and the thick, long necks are similar to those of other Delphinidae sperm (Figs. 9.10 and 9.11). The acrosome:postacrosome ratio is 2:3.

9.4.8 Phocoenidae: *Phocoena spinipinnis* (Burmeister's porpoise)

Sperm suspensions from the caudal epididymus of two adult *Phocoena spinipinnis* (Burmeister's porpoise) accidentally trapped in gillnets were examined by phase contrast and brightfield light microscopy by Beilis *et al.* (2000). Sperm heads are ellipsoidal when viewed *en face* and ensiform (shaped like a sword blade) when viewed from the side. The acrosome:postacrosome ratio was greater than 1:1. The midpiece was ca 3.3 μm long and ca 1.1 μm wide; the principal piece was ca 56.2 μm long.

9.4.9 Kogiidae: *Kogia breviceps* (Pygmy sperm whale)

Endo and Kita (unpublished data) examined epididymal sperm of *Kogia breviceps* collected postmortem and viewed them by LM (Fig. 9.7F) and SEM (Figs. 9.10F and 9.11F). The *en face* aspect of the sperm head was broadly elliptical or "racket shaped" (Fig. 9.11F).

9.4.10 Kogiidae: *Kogia sima* (Dwarf sperm whale)

Using LM (Fig. 9.7G) and SEM (Figs. 9.10G and 9.11G), Endo and Kita (unpublished data) and Meisner *et al.* (2005) examined epididymal sperm of *Kogia sima* collected postmortem. According to Endo and Kita (unpublished) sperm heads viewed *en face* appeared lanceolate (71 percent) or ovate (29 percent) (Fig. 9.11G), while Meisner *et al.* (2005) concluded that the heads of their poorly preserved specimen were "teardrop" shaped. Meisner *et al.* (2005)

found the head of *K. sima* to be only 3.5 μm long in contrast to the 4.4 μm length reported by Endo and Kita (Table 9.1).

9.4.11 Physeteridae: *Physeter catadon/macrocephalus* (Sperm whale)

Yamani (1936) examined sperm from the epididymus of a captured *Physeter catadon* (syn. *P. macrocephalus*; Sperm whale). The heads are described as elliptical with a blunt front end and the basic dimensions presented are: head, 4.9 μm long by 2.7 μm wide; tail, 35.7 μm long; and total length, 40.6 μm.

Matano *et al.* (1976) used *Physeter catadon* as the representative cetacean in their comparative SEM study of mammalian sperm. Measurements from their illustration show the head to be ca 5.2 μm long and ca 1.9 μm wide, with an acrosome:postacrosome ratio of 1:7, and the midpiece to be ca 2 μm long and 0.6 μm broad. The acrosomal region is thin and flat, while the postacrosomal region is very thick. There is an acrosomal band similar to those described for *Lagenorhynchus obliquidens, Tursiops truncatus,* and *Orcinus orca* by Fleming *et al.* (1981), Miller *et al.* (2002), and D.L.M. and E.L.S. (unpublished data). The neck is thin and long (ca 1.6 μm × 0.4 μm) compared to other eutherians, and the midpiece and principal piece are short and thick. The discrepancy in the width of the head [2.7 μm (Yamani 1936) versus 1.9 μm (from measurement of an illustration in Matano *et al.* 1976)] is likely a result of the sperm pictured in Matano *et al.* (1976) being at a slight angle rather than flat. Because sperm of *P. catadon* and *Myotis capaccinii* (long-fingered bat) are distinct from the other 27 species and six orders that they examined, Matano *et al.* (1976) made the questionable suggestion that sperm of Cetacea and Chiroptera have adapted to their unique environments (air and water).

9.4.12 Monodontidae: *Delphinapterus leucas* (Beluga or White whale)

Using LM, SEM and TEM, Miller *et al.* (2002) examined semen collected immediately postmortem from the caudal epididymis of a captive *Delphinaptera leucas*. The heads of *D. leucas* and *Orcinus orca* sperm are very similar in shape and size, appearing almost square with reniform acrosomes (Fig. 9.11a). Sperm heads of *D. leucas* are 3.8 μm long and 3.4 μm wide compared to 4.0 μm long and 3.3 μm wide for *O. orca*. The acrosome:postacrosome ratio of *D. leucas* is somewhat greater than that of *O. orca* (1.5:1 and 1:1, respectively). The *D. leucas* spermatozoal midpiece is ca 3.2 μm long and 1.1 μm wide at its apex and ca 0.5 μm in diameter at Jensen's ring. Cross sections of the upper half of the acrosomal region are straight, not sigmoid.

9.5 CRYOPRESERVATION EFFECTS ON SPERMATOZOAL MORPHOLOGY

Cryopreservation provoked significant ultrastructural changes in sperm of *T. truncatus* and *Lagenorhynchus obliquidens* (D.L.M. and E.L.S., unpublished data), best viewed in sagittal longitudinal sections of the head (Fig. 9.12). The

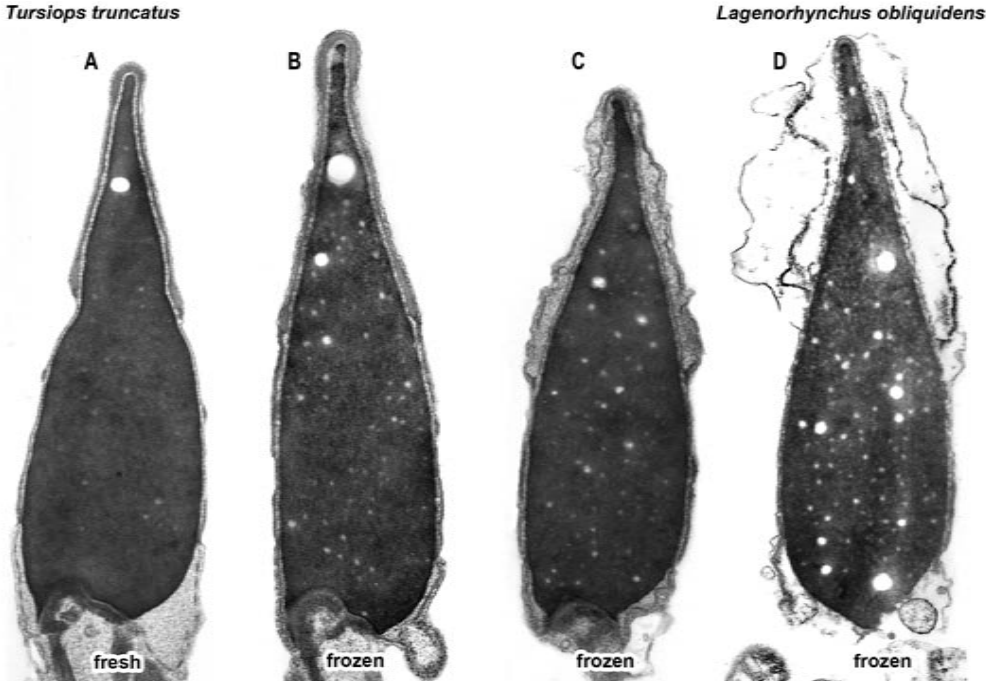

Fig. 9.12 Transmission electron micrographs of fresh and cryopreserved (frozen) sperm of *Tursiops truncatus* (Atlantic bottlenose dolphin) (**A**-**C**) and *Lagenorhynchus obliquidens* (Pacific white-sided dolphin) (**D**) showing increasingly severe damage in the frozen specimens. **A.** fresh sperm; **B.** representative of the least damaged cryopreserved sperm; **C** and **D.** progressively more severely damaged cryopreserved sperm. The plasma membranes of mildly damaged spermatozoa were still predominantly continuous, but were loose and wavy around the acrosome, and occasionally ballooned out from the acrosome in moderately damaged specimens. The plasma membranes became broader and less distinct with escalating damage. The acrosome was expanded and the acrosomal matrix was diffuse in moderately damaged sperm (**C**) and absent in more severely damaged sperm (**D**). The nucleoplasm of cryopreserved sperm appeared denser and more vacuolated (**B**-**D**) than in fresh sperm (**A**). Original.

most pronounced changes were severe ballooning and fragmentation of the plasma membrane in the acrosomal region, which were often preceded or accompanied by swelling of the acrosome and progressive loss of the acrosomal matrix (Fig. 9.12A and D). There was also ballooning, vesiculation and fragmentation of the outer acrosomal membrane (Fig. 9.12D). Additionally, the nucleoplasm of the frozen sperm was more electron dense and more highly vesiculated than the nucleoplasm of fresh sperm.

In undamaged fresh and frozen spermatozoa, plasma membranes were continuous, narrow, and slightly wavy over the acrosome and continuous, narrow, and smooth over the postacrosome. In mildly damaged spermatozoa, plasma membranes remained predominantly continuous around the acrosome, but tended to be loose and wavy instead of closely appressed to the outer acrosomal membrane (Fig 9.12C). Occasionally plasma membranes of sperm with moderately damaged acrosomes were also ballooned. With increasing damage severity, plasma membranes became broad and indistinct. In the acrosomal region of the most damaged specimens, the plasma membranes were grossly discontinuous, vesiculated, or altogether absent (Fig. 9.12D). Except in the most damaged specimens, the plasma membrane remained smooth and closely appressed to the dense lamina of the subacrosomal region.

The acrosomal matrix of undamaged spermatozoa was moderately electron dense and finely and evenly granular (Fig. 9.12A and B). The acrosomes of moderately damaged frozen sperm were swollen and the matrix was diffuse and more coarsely granular (Fig. 9.12C); in such instances, what appeared to be diffuse acrosomal matrix was frequently found between the plasma and the outer acrosomal membranes. In the most damaged sperm, the acrosomal matrix was absent and the acrosomal membranes were ballooned and fragmented (Fig. 9.12D). Interestingly, the very apical portion of the acrosome was comparatively unaffected by cryopreservation (Fig. 9.12C, D). Preliminary observations suggest that spermatozoal regions are differentially susceptible to freezing-induced damage, i.e., the acrosomal region is the most sensitive, followed closely by the neck; the midpiece is moderately sensitive; and the postacrosome and the tail are the least sensitive.

Mogoe *et al.* (1998) examined sperm from the vasa deferens of seven *Balaenoptera acutorostrata* that were diluted with a cryoprotectant and stored in liquid nitrogen. Motility and viability of thawed sperm were 1.0-20 percent and 1.0-11.6 percent, respectively. About 84 percent of sperm had morphological anomalies, including: abnormal or absent heads (69%), folded or bent midpieces (3%), and coiled or folded tails (44%). While these frozen/thawed sperm were highly abnormal, the extent to which defects were due to postmortem collection versus freezing and thawing is unknown. Flemming *et al.* (1981) found that sperm diluted in a glycerol-free cryoprotectant and stored for 10 days in liquid nitrogen retained 95 percent of their motility. It would be interesting to compare the ultrastucture of sperm cryopreserved by techniques resulting in successful artificial insemination (Robeck *et al.* 2005) to those destroyed by cryopreservation, as described above.

9.6 DISCUSSION

Despite great strides, the study of cetacean spermatozoal morphology remains in its infancy, with future advances dependent upon cooperation between field biologists and reproductive anatomy researchers. A strong team effort is required to standardize collection and analysis of suitable specimens. Both Fawcett (1970) and Yasuzumi (1974) suggested that spermatozoal morphology may be correlated less with phylogenetic position than with the environment in which fertilization occurs. We have since gathered evidence that corroborates this theory, as well as documented a few exceptions. While this may be true for some mammals, correlation of sperm morphology and ultrastructure with phylogeny has been well demonstratd for many vertebrate and invertebrte groups (see, for instance, Jamieson, 1999, 2005 and 2006, for amniotes, Chondrichthyes and birds, respectively). Nevertheless, we have but skimmed the surface and much remains to be discovered.

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CHAPTER 10

Fertilization

Yutaka Fukui

10.1 INTRODUCTION

Fertilization in mammals occurs between a mature oocyte ovulated from a specific follicle (Graafian follicle) and a spermatozoon that has undergone capacitation and the acrosome reaction to achieve fertilization capability. *In vitro* fertilization (IVF) also is possible, as demonstrated by live births in humans (Steptoe and Edwards 1978), cattle (Brackett *et al*. 1982) and other mammals. *In vitro* production (IVP) of embryos in domestic animals has been extensively applied in the field to embryo transfer (ET) programs. In humans, IVP of embryos has been limited to establishing pregnancy in infertile couples. Details on fertilization events in cetaceans remain unclear. Research on IVF combined with *in vitro* maturation (IVM) of follicular oocytes (as shown in Chapter **7**) could greatly contribute to our basic understanding of reproductive physiology of cetaceans and be applied to various assisted reproductive technologies (ARTs), such as cryopreservation, artificial insemination (AI), IVP and nuclear transfer (NT), to increase the population and aid in management of marine mammals.

10.2 SPERMATOZOA

In the testis, differentiation of spermatogonia in the seminiferous tubules depends on age and season in seasonal breeders. Spermatocytes located in the basal layers of the tubules move into the lumen during spermatogenesis and transformation from spermatid to spermatozoa occurs to complete spermatogenesis, which is controlled by gonadotropin-releasing hormone (GnRH) released from the hypothalamus, gonadotropins (follicle-stimulating hormone, FSH; luteinizing hormone, LH) released from the pituitary, and steroid hormones (testosterone, T; estradiol-17β, E_2) released from the testis. It is known that in cetaceans, especially baleen whales, sperm production is extremely low during the non-breeding season (feeding period) (Mogoe *et al*.

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2000; Watanabe *et al*. 2004). However, detailed information is lacking on spermatogenesis in baleen whales. Kasuya and Marsh (1984) reported that the peak of the reproductive season of *Globicephala macrorhynchus* (Short-finned pilot whale) was from March to May in the population off southern Japan. From the wide variation in the testicular histology of mature individuals (Kasuya and Marsh 1984), it appears that the timing of spermatogenesis is different among individuals in *G. macrorhynchus* (Kita *et al*. 1999). Mogoe *et al*. (2000) and Watanabe *et al*. (2004) investigated the relationship between hormone concentrations and histology of seminiferous tubules in *Balaenoptera bonaerensis* (Antarctic minke whale) and *Balaenoptera edeni* (syn. *B. brydei*) (Bryde's whale), respectively. They reported that low serum T concentrations reflect the inactivity of spermatogenesis in both baleen whales during the feeding season even though some spermatozoa were present in the seminiferous tubules (Fig. 10.1).

10.2.1 Morphology

Spermatozoal morphology has been dealt with comprehensively in Chapter **9** and will not be further characterized here but scanning electron micrographs

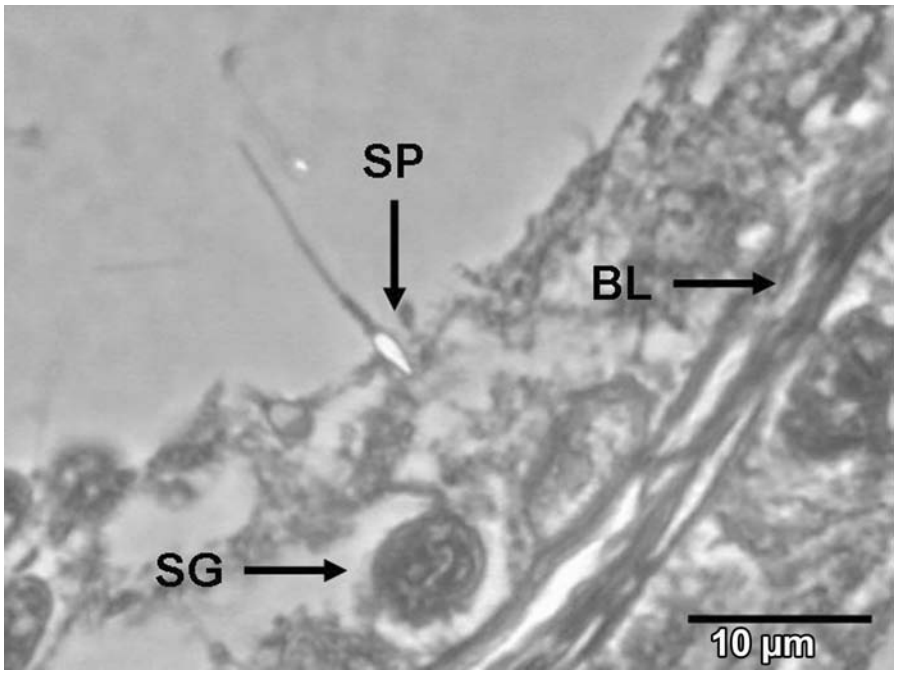

Fig. 10.1 Histological section of a seminiferous tubule in a mature *Balaenoptera edeni* (Bryde's whale). A spermatozoon (SP) has attached to the layer of spermatogonia (SG). BL, basal layer. After Watanabe, H., Mogoe, T., Asada, M., Hayashi, K., Fujise, Y., Ishikawa, H., Ohsumi, S., Miyamoto, A. and Fukui, Y. 2004. Journal of Reproduction and Development 50: 419-427, Fig. 1, F.

of the sperm of the Antarctic minke whale (*Balaenoptera bonaerensis*), described by Mogoe *et al*. (1998a), are illustrated in Fig. 10.2).

10.2.2 Sperm Capacitation

Capacitation is the process whereby sperm obtain the ability to fertilize. It occurs in the female reproductive tract after natural mating or AI. Spermatozoa must be capacitated and the acrosome reacted for sperm to penetrate mature oocytes for the completion of normal fertilization. Induction of *in vitro* capacitation followed by the acrosome reaction in mammalian spermatozoa is a complicated mechanism affected by many factors, such as

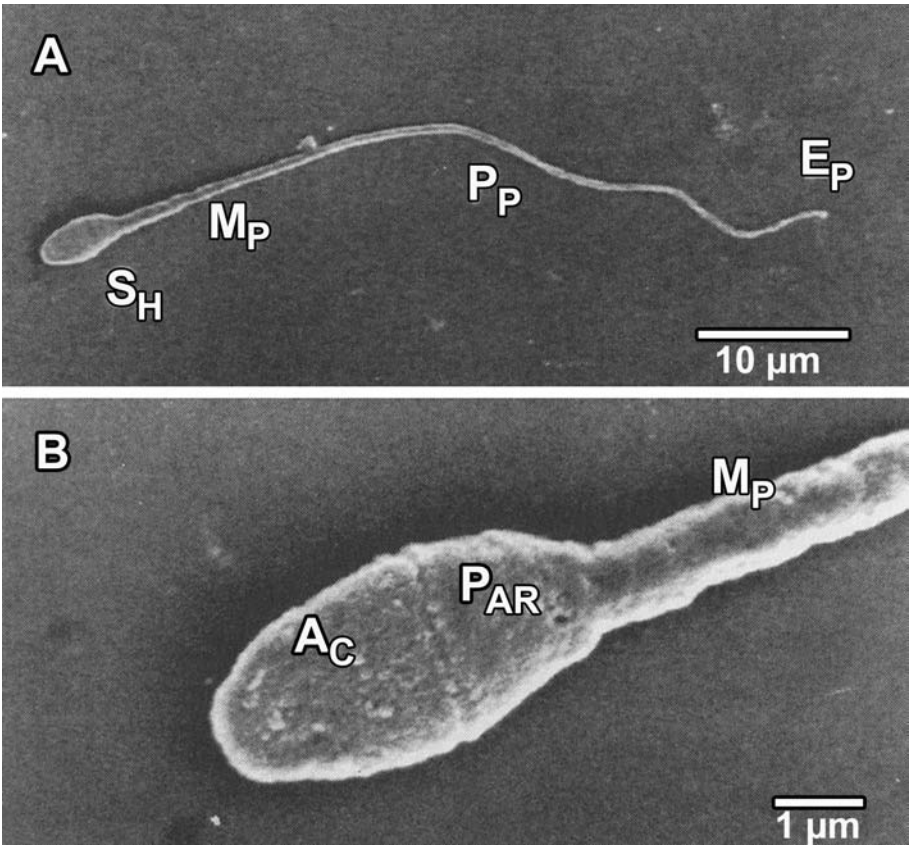

Fig. 10.2 A *Balaenoptera bonaerensis* (Antarctic minke whale) spermatozoon recovered from the vas deferens and observed using scanning electron microscopy. **A**. Picture of a spermatozoon with a conical head-type. S_H: Sperm head, Mp: Midpiece, Pp: Principal piece, Ep: Endpiece. **B**. Enlarged view of the same sperm-head. Ac: Acrosome, P_{AR}: Postacrosomal region. After Mogoe, T., Fukui, Y., Ishikawa, H. and Ohsumi, S. 1988. Journal of Reproduction and Development 44: 95-100, Fig. 1.

sodium and calcium ions, glucose, albumin, and duration of sperm treatment. This mechanism is considered the key to successful IVF in mammalian oocytes; however, the capacitation process is not species-specific. Fleming *et al*. (1981) examined *in vitro* capacitation of frozen-thawed *Tursiops truncatus* (Bottlenose dolphin) spermatozoa in different incubation conditions using zona-free hamster eggs. They found that the spermatozoa were capable of fusing with zona-free hamster eggs only after pre-incubation for 2 h. This supports the suggested need for sperm capacitation and the acrosome reaction before fertilization in dolphins as in other mammals. Fleming *et al*. (1981) also observed a vigorous 'activated' type of tail movement characterized by a high-amplitude whiplash beating (so-called "hyperactivation") of spermatozoan tails. This movement has been described for spermatozoa of other mammals in association with capacitation and/or the acrosome reaction (Yanagimachi 1970).

To induce sperm capacitation *in vitro*, caffeine, heparin, and/or calcium ionophore have been used successfully, but there is no report on *in vitro* capacitation methods for whale spermatozoa. Caffeine and heparin or both have been used successfully for bovine sperm capacitation (Niwa and Ohgoda 1988; Fukui 1990). Addition of caffeine tended to improve sperm motility and penetration and fertilization rates; however, neither caffeine nor heparin were effective on *Balaenoptera bonaerensis* (Antarctic minke whale) sperm capacitation (Fukui *et al*. 1997a). Recently, H. Tateno *et al*. [unpublished data cited by Fukui (2002)] showed the first evidence of penetration of frozen-thawed *B. bonaerensis* spermatozoa into zona-free hamster oocytes, indicating the possibility of induction of sperm capacitation and the acrosome reaction in *B. bonaerensis* spermatozoa pre-treated with Percoll centrifugation and calcium ionophore (10 mM, 7 min). H. Tateno *et al*. (unpublished data) demonstrated that 4 of 93 hamster oocytes had been penetrated by *B. bonaerensis* spermatozoon. They did this by demonstrating two sets of chromosomes on a hamster ootid; one set from a *B. bonaerensis* spermatozoan and the other from the hamster oocyte. Although the number of chromosomes in *B. bonaerensis* is the same (2n =44) as in hamsters, the two sets of the chromosomes could be distinguished by the larger size of the acrocentric chromosome and the subtelocentric chromosomes, which are characteristic of the *B. bonaerensis* karyotype. The success of this induction method of *in vitro* capacitation of *B. bonaerensis* spermatozoa has led to attempted IVF in our laboratory. The *B. bonaerensis* spermatozoa used in our studies (Fukui *et al*. 1997a; Asada *et al*. 2001a) were obtained postmortem from the vas deferens of wild-caught individuals.

Spermatozoa were collected from the vas deferens, which is situated proximal to the accessory reproductive tracts such as the prostate gland and seminal vesicles which contain seminal plasma which is thought to have anti-capacitation factors that inhibit fertilization. Therefore, spermatozoa from the vas deferens have not been influenced by components of the seminal plasma and *in vitro* capacitation might be easily accomplished by centrifugation or perhaps by incubation as described above.

10.2.3 Cryopreservation of Spermatozoa

Sperm cryopreservation has been successfully applied to the domestic animal industry and to wildlife conservation programs. Specifically, the use of frozen semen has greatly enhanced worldwide AI programs. Hill and Gilmartin (1977) were the first to attempt cryopreservation of cetacean spermatozoa in *Tursiops truncatus*. Fleming *et al*. (1981) and Keller (1986) successfully collected semen from captive dolphins by electro-ejaculation and hand service, respectively. Since then, cryopreservation of ejaculated semen has been reported in some dolphins (Schroeder and Keller 1990; Yoshioka 1994; Robeck and O'Brien 2004) and successful birth by AI using fresh and/or frozen-thawed spermatozoa has been achieved in *T. truncatus* (Robeck *et al*. 2001, 2005), *Lagenorhynchus obliquidens* (Robeck *et al*. 2003) and *Orcinus orca* (Robeck *et al*. 2004). Various freezing extenders (diluents) have been used for cryopreservation of *T. truncatus* spermatozoa. Schroeder and Keller (1990) used diluents containing 20% egg yolk, 6% glycerol and 11% fructose or lactose, frozen in pellet-form, and observed 60% progressive sperm motility in both diluents after thawing. Robeck and O'Brien (2004) froze Bottlenose dolphin spermatozoa in straw-form by a two-step dilution (21 and 5°C) using three diluents and found that the sperm motility index (SMI: total motility × percentage progressive motility × kinetic rating; scale, 0-5, where 0 = no movement and 5 = forward progressive movement) of the post-thawing spermatozoa was significantly ($P < 0.005$) higher in a diluent containing a final concentration of 3% glycerol (TYB: Test Yolk Buffer, Refrigeration Media; Irvine Scientific, Santa Ana, CA) than in the other two diluents, PDV (Platz Diluent Variant) and AE (Androhep) (53.7 ± 9.3, 49.6 ± 8.9 and 44.8 ± 10.1%, respectively). The interesting point of preparing the diluents is that osmolarity of the diluents is slightly high (320 to 350 mOsM) compared with that of the diluents for domestic animals (approximately 300 mOsM). For cryopreservation of *O. orca* spermatozoa, Robeck *et al*. (2004) used a commercially available bovine extender, Biladyl (Fraction A: 1,210 g Tris, 690 g citric acid, 5 g fructose, and 20% egg yolk per 500 ml; Minitube of America). Ejaculated semen was diluted three-fold at 21°C, cooled to 5°C over 1 h and then placed into an ice-water bath (2°C) for 1 h. A second three-fold dilution was carried out with Fraction B (Fraction A + 14% glycerol) and the suspension was kept for another 30 min at 2°C. Robeck *et al*. (2004) demonstrated good post-thawing total motility (50%), progressive motility (94%), and kinetic rating (3.5), and obtained one new-born calf by AI into the uterus using cryopreserved spermatozoa from five trials with three *O. orca*.

Unfortunately, only postmortem sperm collection has been successful in baleen whales, such as *Balaenoptera bonaerensis*. Fukui *et al*. (1996) successfully collected spermatozoa from the vas deferens of 21 out of 22 mature *B. bonaerensis* captured during the feeding season. Testicular weight ranged from 615 to 2,150 g (mean: 1,466 ± 97.7 g). The sperm samples were diluted ten-fold at 30°C with a diluent consisting of Tris (200 mM), glucose (18.5 mM), citric acid (63.1 mM), egg yolk powder (3%, w/v), and glycerol (3%, v/v). Methods

for dilution, cooling, and freezing were adopted from those reported for ram semen (Evans and Maxwell 1987). After thawing at 37°C, motile spermatozoa (2-40%) were observed in 10 out of the 21 samples. The sample with the highest motility was examined for motility and velocity using a computerized sperm analyzer, and it was demonstrated that 43% of spermatozoa were motile, 25% showed rapid velocity, and 9% were progressively motile (Fukui *et al*. 1996).

In a second study (Mogoe *et al*. 1998b) with 61 mature *Balaenoptera bonaerensis*, spermatozoa were successfully recovered from 57 males by manually compressing the vas deferens. Twenty-one of the 57 specimens had motile (2-70%) spermatozoa but only 13 of the specimens were considered suitable for freezing. Spermatozoa of these 13 whales were diluted (five-fold) with Tris-based diluent (300 mM Tris, 27.5 mM glucose, 90 mM citric acid, 15% egg yolk, and 5% glycerol) in cryo-microtubes at –80°C. One-half of each of the 13 specimens was stored in liquid nitrogen for comparison of post-thawing motility. After thawing, it was shown that there was no significant difference in the post-thawing motility between the two storage temperatures (31.3 and 35.5%, respectively). The recovery rate of motile spermatozoa after freezing and thawing was high (about 80%) in comparison with the post-thawing survival rate (about 50%) of mammalian spermatozoa cryopreserved with the best method to date. Freshly diluted spermatozoa have higher longevity than frozen-thawed spermatozoa. Mogoe *et al*. (1998b) showed that spermatozoa diluted with Tris-based diluent and D-PBS maintained their motility for about 3 wk following liquid storage at 5°C, similar to chilled canine spermatozoa (Ponglowhapan *et al*. 2004).

Fukui *et al*. (1996), Mogoe *et al*. (1998b), and Robeck *et al*. (2004) have used diluents adapted for ovine and bovine spermatozoa in *Balaenoptera bonaerensis* and *Tursiops truncatus*, respectively. Development of an optimal diluent for specific species of dolphin or whale spermatozoa is needed. For mammalian semen extenders, fructose and glucose are the most commonly used sugars. Fructose is thought to be a major energy source for ejaculated mammalian spermatozoa, whereas glucose, which is not usually found in the seminal plasma of many mammalian species, is used through glycolysis. The preference for both sugars may vary among mammalian species. Measurement of sugar concentrations in semen or seminal plasma and metabolism and utilization of glucose or fructose in dolphin and whale spermatozoa before and after freezing is needed to understand sperm physiology and develop a new diluent for cryopreservation of marine mammal spermatozoa.

10.3 *IN VITRO* FERTILIZATION

Successful IVF using *in vivo* or *in vitro* matured oocytes has been achieved in almost all domestic animals and in some wild animals. If *in vitro* matured oocytes are used for IVF, both completed maturity of oocytes (nucleus and cytoplasm) and capacitated and acrosome-reacted spermatozoa have to coincide. Also, fresh, undiluted or diluted spermatozoa rather than frozen-

thawed spermatozoa are desired to achieve a high IVF efficiency. To date, there are no reports of successful IVF in dolphins or whales beyond our studies in *B. bonaerensis* using fresh-diluted (Asada *et al*. 2001a) or frozen-thawed spermatozoa (Fukui *et al*. 1997a). Fukui *et al*. (1997a), using 34 immature and mature *B. bonaerensis*, investigated the effects of IVM culture duration (4 or 5 d) and the addition of caffeine and/or heparin to the sperm capacitation medium reported by Fukui (1990). Matured oocytes comprised 157 (30.1%) of 522 total oocytes and the proportion of these matured oocytes penetrated by spermatozoa (55.1 (32.4%); $P < 0.05$) or fertilized with female and male pronuclei (40.4 (20.6%); $P < 0.01$) was higher when cultured for 5 d versus 4 d. Addition of caffeine enhanced the proportion showing penetration (50.0 vs 39.4%) and pronuclear formation (36.0 vs 26.8%), but the differences were not significant ($P < 0.21$ and $P < 0.15$, respectively). Heparin treatment did not significantly effect the normal fertilization rate. Asada *et al*. (2001a) compared the effects of 20% fetal whale serum (FWS) and 0.6% bovine serum albumin (BSA) in the fertilization medium reported by Fukui (1990) on the IVF rates of *in vitro* matured oocytes from prepubertal and adult *B. bonaerensis* (Fig. 7.5 in Chapter **7**) and found that the proportion of sperm penetration (63.4 and 58.5%, respectively) and normally fertilized oocytes (34.1 and 22.0%, respectively) with two pronuclei and a sperm-tail were not significantly different between FWS and BSA. Therefore, sexual maturity of whales did not affect sperm penetration and pronuclei formation of *in vitro* matured and inseminated Antarctic minke whale oocytes.

As shown above, successful IVF in *Balaenoptera bonaerensis* is limited due to the inadequate supply of oocytes, the low maturation (about 30%) of oocytes, and the low number of motile spermatozoa recovered from the vas deferens. Intracytoplasmic sperm injection (ICSI) could be an alternative method to produce normal fertilized oocytes and embryos in *B. bonaerensis* and other mammalian species as described in a later section of this chapter. Asada *et al*. (2001b) first attempted ICSI into *in vitro* matured oocytes that were cryopreserved at the germinal vesicle (GV) stage and cultured for 5 d (Fig. 10.3). Before ICSI, frozen-thawed *B. bonaerensis* spermatozoa were pre-treated with 5 mM ditiothreitol (DTT) for 60 min. After ICSI, the oocytes were activated with 7% ethanol for 5 min. Oocyte activation after ICSI did not produce a significant difference in survival. Pronucleus formation and embryonic development up to the 2- and 4-cell stages were obtained after ICSI using spermatozoa pre-treated with DTT (Fig. 10.4). This suggests that DTT treatment is necessary for successful ICSI in *B. bonaerensis* spermatozoa. Unfortunately, no further embryonic development beyond the 4-cell stage was observed using 5 d of IVC following ICSI.

10.4 *IN VITRO* CULTURE OF EMBRYOS

Even with the best methods for IVM and IVF, including ICSI, it remains unknown whether *in vitro* matured and fertilized *Balaenoptera bonaerensis* oocytes would develop to normal embryos in any culture system. As described

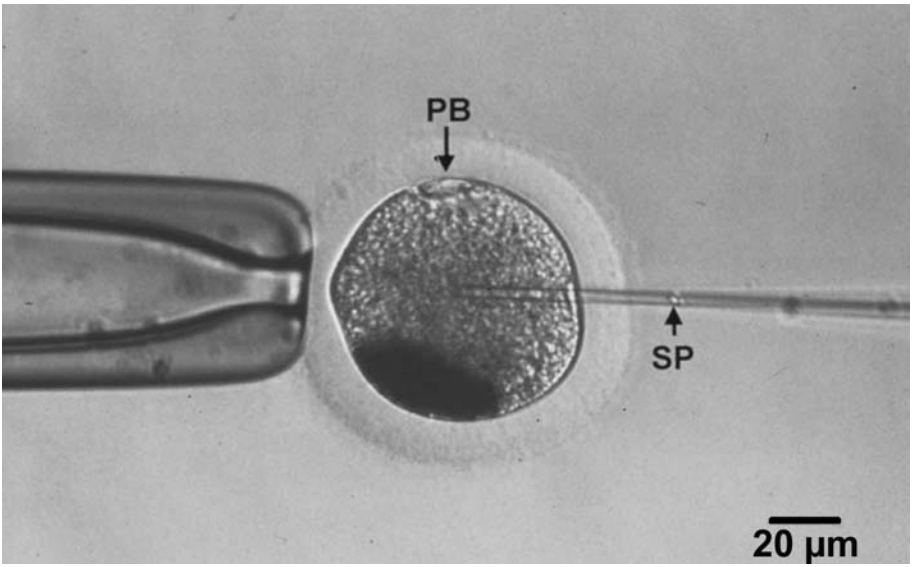

Fig. 10.3 A thawed cryopreserved *Balaenoptera bonaerensis* (Antarctic minke whale) maturing oocyte being intra-cytoplasmically injected with a spermatozoon. A tail-cut sperm head (SP) is in the injection pipette, and the first polar body (PB) is present. After Asada, M., Wei, H., Nagayama, R., Tetsuka, M., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2001b. Zygote 9: 299-307, Fig. 1.

earlier, only 20-30% of IVM cultured oocytes matured to the M-II stage, and of those, 40-50% of the *in vitro* inseminated oocytes were naturally fertilized. Therefore, at present, the maximum expected proportion of cleaved or further developed (beyond the 4-cell stage) embryos following IVM and IVF would be 10%. However, when natural fertilization occurs under the present methods, cleavage and subsequent development of those oocytes could be possible if appropriate *in vitro* culture (IVC) conditions were provided for the *in vitro* fertilized whale embryos. Fukui *et al*. (1997a) cultured 448 *in vitro* inseminated *B. bonaerensis* oocytes for 14 d at 37°C in two culture conditions (with or without co-culture of cumulus cells previously used for IVM culture) using Medium 199 supplemented with 20% FWS. Within 7 d of *in vitro* insemination, a few cleaved (2-16 cell stages) embryos were observed. The proportions of cleaved oocytes were not significantly different between the co-culture and non co-culture systems (6 and 5%, respectively).

Little information is available for embryonic development, especially in an IVC system for the first cleavage to 2-cell stage and development to the blastocyst stage. Some of the embryos developed to the morula stage (more than 16 and 32 cells) (Fukui *et al*. 1997a) but no blastocyst stage embryos were observed. Asada *et al*. (2001a) improved the IVM rate to 31.8% and obtained a 15.4% cleavage rate using freshly-diluted spermatozoa for IVF, including 4.2% of morula stage embryos (Fig. 7.6 in Chapter **7**) when the highest grade IVM

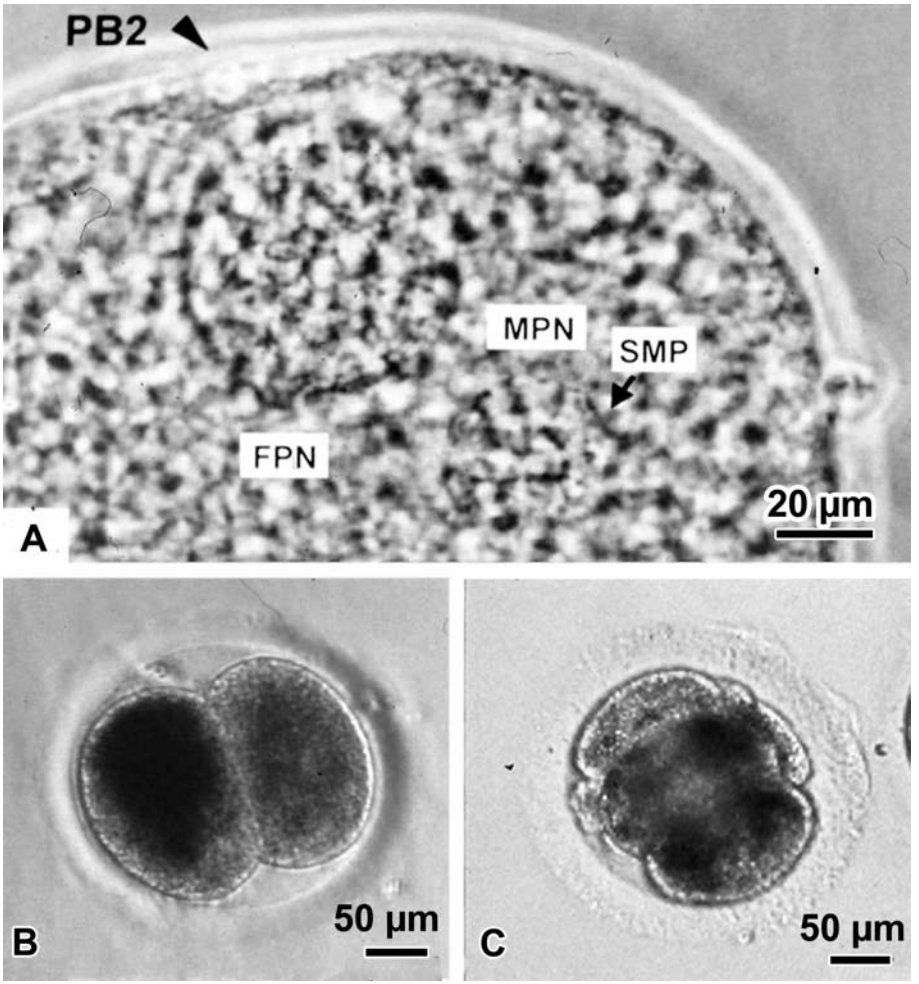

Fig. 10.4 Pronucleus formation and cleavage following injection of a spermatozoon pre-treated with dithiothreitol (5 mM, 1 h) into a frozen-thawed *Balaenoptera bonaerensis* (Antarctic minke whale) oocyte. **A**. Formation of female (FPN) and a male pronuclei (MPN) was observed in the cytoplasm (SMP, sperm mid-piece; arrowhead, the second polar body). **B**. A 2-cell stage embryo obtained 30 h after sperm injection. **C**. A 4-cell stage embryo obtained 72 h after sperm injection. After Asada, M., Wei, H., Nagayama, R., Tetsuka, M., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2001b. Zygote 9: 299-307, Fig. 3.

oocytes were used for IVF. Again, development to the blastocyst stage of the whale oocytes was not observed. This lack of further development may be as seen in other mammalian embryos (bovine, 8-16 cell stages; porcine, 4-cell stage; mice, 2-cell stage) where there is a cell-block stage that occurs during early cleavage. Further investigation into improved culture media to gain viable whale embryos *in vitro* for the ultimate goal of transfer to recipient free-

ranging whales and thus births of calves in their natural environment is needed. Success in this endeavor may greatly enhance conservation efforts for highly endangered cetacean species though a strict limitation of culling is also needed.

10.5 ASSISTED REPRODUCTIVE TECHNOLOGIES

Impressive progress on ARTs has been made since the 1950s, especially in domestic animals and humans. Artificial Insemination (AI) has allowed the broad-scale distribution of genes (sperm) from outstanding, genetically superior sires, especially in cattle. Embryo transfer (ET) has permitted large numbers of offspring to be produced from dams normally capable of producing only a few young during a normal lifetime. *In vitro* fertilization (IVF), combined with ET, has allowed thousands of human couples to successfully combat infertility. Furthermore, IVP of embryos, combined with oocyte pick-up (OPU) from live animals and the culture systems for IVM, IVF including ICSI, and IVC have been applied to bovine, porcine and other domestic animals. Nuclear transfer (NT) or cloning is a process by which the nucleus is moved from a donor cell to an enucleated recipient cell to create an exact genetic match of the donor. Since the first successful report by Wilmut *et al*. (1997) of cloning in sheep, NT has received widespread attention in the livestock industry because of the potential for rapidly disseminating the genes of outstanding individuals and the production of unique genotypes benefiting biotechnologies, including the production of human pharmaceuticals (Pukazhenthi and Wildt 2004). Gene transfer (GT) also has progressed to produce genetically controlled animals (so called "transgenic animals") for the production of meat, milk and new proteins. A combination of NT and GT techniques could potentially promote even more rapid progress in animal husbandry. Compared with ARTs in livestock and humans, management and conservation efforts of wildlife species generally entails more complex ideals and logistics, especially in marine mammals such as whales and dolphins.

10.5.1 Artificial Insemination (AI)

Artificial insemination (AI) and sperm preservation show great promise for use in population management of *Tursiops truncatus*, *Orcinus orca* and other cetaceans. Induction and synchronization of ovulation are achieved by injections of pregnancy mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) (Sawyer-Steffan *et al*. 1983; Schroeder and Keller 1990; Yoshioka 1994) or by progesterone-analog treatment (Robeck *et al*. 2004). Schroeder and Keller (1990) induced ovulation in 14 out of 20 bottlenose dolphins using two injections of PMSG (1,600 IU followed by 800 or 1,000 IU) 2 d apart followed 5 d later by one injection of hCG (1,000 or 3,000 IU). Artificial insemination with fresh or frozen-thawed semen was performed 8 and 24 h after induced ovulation using a flexible fiber-optic laryngoscope (50 cm long and 6.5 mm in diameter) with remote end-tip control. Semen was

placed into the spermathecal recess between the external opening of the pseudo-cervix and the external opening of the true cervix. Unfortunately, the two diagnosed pregnancies from AI spontaneously terminated after 10 and 14 wk (Schroeder and Keller 1990).

Recently, successful births by AI using fresh (Robeck *et al*. 2001) and frozen-thawed spermatozoa (Robeck *et al*. 2003; Robeck *et al*. 2004; Robeck *et al*. 2005), as well as flow cytoplasmic analysis of *Tursiops truncatus* spermatozoa (Robeck and O'Brien 2004), have been reported. Robeck and O'Brien (2004) examined the effects of cryopreservation methods and pre-cryopreservation storage on motility, viability, and acrosome integrity of *T. truncatus* spermatozoa. They found that the initial characteristics of ejaculated spermatozoa were maintained (> 85% motility) and that transport of semen for sex pre-selection and cryopreservation within 24 h may be feasible. Furthermore, Robeck *et al*. (2004) reported in *O. orca* that AI during 8 estrous cycles resulted in 3 pregnancies (38%), two from liquid-stored and one from cyropreserved spermatozoa. The achievement in *O. orca* and *T. truncatus* was due to the recent development of hormonal treatment for induction or synchronization of ovulation and to ultrasonographic imaging techniques that monitor follicular development to determine the most appropriate time for AI (Schroeder and Keller 1990; Brook 2001; Robeck *et al*. 2004). Successful development of AI in these marine species would enable long-term genetic management and maximization of genetic diversity without the need for animal transport. Offspring of pre-determined sex have been produced in many livestock species following the sorting of fresh semen and use of ARTs (Johnson 2000). A prerequisite for successful AI in any species is a fundamental understanding of the species' reproductive physiology, such as seasonality and endocrinology. For application in the field, two major tools have to be established: a precise method for ovulation induction during any season and preservation of semen. Determination of the optimal timing of AI related to ovulation time may be the most important factor for successful AI.

Artificial insemination has not been attempted in large baleen whales, such as *Balaenoptera borealis* (Sei whale), *B. edeni* or *B. bonaerensis*. At present, *B. bonaerensis* are collected by the Institute of Cetacean Research, Japan, by special permit from the International Whaling Commission (IWC). Because semen is collected from these baleen whales postmortem, the only possible sources of live spermatozoa are the vas deferens or the epididymis. Even if this is successful, the volume of semen suspension and numbers of spermatozoa collected often are not sufficient for standard AI, and the only possible utilization is for conventional IVF or ICSI for IVF.

10.5.2 Intracytoplasmic Sperm Injection (ICSI)

Attempted IVF in whales has been limited by the inadequate supply of matured oocytes, the difficulties of *in vitro* oocyte maturation and low motility of the spermatozoa collected from the vas deferens of killed whales. Therefore, ICSI has been used as an alternative means of basic research in whale

fertilization. ICSI has been used in many mammalian species, such as hamsters (Uehara and Yanagimachi 1976), rabbits (Keefer 1989), cattle (Goto *et al*. 1990), humans (Palermo *et al*. 1992), sheep (Catt and Rhodes, 1995), mice (Kimura and Yanagimachi 1995), horses (Squires *et al*. 1996), and pigs (Kim *et al*. 1999). In *Balaenoptera bonaerensis*, Asada *et al*. (2001b) attempted ICSI on *in vitro* matured oocytes following freezing at the GV stage, thawing, and IVM culture. The spermatozoa were collected from the vas deferens, frozen, pretreated with 5 mM DTT for 60 min before ICSI, and injected. They obtained normally fertilized oocytes and cleaved (2- to 4-cell stage) embryos as shown in Figure 10.4. This was the first report of producing cleaved minke whale embryos by the ICSI technique using frozen-thawed and *in vitro* matured whale oocytes.

The ICSI technologies can be used to examine fertilization ability of individual spermatozoa using interspecific matured oocytes, such as those from mice or hamsters. By applying this "Hamster Test" using zona-free hamster matured oocytes, the "ICSI Test" may provide an alternative method for determining the ability of sperm to form male pronuclei and for evaluating the sperm-born oocyte-activating factor (SOAF) for use in activation of matured oocytes. H. Tateno (unpublished data) microinjected *Balaeonoptera bonaerensis* frozen-thawed spermatozoa into matured mouse oocytes using the piezo-ICSI method and examined the subsequent chromosome status. Out of 57 mouse oocytes, 40 (70.2%) oocytes were normally fertilized with two pronuclei. Of those, chromosomes from 33 oocytes were successfully analyzed (Fig. 10.5). Abnormal chromosomes were observed in seven oocytes. Eight mouse oocytes (14%) were activated, but whale sperm heads remained in the enlarged stage and did not form pronuclei (Fig. 10.6). The remaining nine oocytes (15.8%) were not activated beyond the metaphase stage, and the whale sperm showed premature chromosome condensation (Fig. 10.7). This study showed that *B. bonaerensis* frozen-thawed spermatozoa have kept SOAF and a comparatively high integrity of male chromosomes.

Recently, our co-workers (Amemiya *et al*. 2004) examined activation of mouse oocytes by microinjected mouse, bull, and *B. bonaerensis* spermatozoa and showed that the proportions of activated mouse oocytes were 90.5, 84.6 and 76.5%, respectively. The activation rates of mouse oocytes microinjected with frozen-thawed whale spermatozoa by H. Tateno (unpublished data) and Amemiya *et al*. (2004) were similar (70 and 77%, respectively). Amemiya *et al*. (2004) also attempted the mouse oocyte activation assay using different *B. bonaerensis* spermatogenic cells (Fig. 10.8). Late-stage elongating spermatids (stages 15-19) and testicular spermatozoa triggered resumption of meiosis by mouse oocytes at similar rates (68.0 and 62.5%, respectively). The proportion of oocytes activated by the early-stage elongating spermatids (stages 8-14) was significantly ($P < 0.05$) lower (25.0%) than those activated by late-stage elongating spermatids and testicular spermatozoa. The round spermatids (stages 1-7) did not activate mouse oocytes (0%). These results indicate that the spermatogenic cells of *B. bonaerensis* acquire the oocyte-activating capacity

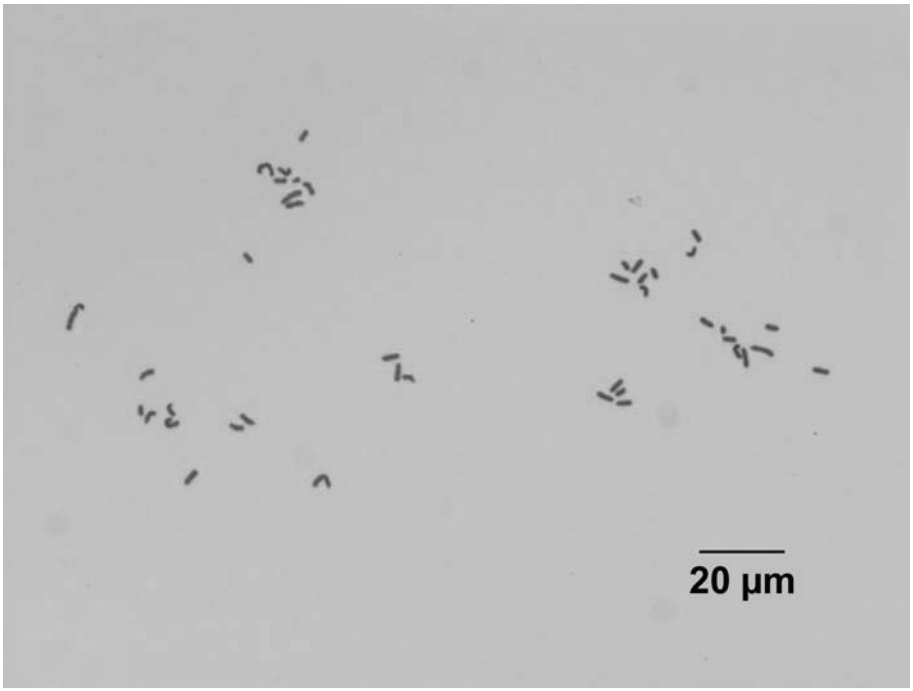

Fig. 10.5 Chromosome preparation of a hybrid zygote between a mouse oocyte and a *Balaenoptera bonaerensis* (Antarctic minke whale) spermatozoon. Two mitotic chromosome sets (left, from minke whale spermatozoon, n=22; right, from mouse oocyte, n=20) are clearly observed (H. Tateno, unpublished data). Original.

at a relatively early elongating spermatid stage. The initial timing of SOAF acquirement in *B. bonaerensis* spermatogenic cells was similar to that in mice and rats, but much later than that in hamsters, rabbits, monkeys, and humans. These studies have showed that intracytoplasmic injection of elongating spermatids or spermatozoa recovered from the vas deferens of whales could be used to produce *in vitro* fertilized embryos in place of standard IVF procedures.

Recent advances have been made in sexing mammalian spermatozoa on the basis of well-known differences in DNA content in X- compared with Y-bearing spermatozoa (Johnson 2000; Garner 2001). Most effort has been directed towards commercial uses, especially for livestock (Garner 2001); however, preliminary studies have revealed some differences in DNA content for X- and Y-bearing spermatozoa in elk, elephants, and camels (Johnson 2000). The sperm motility and density of semen collected from live and dead wild animals often are too low for standard AI or even IVF protocols. If perfected in the future, ICSI using X- and Y- bearing spermatozoa may prove to be a promising tool to manipulate sex ratios in wild animals, including marine mammals, and perhaps aid in management of endangered species.

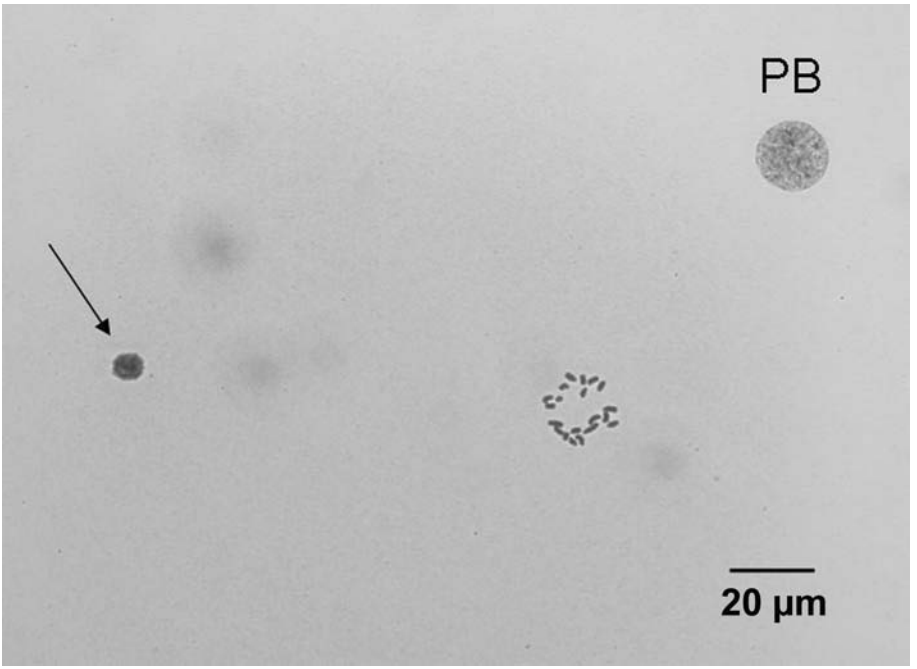

Fig. 10.6 Mitotic chromosome set from a mouse oocyte and a decondensed *Balaenoptera bonaerensis* (Antarctic minke whale) sperm nucleus (arrow). The mouse oocyte was activated following sperm injection and developed to the first cleavage metaphase, while the whale sperm nucleus failed to transform into a pronucleus. PB, nucleus of the second polar body (H. Tateno, unpublished data). Original.

10.5.3 *In vitro* Production (IVP) of Embryos

In vitro production (IVP) consists of IVM of immature follicular oocytes followed by IVF, including micro-injection of sperm into the oocytes, such as ICSI, and IVC to produce the preimplantation stage of embryos. As described in Chapter **7** and in this Chapter, IVP is one of the soundest technologies in the ARTs relating to ET in the field of human clinics and animal husbandry. Today, human and domestic animal blastocysts produced *in vitro* have been used for immediate transfer or for transfer after cryopreservation. Fukui *et al*. (1977a) first attempted *in vitro* embryo production by IVM of *Balaenoptera bonaerensis* oocytes followed by IVF and IVC, but the proportion of cleaved oocytes was only 5.8% by co-culture with follicular cells after IVF. The blastocyst stage of dolphin or whale embryos has not been produced *in vitro*, although some cleaved embryos up to the morula stage have been obtained (Fukui *et al*. 1977a; Asada *et al*. 2001a). *In vitro* development to blastocysts or at least to the compacted morula is desired for transfer into the uterus of recipient whales without surgery. Unfortunately, it would be difficult to restrain recipient whales in the open sea, and many problems such as the

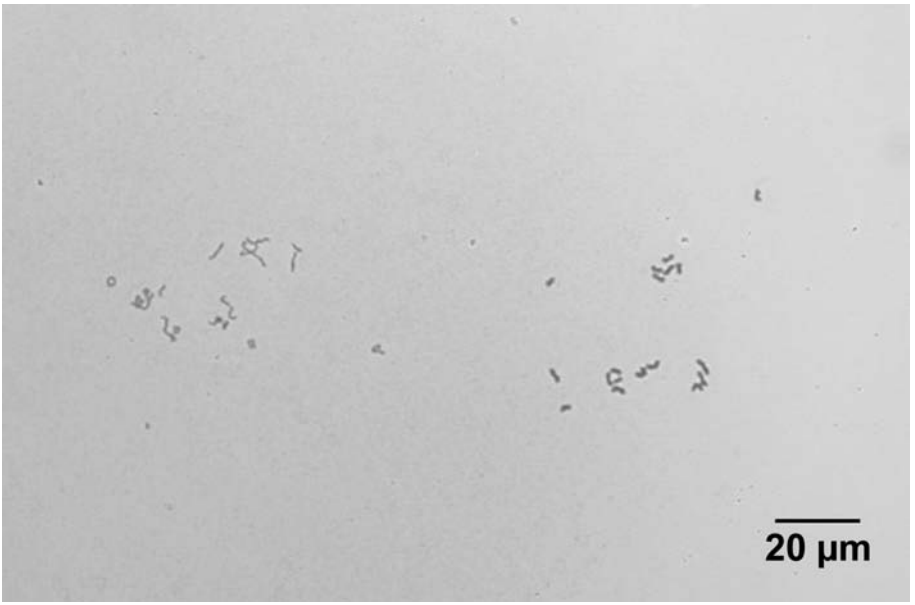

Fig. 10.7 *Balaenoptera bonaerensis* (Antarctic minke whale) sperm nuclei underwent premature chromosome condensation (left), while mouse oocyte chromosomes remained at the second metaphase (right). (H. Tateno, unpublished data). Original.

methods of transfer of embryos and monitoring of the estrous cycle in female whales have to be solved. Therefore, before attempting ET into free-ranging baleen whales, it may be better to attempt ET in dolphins at aquaria using trained behaviors. The premise of this is that trained behaviors already are used in captive aquaria for semen collection from live *Tursiops truncatus* (Yoshioka 1994; Robeck and O'Brien 2004) and *Orcinus orca* (Robeck *et al*. 2004) for AI. Even if ET using freshly produced embryos is not performed, the embryos could be cryopreserved by a conventional slow freezing or ultra-rapid freezing method (vitrification, in Chapter **7**), and examined for viability by the following IVC.

Development of techniques for IVP by IVM/IVF (ICSI)/IVC in dolphins and whales, would provide important information on the basic reproductive events (e.g. follicular development and oocyte maturation, sperm capacitation, mechanism of fertilization, and embryonic development) and also could be applied to population management programs for cetaceans, especially for endangered species.

10.5.4 Nuclear Transfer (NT)

Since the first convincing demonstration of somatic cell nuclear transfer (SCNT) in sheep ("Dolly") (Wilmut *et al*. 1997), many different somatic cell types, such as cumulus or granulosa, oviductal, uterine, fetal or adult skin

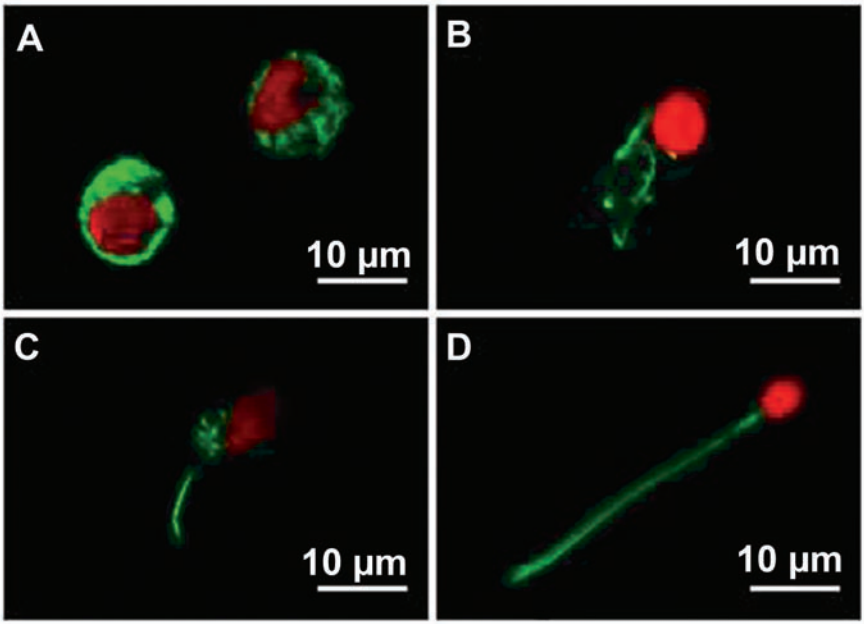

Fig. 10.8 *Balaenoptera bonaerensis* (Antarctic minke whale) testis-derived spermatogenic cells after propidium iodide staining and immuno-staining against α-tubulin. Two fluorescent images were combined, one taken under 514-nm UV light (cell nucleus, red) and one taken under 488-nm UV light (α-tubulin, green) were combined: **A**. A round spermatid (Gorgi/cap phase), **B**. An early-stage elongating spermatid (acrosome phase), **C**. A late-stage elongating spermatid (maturation phase), **D**. A testicular spermatozoon. After Amemiya, K., Iwanami, Y., Terao, T., Fukui, Y., Ishikawa, h., Ohsumi, S., Hirabayashi, M. and Hochi, S. 2004. Journal of Mammalian Ova Research 21:149-156, Fig. 2.

fibroblasts, liver cells, spleen cells, muscular cells, and macrophages, have been used as nuclear donor sources in domestic animal NT. Although the SCNT may be one of the most cost-effective approaches for improving productive and genetic merits in domestic animals, the efficiency (pregnancy and offspring) is still low due to high abortion rate, abnormal placenta formation, and low parturition rate with a high rate of abnormal offspring born. Recent studies have concentrated on defining several important factors influencing the efficiency of SCNT, such as donor cell type, cell cycle of donor cell, DNA remodeling, epigenetic reprogramming, methylation pattern, activation method, and culture method. Beaujean *et al*. (2004) observed that with sheep almost half of the SCNT embryos that survive to the blastocyst stage present abnormally methylated trophectoderm cells, and they concluded that both remodeling of DNA and epigenetic reprogramming appear critical for development of NT embryos.

The techniques of SCNT provide not only a valuable tool to multiply animals with the same genetic traits, but also a prospective alternative to save

endangered animal species (West and Damiani 2000). However, the lack of available species-specific component recipient cytoplasm and the lack of available matured oocytes from endangered animals, including large baleen whales, have been considered major limitations for performing SCNT. Interspecies SCNT, which is involved in transferring cell nuclei of one species into enucleated matured oocytes of another species, may be an alternative approach to clone animal species whose matured oocytes are difficult to obtain or have a low (approximately 30%) maturation rate, as in *Balaenoptera bonaerensis* follicular oocytes (Fukui *et al*. 1997b; Asada *et al*. 2001a). Previous studies on interspecies SCNT have shown that oocyte cytoplasm from cattle, sheep, and rabbits are able to dedifferentiate somatic cell nuclei from sheep, pig, monkey, rat (Dominko *et al*. 1999), cat (Wen *et al*. 2003), and *Ailuropoda melanoleuca* (Giant panda) (Chen *et al*. 2002) and to support early development of these interspecies cloned embryos to blastocysts. Lately, the successes of cloning *Bos gaurus* (gaur) (Lanza *et al*. 2000) and *Ovis mosimon* (muflon) (Loi *et al*. 2001) have demonstrated that it is practical to clone animals using the SCNT techniques.

In our recent study (Ikumi *et al*. 2004) on the interspecies SCNT using granulose-cumulus cells as donor cells, we used *in vitro* matured bovine or porcine oocytes as recipient cytoplasm to investigate the developmental ability of *B. bonaerensis* embryos (Fig. 10.9). There were no significant differences ($P < 0.05$) among the proportions of pseudo-pronucleus (PPN) formation of whale SCNT and interspecies SCNT oocytes. Furthermore, no significant difference ($P < 0.05$) was found in the cleavage rates of whale SCNT embryos between 6-dimethylaminopurin and cycloheximide as secondary activation treatments. There was no significant difference ($P < 0.05$) in the cleavage rates of whale SCNT embryos between the two donor cell types (viable and non-viable). Two- to four-cell stages of whale SCNT embryos were obtained in bovine (42-47%) and porcine (25-43%) cytoplasm, but none of the embryos reached the blastocyst stage. However, the cleaved whale embryos were confirmed to have whale genomic DNA (Figs. 10.10 and 10.11). Further, the results showed that both bovine and porcine oocyte cytoplasm had the potential to form PPN and to produce cleaved whale SCNT embryos, regardless of the survivability of donor cells.

Many factors related to embryonic development contributed to the fact that none of the cleaved whale-bovine and whale-porcine interspecies SCNT embryos reached the blastocyst stage. One such factor is that the timing of activation of the embryonic genome is unclear in the whale. Another factor is that the developmental failure of whale interspecies SCNT embryos may be related to the so-called "cell block" which is species-specific. In cattle, the transition from maternal to embryonic control occurs at the 8-16 cell stages (8-cell block), and in pigs, it occurs at 4-8 cell stages (4-cell block). Furthermore, it is a well-known fact that mitochondria have great variation between species. Mitochondria bear the responsibility of energy production and cellular respiration, and mitochondrial DNA (mtDNA) plays an important

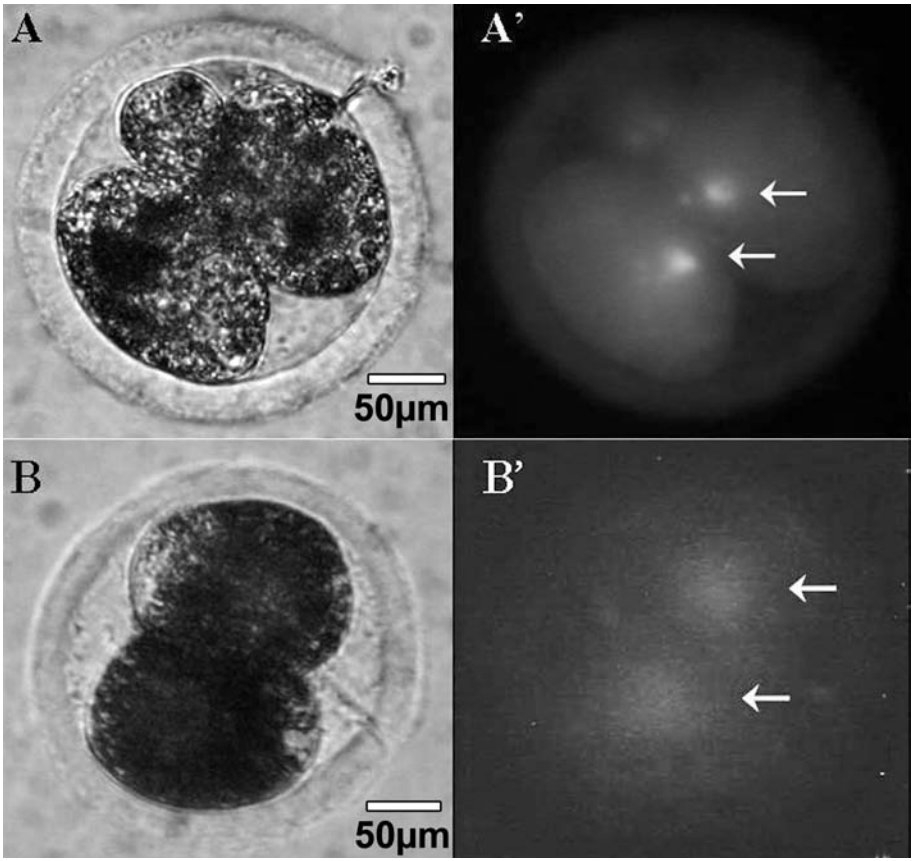

Fig. 10.9 Interspecies somatic cell nuclear transfer (SCNT) derived from *Balaenoptera bonaerensis* (Antarctic minke whale) donor cells. **A**. A 2-cell stage whale-bovine interspecies SCNT embryo at 72 h after activation. **A'.** UV view of A. **B**. A 2-cell-stage whale-porcine interspecies SCNT embryo at 48 h after activation. **B'.** UV view of B. Arrows show nuclei. After Ikumi, S., Sawai, K., Takeuchi, Y., Iwayama, H., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2004. Cloning and Stem Cells 6: 284-293, Fig. 2.

role in nuclear-cytoplasmic incompatibilities (Gomez *et al*. 2003). In interspecies nuclear transfer, it was suggested that mtDNA transferred into recipient cytoplasm by NT might influence the developmental ability of the embryos. The three species (cattle, pig, whale) used in our study (Ikumi *et al*. 2004) as recipient oocytes or donor cells were not genetically close. Chen *et al*. (2002), who produced blastocysts from panda-rabbit cloned embryos, suggested that rabbits might not be proper recipients for interspecies cloned *Ailuropoda melanoleuca* embryos. Thus, the low developmental ability of whale interspecies SCNT embryos might be due to the unsuitability of the mitochondria between the recipient bovine or porcine oocytes and donor cells. Furthermore, the incomplete reprogramming of donor nuclei, DNA

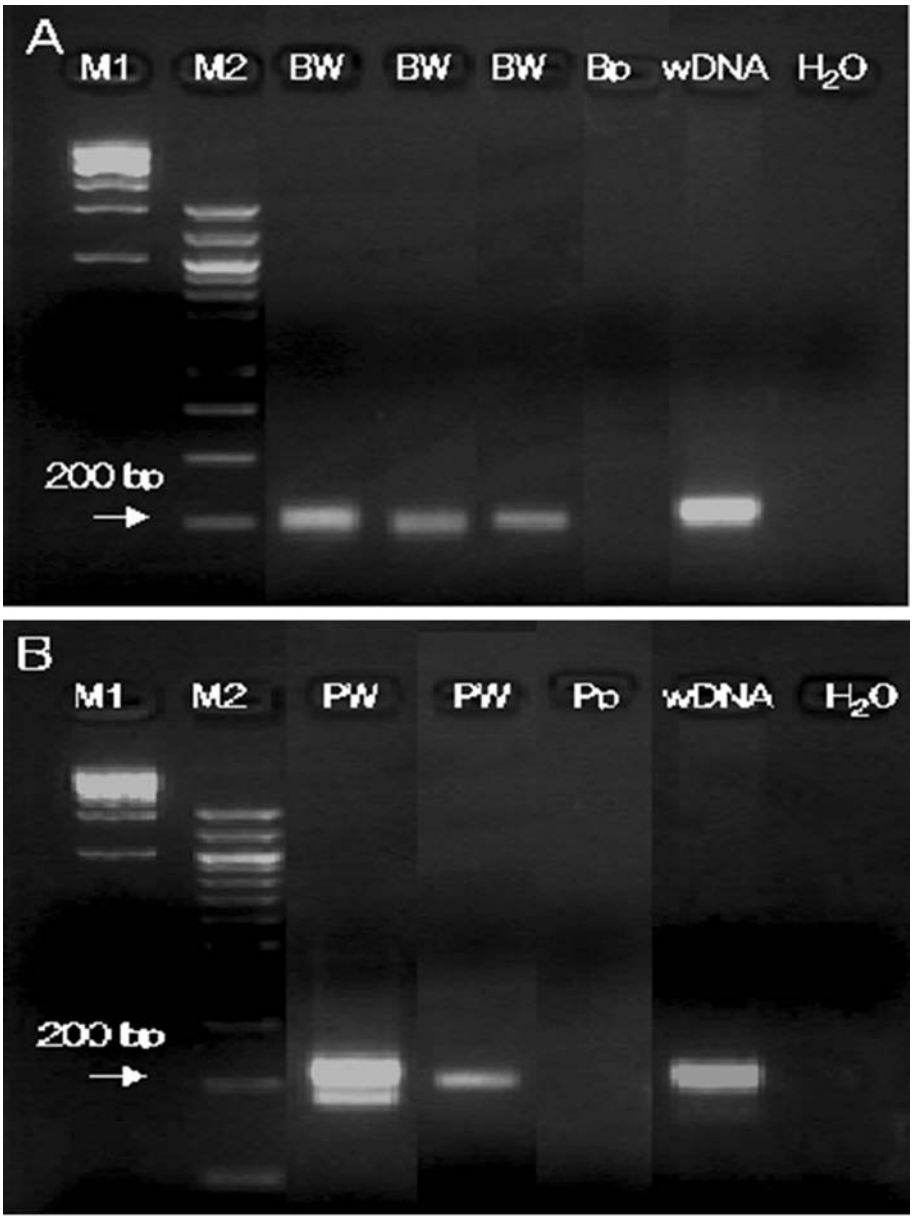

Fig. 10.10 PCR results on signals of *Balaenoptera bonaerensis* (Antarctic minke whale) micro-satellite marker. M1, 1-kbp DNA marker; M2, 100-bp DNA marker; wDNA, whale DNA. **A**. BW, whale-bovine interspecies somatic cell nuclear transfer (SCNT) embryos; Bp, bovine parthenote. **B**. PW, whale-porcine interspecies SCNT embryos; Pp, porcine parthenote. After Ikumi, S., Sawai, K., Takeuchi, Y., Iwayama, H., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2004. Cloning and Stem Cells 6: 284-293, Fig. 3.

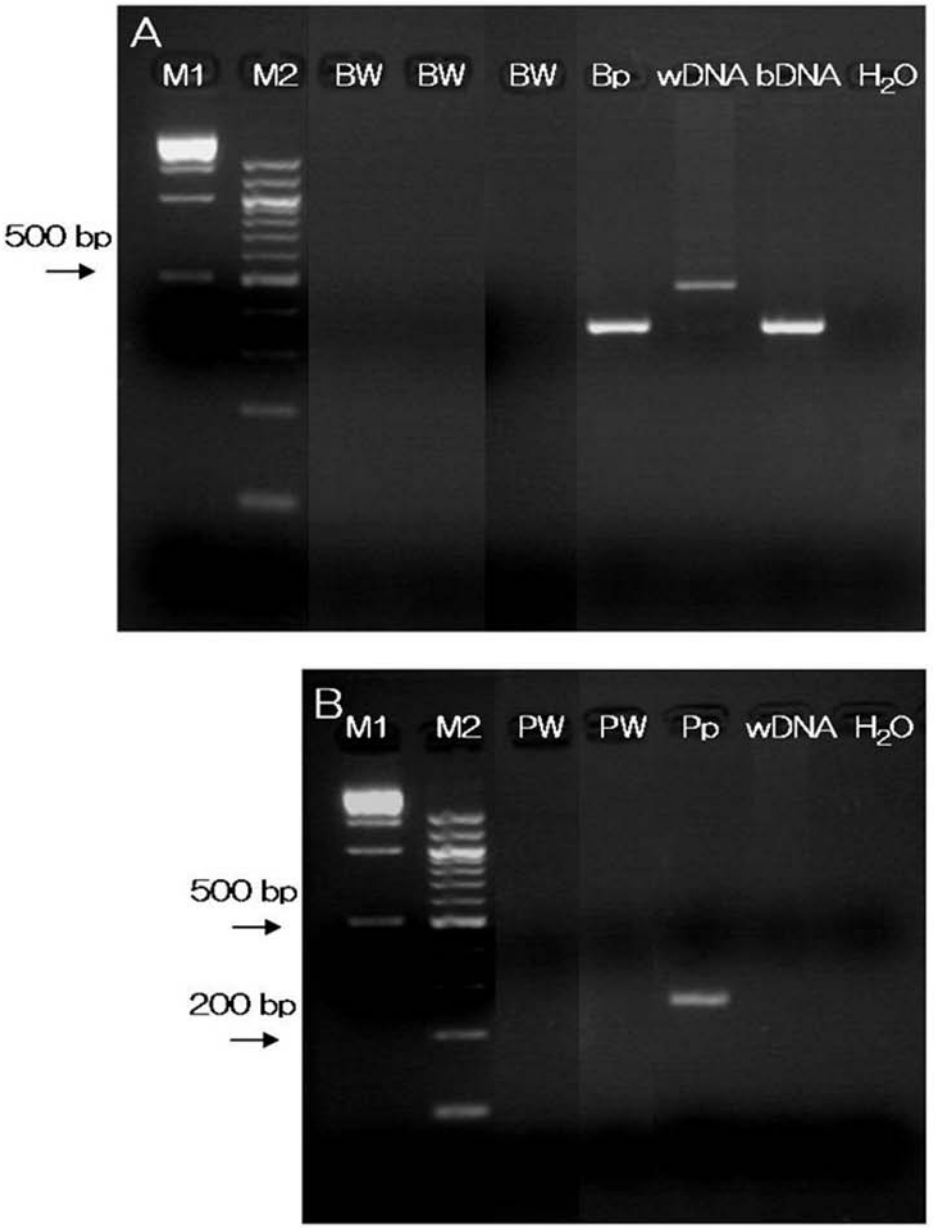

Fig. 10.11 PCR results on signals of bovine or porcine genes. M1, 1-kbp DNA marker; M2, 100-bp DNA marker; wDNA, whale DNA. **A**. BW, whale-bovine interspecies somatic cell nuclear transfer (SCNT) embryos; Bp, bovine parthenote. **B**. PW, whale-porcine interspecies SCNT embryos; Pp, porcine parthenote. After Ikumi, S., Sawai, K., Takeuchi, Y., Iwayama, H., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2004. Cloning and Stem Cells 6: 284-293, Fig. 4.

fragmentations and abnormal methylation patterns of cloned embryos are serious problems for normal embryogenesis. Therefore, a future study on the donor cell reprogramming process at the molecular level is needed to gain in-depth understanding of intra- and interspecies SCNT embryos in the whale. Dominko *et al*. (1999) found that bovine oocyte cytoplasm may be a suitable host for dedifferentiation of somatic nuclei of various mammals, including humans. The embryonic cell lines grown from the interspecies embryos could be used with increasing success of embryonic stem (ES) cell technology.

Embryonic stem cells derived from the inner cell mass (ICM) of blastocysts are self-renewing, pluripotent, and capable of differentiating into any of the cell types found in the adult body. Since the first reports of ES cells from mouse blastocysts (Evans and Kaufman 1981; Martin 1981), ES cell lines have been established in rhesus monkeys (Thomson *et al*. 1995) and humans (Thomson *et al*. 1998). Once ES cell lines from a blastocyst are established, the cells could be cultured to produce a large number of cells to freeze for use as donor cells in NT programs. Unfortunately, no blastocysts have been produced in whales and dolphins, as described in the above sections. Much research is needed to produce cetacean blastocysts *in vitro* for establishing ES cell lines by ARTs combined with IVF, ICSI, IVP and SCNT technologies.

10.5.5 Gene Transfer (GT)

Production of transgenic (TG) animals by introducing exogenous DNA into the pronucleus at the zygote stage and IVF or ICSI with DNA-absorbed spermatozoa has been attempted in mice and domestic animals. Although TG sheep, goats, pigs and cattle can now be routinely produced, the efficiency remains low, particularly for cattle. The application of TG technology to farm animals has promised to improve productivity from the animals and has resulted in a new industry, *e.g.*, the successful expression of foreign proteins in the mammary gland for the pharmaceutical industry (Murray 1999).

Additionally, genetic linkage maps have been developed for a number of livestock species, including cattle, sheep, and pigs, as well as for humans. The total number of genes in the mammalian genome is estimated to be from 30,000 to 70,000. New technologies are being developed in the field of proteonics that will dramatically increase the rate at which protein structure and function information is generated (Kappes 1999). A number of recent advances in genomic research, combined with ARTs, such as SCNT technologies, may change and improve the efficiency in reproduction and population management of cetaceans as well as livestock production in the future.

10.6 ACKNOWLEDGMENTS

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CHAPTER 11

Embryogenesis and Development in *Stenella attenuata* and Other Cetaceans

J.G.M. Thewissen[1] *and John Heyning*[2]

11.1 INTRODUCTION

The prenatal development of most species of cetaceans is poorly known because descriptions were based on fortuitous recoveries of one or a few embryos of one species, and it was impossible to acquire complete ontogenetic series. However, these occasional and inconsistent discoveries have played an important role in the development of the biological sciences. For instance, the discovery that dolphins have hind limb buds was initially controversial (Kükenthal 1893, 1895; Guldberg 1894, 1899); however, they served to boost the nascent evolutionary theory which had been interested in whale evolution since its origin (Darwin 1859, p. 450-456). At present, the best-studied embryological collection of cetaceans is located at the Senckenbergische Anatomie of the Wolfgang Goethe Universität in Frankfurt, Germany. Many recent studies of cetacean embryology are based on this collection (references by Klima and Oelschläger and co-workers), and it has spawned a new interest in theoretical works into broader evolutionary topics, such as developmental control in evolution (Bejder and Hall 2002; Thewissen and Williams 2002). In spite of its great importance, most species of cetaceans in the Frankfurt collection are represented by only a few specimens. A relatively complete ontogenetic series for three delphinid species in the Frankfurt Collection was described by Štěrba *et al.* (2000). These authors divided embryonic and early fetal dolphin development into twelve stages using criteria originally designed for staging land mammal embryos. However, Štěrba *et al.* (2000) did not have material documenting the earliest stages of development (their Stages

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1 and 2), leading them to make assumptions about probable morphologies of early embryos. Although very useful as a descriptive study, Štěrba's *et al.*'s (2000) work is also idiosyncratic. Several of their stages are defined on the basis of characteristics not present in cetaceans (such as Stage 11: haircoat all over body), making objective characterization of these stages impossible. Furthermore, Štěrba *et al.* (2000) rigidly apply their numbering system to the delphinids even though heterochronic events in cetacean evolution have altered the order in which key characters appear in ontogeny. As a result, the sequence of numbers is counterintuitive: Stage 12 occurs between Stage 9 and 10 in developmental time.

In spite of these details, Štěrba *et al.*'s (2000) work remains the best general descriptive paper for development in *Stenella*. Here, we only add to it by using a more widely accepted staging system, the Carnegie System. In addition, we present, in a tabular form (Table 11.1), a literature review of cetacean embryology based on our staging system in order to facilitate easy entry into the literature. Our staging system forms the basis for ongoing protein expression work (Thewissen *et al.* 2006).

Our description of the stages in the development of *Stenella attenuata* (Pantropical spotted dolphin) follows that of the Carnegie staging system, with minor modifications. The Carnegie system was designed for human embryos (O'Rahilly and Müller 1987) and is widely used in describing the embryonic development of mammalian species. It is especially appropriate for use in dolphins because these cetaceans are similar in (adult) size to humans and have a similar gestation period (11 months, Perrin 2002). There is reasonable agreement between the Carnegie staging system as applied by us in *Stenella*, and the staging system used by Štěrba for delphinids, but there also are important differences. Table 11.2 compares individual stages in these systems. The Carnegie system addresses only the embryonic period of human prenatal development. Since our collection also contains many fetuses, and since many significant cetacean features arise in the fetal period, we have extended our staging system into this period using some of the criteria previously identified by Štěrba *et al.* (2000) and calling these later stages Fetal Stages. The discussion in this paper is restricted to prenatal development, with special emphasis of the embryonic period (the initial 8 weeks after fertilization). There is vast literature on postnatal development and growth, summarized in part by Bryden (1972).

11.2 METHODS

Stenella embryos and fetuses were obtained from the Los Angeles Natural History Museum (LACM) and were drawn, photographed, and measured. The staging system described below is based on this study. Smaller specimens (up to Carnegie 20) were usually preserved in ethanol, whereas larger specimens were usually preserved in formalin. All of these specimens had been in

Table 11.1 A bibliography of cetacean embryology. Listed are the the most important works that have as their primary objective a description of cetacean embryology. Cetacean embryology goes back to the first half of the 18th century (see partial reviews by Eales 1950 and Karlsen 1962), although no useful descriptions were published by the early authors.

Genus	*Organ system*	*Reference*
Balaena	heart	Tarpley *et al*. 1997
Balaenoptera	brain	Friant 1957
Balaenoptera	brain	Friant 1967
Balaenoptera	brain stem	Jansen and Korneliussen 1977
Balaenoptera	brain stem	Jansen and Osen 1984
Balaenoptera	brain stem	Korneliussen and Jansen 1964
Balaenoptera	cerebellum	Jansen 1950
Balaenoptera	cerebellum	Korneliussen 1967
Balaenoptera	cerebellum	Korneliussen and Jansen 1966
Balaenoptera	chondrocranium	Burlet 1914b
Balaenoptera	clavicle	Klima 1990a
Balaenoptera	dentition	Dissel-Scherft and Vervoort 1954
Balaenoptera	dentition	Eschricht 1849
Balaenoptera	dentition	Leche 1892
Balaenoptera	dentition and mandible	Karlsen 1962
Balaenoptera	dentition, no description	Leche 1895
Balaenoptera	digestive tract	Amasaki *et al*. 1989a
Balaenoptera	ear	Solntseva 1985
Balaenoptera	ear	Solntseva 1990
Balaenoptera	ear	Solntseva 1999
Balaenoptera	external genitalia	Amasaki *et al*. 1989b
Balaenoptera	external morphology	Gill 1927
Balaenoptera	fore- and hindlimbs	Amasaki *et al*. 1989c
Balaenoptera	integument	Lick (unpubl) 1987
Balaenoptera	mandible and teeth	Julin 1880
Balaenoptera	nasal skull	Klima 1995
Balaenoptera	nasal skull	Klima 1999
Balaenoptera	olfactory system	Oelschlager 1989
Balaenoptera	oocyte through morula	Asada *et al*. 2001
Balaenoptera	overview	Schulte 1912
Balaenoptera	pelvic rudiments	Hosokawa 1951
Balaenoptera	skin	Naaktgeboren 1960
Balaenoptera	skull	Ridewood 1922
Balaenoptera	teeth	Kukenthal 1891
Balaenoptera	teeth	Pouchet and Chabry 1882
Balaenoptera	teeth and baleen	Ishikawa and Amasaki 1995
Balaenoptera	vertebral column	Burlet 1917a
Balaenoptera	vestibular organ	Solntseva 1998
Balaenoptera	vestibular organ	Solntseva 2002
Delphinapterus	ear	Solntseva 1990
Delphinapterus	ear	Solntseva 1999
Delphinapterus	vestibular organ	Solntseva 2002

Table 11.1 Contd. ...

Table 11.1 Contd. ...

delphinid	vestibular organ	Solntseva 1999
Delphinus	clavicle	Klima 1990a
Delphinus	gubernaculum	Schoot, van der 1995
Delphinus	head pigmentation	Perrin 1997
Delphinus	hind limb, genitals, mammary gland	Guldberg 1899
Delphinus	middle ear	Kinkel *et al*. 2001
Delphinus	nasal skull	Klima 1995
Delphinus	nasal skull	Klima 1999
Delphinus	overview	Sterba *et al*. 2000
Delphinus	skeleton	Buffrenil and Collet 1983
Delphinus	skin	Meyer *et al*. 1995
dolphin	brain growth	Pirlot and Kamiya 1982
Eschrichtius	clavicle	Klima 1990a
Eschrichtius	gubernaculum	Schoot, van der 1995
Globicephala	chondrocranium	Schreiber 1915
Globicephala	neck vertebrae	Ogden *et al*. 1981
Lagenorhynchus	chondrocranium	Burlet 1914a
Lagenorhynchus	clavicle	Klima 1990a
Lagenorhynchus	nasal skull	Klima 1987
Lagenorhynchus	nasal skull	Klima 1995
Lagenorhynchus	nasal skull	Klima 1999
Lagenorhynchus	nasal skull	Klima and van Bree 1990
Lagenorhynchus	nervus terminalis	Buhl and Oelschlager 1986
Lagenorhynchus	olfactory system	Oelschlager *et al*. 1987
Lagenorhynchus	overview	Guldberg and Nansen 1894
Lagonorhynchus	nasal skull	Klima and van Bree 1985
Megaptera	brain	Anthony 1925
Megaptera	brain	Riese 1928
Megaptera	chondrocranium	Honigman 1917
Megaptera	clavicle	Klima 1990a
Megaptera	dentition	Eschricht 1849
Megaptera	forebrain	Riese 1936
Megaptera	hind limbs, fluke	Ogawa 1953
Megaptera	nasal skull	Klima 1995
Megaptera	nasal skull	Klima 1999
Megaptera	olfactory system	Oelschlager 1989
Megaptera	overview incl. hind limbs	Kukenthal 1914
Megaptera	skull	Ridewood 1922
Megaptera	sternum and clavicle	Klima 1978
Megaptera		Stump *et al*. 1960
Monodon	clavicle	Klima 1990a
Monodon	head	Eales 1951
Monodon	nasal skull	Klima 1987
Monodon	nasal skull	Klima 1995
Monodon	nasal skull	Klima 1999
Monodon	nasal skull	Klima and van Bree 1985

Table 11.1 Contd. ...

Table 11.1 Contd. ...

Monodon	nervus terminalis	Buhl and Oelschlager 1986
Monodon	olfactory system	Oelschlager *et al*. 1987
mysticete	brain	Guldberg 1885
mysticete	olfactory system	Oelschlager 1989
mysticete	dentition	Leche 1893
mysticete	dentition	Weber 1886
odontocete	olfactory system	Oelschlager and Buhl 1984
Orca	overview	Guldberg and Nansen 1894
Phocaena	brain	Buhl and Oelschlager 1988
Phocaena	brain	Friant 1952
Phocaena	brain	Friant 1967
Phocaena	chondrocranium	Burlet 1913a
Phocaena	chondrocranium	Burlet 1913b
Phocaena	chondrocranium	Burlet 1917b
Phocaena	dentition, no description	Leche 1892
Phocaena	general description	Muller 1921
Phocaena	gubernaculum	Schoot, van der 1995
Phocaena	hind limb, mammary gland	Guldberg 1899
Phocaena	hind limbs	Guldberg 1894
Phocaena	hind limbs	Kukenthal 1895
Phocaena	integument	Lick (unpubl) 1987
Phocaena	nasal skull	Klima 1987
Phocaena	nasal skull	Klima 1995
Phocaena	nasal skull	Klima 1999
Phocaena	nasal skull	Klima and van Bree 1985
Phocaena	nasal skull	Oelschlager and Buhl 1985a
Phocaena	nervus terminalis	Buhl and Oelschlager 1986
Phocaena	olfactory system	Oelschlager and Buhl 1985b
Phocaena	olfactory system	Oelschlager *et al*. 1987
Phocaena	overview	Guldberg and Nansen 1894
Phocaena	overview	Sterba *et al*. 2000
Phocaena	teeth	Leche 1895
Phocaena	teeth, hind limb	Kukenthal 1893
Phocaena	clavicle	Klima 1990
Physeter	brain	Oelschlager and Kemp 1998
Physeter	clavicle	Klima 1990a
Physeter	external morphology	Beddard 1919
Physeter	external morphology; viscera	Beddard 1915
Physeter	gubernaculum	Schoot, van der 1995
Physeter	integument	Lick (unpubl) 1987
Physeter	nasal skull	Klima 1987
Physeter	nasal skull	Klima 1990b
Physeter	nasal skull	Klima 1995
Physeter	nasal skull	Klima 1999
Physeter	nervus terminalis	Buhl and Oelschlager 1986
Physeter	olfactory system	Oelschlager *et al*. 1987

Table 11.1 Contd. ...

Table 11.1 Contd. ...

Physeter	overview	Kukenthal 1914
Physeter	skull	Kuzmin 1976
Physeter	nasal skull	Klima *et al*. 1986
Platanista	overview	Kukenthal 1914
Sotalia	overview	Kukenthal 1914
Stenella	brain	Kamiya and Pirlot 1974
Stenella	clavicle	Klima 1990a
Stenella	ear	Solntseva 1983
Stenella	ear	Solntseva 1990
Stenella	ear	Solntseva 1999
Stenella	forelimb	Calzada and Aguilar 1996
Stenella	forelimb	Sedmera *et al*. 1997b
Stenella	general	Sinclair 1962
Stenella	general cross-sections	Hosokawa 1955
Stenella	head pigmentation	Perrin 1997
Stenella	heart	Sedmera *et al*. 2003
Stenella	hind limb	Sedmera *et al*. 1997a
Stenella	hind limbs, fluke	Ogawa 1953
Stenella	hind limb	Thewissen *et al.* 2006
Stenella	lungs	Drabek and Kooyman 1983
Stenella	middle ear	Kinkel *et al*. 2001
Stenella	nasal skull	Klima 1987
Stenella	nasal skull	Klima 1995
Stenella	nasal skull	Klima 1999
Stenella	nervus terminalis	Buhl and Oelschlager 1986
Stenella	olfactory system	Oelschlager *et al*. 1987
Stenella	overview	Sterba *et al*. 2000
Stenella	skeleton	Ito and Miyazaki 1990
Stenella	skin	Meyer *et al*. 1995
Stenella	sternum and clavicle	Klima 1978
Stenella	teeth	Misek *et al.* 1996
Stenella	vestibular organ	Solntseva 1996
Stenella	vestibular organ	Solntseva 2002

Table 11.2 Comparison of cetacean prenatal stages in this study, Carnegie (C) and Fetal (F), with those of Štěrba *et al.* (2000) (S).

This study	*Štěrba et al.*
C9+C10	S1
C11+C12+C13	S2
C14+C15	S3
C16	S4
C17	S5
C18+19	S6
F20	S7+8
F21	S9
F22	S12
F23	S10+11

fixative for at least 15 years, and considerable shrinkage likely occurred. Measurements listed below are uncorrected for shrinkage and fresh embryos were probably larger. Dimensions for Carnegie stages 8 to 10 are indicated as total length (TL), as these embryos are small and straight and can be easily measured with an ocular micrometer. Dimensions during Carnegie stage 11-18 are given as crown-rump lengths (CRL) which are easily measured without handling delicate specimens excessively. During stages 19-23, TL is the preferred method of size determination because these embryos and fetuses move and curvature varies, making CRL a less than adequate indicator of size.

This study represents the first phase in a large project that intends to document cetacean development in detail. At present, embryos are being thin-sectioned and stained. We are creating the Digital Library of Dolphin Development, which will contain downloadable JPG and TIF images of all sections of all embryos (many thousands of histological sections for some of the larger specimens). Hosting these on a website (http://www.neoucom.edu/dldd) will allow researchers to study embryonic development in detail and we will interface the data files with user-friendly web pages in order to highlight some of the more salient points of dolphin development. In addition, our collection of Stenella specimens will form the basis for research on the gene control of development using antibodies (Thewissen et al., 2006).

11.3 DESCRIPTION OF EMBRYONIC AND FETAL STAGES

11.3.1 Carnegie Stage 8

Diagnostic criteria. Embryo consisting of an oval disc with a primitive streak, no somites.

Material. LACM 94744, 94766, 94723, 94775, 94803, 94791

Dimensions. Total length (TL): 1.5-3.2 mm.

Discussion. The earliest stages represented in our collection represent Carnegie 8. These embryos do not differ morphologically from other mammalian embryos at this stage.

11.3.2 Carnegie Stage 9

Diagostic criteria. From one to six somites.

Material. LACM 94633, 94737.

Dimensions. TL: 6.0 mm.

Discussion. A single embryo in our collection pertains to this stage. It is flat with a pronounced neural groove and shows early somite formation, similar to other mammalian embryos (O'Rahilly and Müller 1987).

11.3.3 Carnegie Stage 10

Diagnostic criteria. More than six somites, pharyngeal arches not visible. rostral neuropore near closing or closed, embryo straight or folding onto itself (Fig. 11.1A-B).

Material. LACM 94631, 94633, 94648, 94727, 94730, 94749, 94786, 94792, 94802.

Dimensions. TL: 3.9-6.0 mm.

Discussion. Neurulation is in progress at this stage, and the neural groove is widely open. There are no pharyngeal arches. The head is forming.

In LACM 94730, there are 13 visible somites and there is a faint optic placode but no cardiac bulge. The yolk stalk protrudes widely from its ventral surface. Embryos of this stage can be straight (LACM 94648) or occasionally show the spiraling that develops fully in Carnegie 11 (LACM 94730).

11.3.4 Carnegie Stage 11

Diagnostic criteria. Pharyngeal arches I, II, and III visible, pharyngeal cleft 1 and 2 visible, rostral neuropore closed. Forelimb bud present in late embryos of this stage (Fig. 11.1C-D).

Material. LACM 94674, 94678, 94700, 94705, 94714, 94731, 94737, 94741, 94751, 94753, 94781, 94787, 94783, 94813

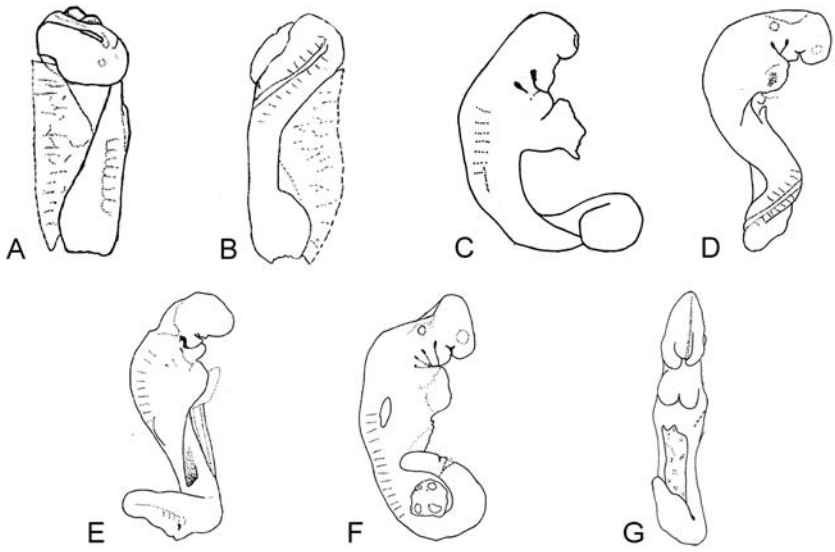

Fig. 11.1 Early embryos of *Stenella attenuata*. **A-B**. Carnegie Stage 10, displaying the beginnings of spiraling (LACM 94730). **C**. Carnegie Stage 11 (LACM 94741). **D**. Carnegie Stage 11 (LACM 94674). **E**. Carnegie Stage 12 (LACM 94789). **F**. Carnegie Stage 13 (LACM 94657). **G**. Carnegie Stage 13 in ventral view (LACM 94815). Not to scale. Original.

Dimensions. Crown-Rump Length (CRL): 3.3-6.5 mm

Discussion. This stage is characterized by the beginning of facial development. The frontal process and maxillary prominence and mandibular prominences are beginning development. The nasal placode is visible at this stage. Otic vesicle is present in older embryos of this stage (LACM 94674). Pharyngeal cleft 3 may appear occasionally late during this stage (LACM 94674).

More than 20 somites are visible in LACM 94674. This exceeds the number of somites in human Carnegie 11 embryos (O'Rahilly and Müller 1987). *Stenella* consistently has more somites than humans at comparable Carnegie stages. We assume that this is correlated to the vast difference in adult vertebrae between these species. The cardiac bulge is present in most of these specimens. There are no limb buds in early embryos of this stage but the forelimb bud develops in late Carnegie 11 embryos (LACM 94705).

In early embryos of this stage (LACM 94787), the tail is folded onto the right flank (Fig. 11.1C), while the body is more or less straight, as in Carnegie 10. In later embryos, the tail forms a broad spiral (Fig. 11.1D). Spiraling starts caudally (LACM 94741) and progresses cranially (LACM 94674), and is counterclockwise in caudal view.

11.3.5 Carnegie Stage 12

Diagnostic criteria. Pharyngeal cleft 1-3 visible, forelimb bud present (Fig. 11.1E).

Material. LACM 94600, 94642, 94713, 94734, 94767, 94789, 94804, 94789.

Dimensions. CRL: 5.2-5.5 mm.

Discussion. Pharyngeal cleft 3 appears at this stage and somites are visible throughout the length of the embryo. Forelimb buds are clear at this stage, but there are no hind limb buds. As in Carnegie 11, the tail of these embryos spirals.

11.3.6 Carnegie Stage 13

Diagnostic criteria. Pharyngeal arch I-IV visible, pharyngeal cleft 1-4 visible, hindlimb bud present, lens placode convex and not indented (Figs. 11.1F-G, 11.2A, 11.3A).

Material. LACM 94619, 94628, 94632, 94643, 94656, 94657, 94707, 94733, 94735, 94785, 94797, 94815,

Dimensions. CRL: 5.4-8.6 mm, forelimb bud length: 0.20-0.39 mm.

Discussion. Pharyngeal cleft 4 is clearly visible at this stage (Fig. 11.2A). The forelimb bud is a flat, paddle-shaped structure. Hind limb buds are located on the ventral body wall, ventral to somite 43 (Fig. 11.1G). The embryos of this stage do not exhibit spiral twisting, but their tails are folded back onto the abdomen and may cross the abdomen on the left or right side. Early embryos

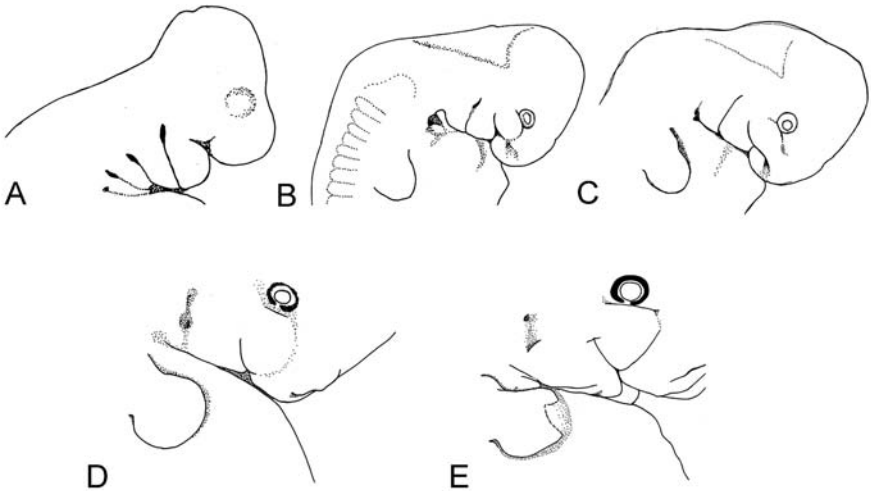

Fig. 11.2 Face of *Stenella attenuata* embryos. **A**. Carnegie Stage 13 (LACM 94657). **B**. Carnegie Stage 14 (LACM 94594). **C**. Carnegie Stage 15 (LACM 94746). **D**. Detail of Carnegie Stage 16 (LACM 94651). **E**. Detail of Carnegie Stage 16 (LACM 94770). Not to scale. Original.

of this stage have a convex back, but the back is concave in later embryos (LACM 94815, 94628, 94735, and 94619).

Štěrba *et al*. (2000) equated their Stage 3 with Carnegie Stage 13, the youngest stage for which they had specimens. They characterized this stage as having four brachial bars, anterior and posterior limb buds, and a tail bud. Stage 2 of Štěrba *et al*. (2000) was characterized by presence of 1-7 somites. This implies that embryos with more than 7 somites and fewer than four branchial arches cannot be assigned in their system. This includes all embryos identified by us as Carnegie 10-12.

11.3.7 Carnegie Stage 14

Diagnostic criteria. Lens vesicle not closed, lens placode indented, optic cup formed, branchial clefts 4-6 fusing, tapering limb buds (Figs. 11.2B, 11.3B, 11.4A-B).

Material. LACM 94594, 94617, 94637, 94641, 94649, 94701, 94702, 94726, 94738, 94739, 94758, 94776, 94782, 94794, 94814, 94819, 94821.

Dimensions. CRL: 6.7-12.6 mm., forelimb bud length: 0.96-1.49 mm.

Discussion. Carnegie 14 embryos are best differentiated based on the development of their eye, as studied in histological section (Fig. 11.3B). The lens placode is indented, the lens vesicle is not closed, the optic cup is forming, and the eye lacks all pigmentation. The nasal prominence is indistinct in earlier embryos of this stage (LACM 94617), but becomes pronounced later (LACM 94701). The nasal pit appears during this stage.

Branchial clefts 4 through 6 are fusing and recognizable as a single depression in the side of the embryo. In most of these embryos, the external auditory meatus is distinct from the first branchial cleft.

The forelimb bud of Carnegie 14 embryos differs from that of Carnegie 13 embryos in being oriented in the parasagittal plane. Carnegie 14 embryos are curved in one plane, they do not spiral. They have a convex back, and a strongly convex neck. Somites are poorly visible in later embryos of this stage.

There are clearly visible fore- and hind-limb buds. The forelimbs taper, there is no handplate. The genital tubercle is prominent.

Stage 3 of Štěrba *et al.* (2000) combines Carnegie 14 and Carnegie 15. These authors estimated an age range of 22-28 days for Stage 3 embryos.

11.3.8 Carnegie Stage 15

Diagnostic criteria. Lens vesicle closed, beginning of eye pigmentation, nasal pit clear, handplate forms (Figs. 11.2C, 11.4C-D).

Material. LACM 94613, 94645, 94706, 94710, 94746, 94748, 94726, 94773, 94778, 94780, 94808, 94810.

Dimensions. CRL: 8.6-10.4 mm, forelimb bud length: 1.1-1.5 mm.

Discussion. On histological section, it is apparent that the optic cup is well-developed and the lens vesicle is detached from the surface epithelium. Initial stages of eye pigmentation occur during this stage. Branchial clefts 1 and 2 are strongly reduced, and only a small dimple remains for branchial clefts 4-6. Carnegie 15 embryos differ from Carnegie 14 embryos by the presence of a flattened handplate, and unlike Carnegie 16 embryos, the digits are not distinct in the hand. *Stenella* embryos of this stage have larger hindlimb buds than preceding or subsequent stages. The hindlimb buds are variable in shape, with some tapering distally, whereas others widen distally. They are much smaller than the forelimb buds. In general shape, Carnegie 14 and 15 embryos are convex, curled in a flat plane.

11.3.9 Carnegie Stage 16

Diagnostic criteria. Branchial clefts absent, eye pigmentation distinct, hand plate distinct (Figs. 11.2D-E, 11.4E-F).

Material. LACM 94651, 94728, 94747, 94752, 94756, 94759, 94722, 94770, 94777, 94784, 94793, 94807.

Dimensions. CRL: 11.2-15.8 mm. Forelimb bud length: 1.2-1.6 mm.

Discussion. The lens is translucent in preserved embryos of this stage, and the nose opening is asymmetrical. Branchial cleft 1 is represented by two distinct depressions, the superior of which is the external auditory meatus (Fig. 11.2D). The handplate is the best external diagnostic feature for these embryos; it is distinct but lacks digital rays. Somites are occasionally visible

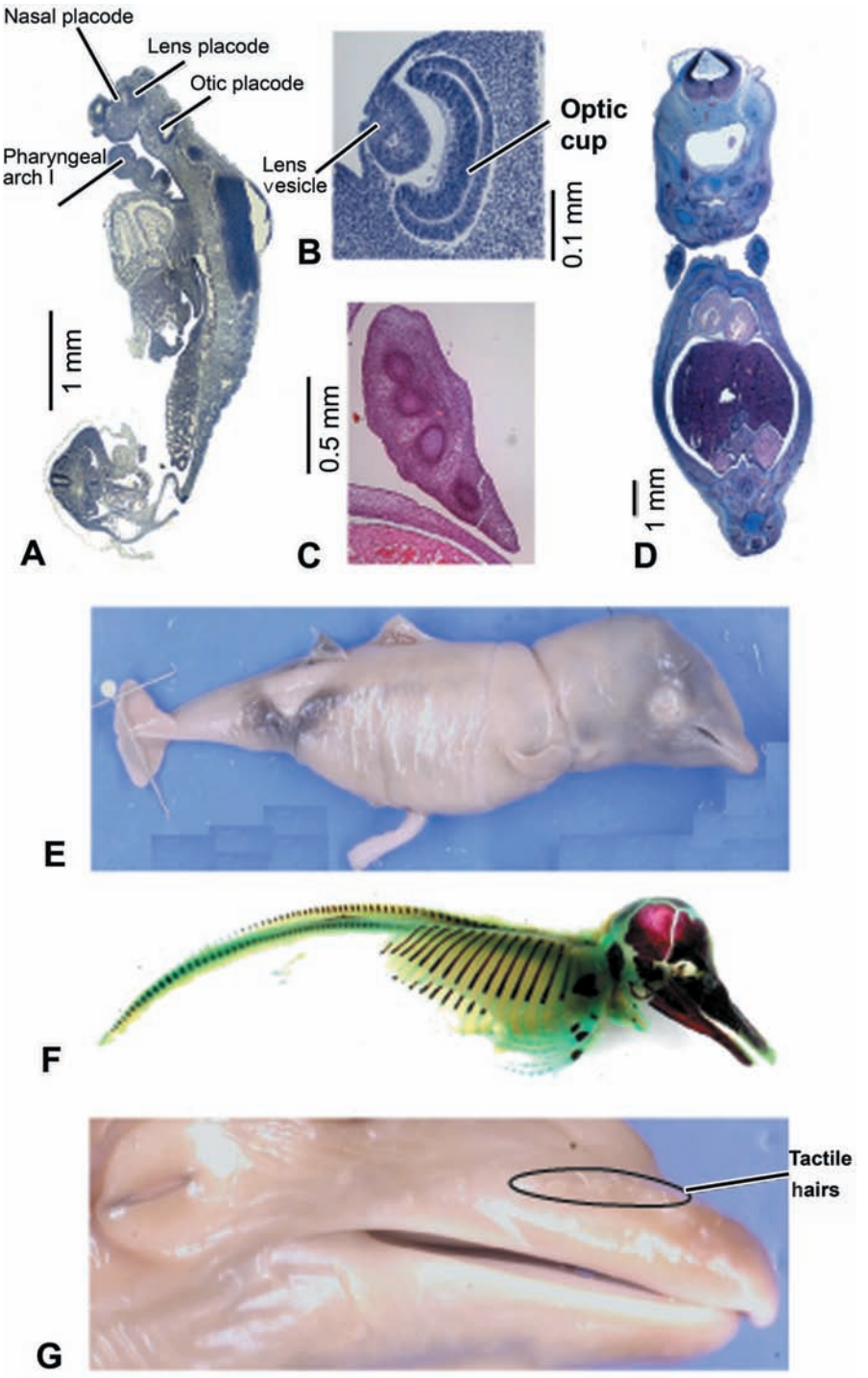

Fig. 11.3 Contd. ...

through the skin. The hindlimb buds have stopped their growth and are smaller than the genital tubercle. Carnegie 16 embryos are similar in their convex shape to Carnegie 15 embryos, but the neck of the former is distinctly more convex than at the earlier stage. Carnegie 16 embryos in humans are mainly characterized by nasal pits, auricular hillocks, and features of the hind limbs (O'Rahilly and Müller 1987). All of these features are greatly modified in cetaceans and cannot be used to characterize this stage. Carnegie 16 embryos were catalogued as Stage 4 embryos by Štěrba *et al.* (2000), based on eye pigmentation and presence of a handplate. These authors noted the appearance of the first cartilagenous tissue at this stage and proposed a mean age of 30 days for these embryos.

11.3.10 Carnegie Stage 17

Diagnostic criteria. Digital rays formed in hand, digit 3 longer than digit 2, initial outgrowth of fluke and dorsal fin (Figs. 11.3 C, 11.4G, 11.5A).

Material. LACM 94601, 94621, 94630, 94639, 94650, 94661, 94667, 94670, 94673, 94681, 94688, 94693, 94715, 94745, 94771, 94798, 94694, 94697, 94698, 94820.

Dimensions. CRL: 12.2-23.0 mm, forelimb 2.9-4.3 mm.

Discussion. The eyelids are forming in these embryos and the nasal opening is asymmetrical. The porus acousticus is all that remains of branchial cleft 1.

Externally, these embryos are best distinguished by the presence of digits in the handplate (Fig. 11.5A). There are five digital rays in the forelimb and interdigital areas are not indented. Unlike in Carnegie 18, digit 3 is longer than digit 2 during Carnegie 17. The left forelimb of LACM 94670 is longer than the right forelimb. Rudiments of the hindlimb buds may still occur lateral to the genital tubercle at this stage; they are not present during Carnegie 18. During Carnegie 17, the viscera have herniated through the umbilicus. The tail displays the beginning of lateral outgrowths that form the fluke (Fig. 11.5D), and on the back, some embryos show the beginnings of the dorsal fin. Embryos in C17 are less convex than earlier embryos.

Carnegie Stage 17 coincides with Stage 5 of Štěrba *et al*. (2000). These authors list "pinna present" as a characteristic of Stage 5 on page 56.

Fig. 11.3 Contd. ...

Fig. 11.3 Color images of *Stenella attenuata* embryos and fetuses. **A**. parasagittal section through Carnegie 13 (LACM 94657, section 73b). **B**. Eye of Carnegie 14 (LACM 94594, section 45). **C**. Section through left flipper of Carnegie Stage 17 (LACM 94715, section 337). **D**. Frontal section through Carnegie 18 (LACM 94534, section 263). **E**. Fetal Stage 22 (LACM 94310), TL=182 mm **F**. Clear-and-Stain of Fetal Stage 23 (LACM 94285), TL=218 mm **G**. Fetal Stage 23, close-up of face showing loss of epidermal seal of eye and tactile hair (LACM 94285), TL=218 mm. Original.

However, in delphinids the pinna never develops (as indicated by the same authors on p. 40). Štěrba *et al.* (2000) estimate the age of these embryos as 32-42 days.

11.3.11 Carnegie Stage 18

Diagnostic criteria. Second and third fingers similar in length (Fig. 11.4G).

Material. LACM 94605, 94623, 94634, 94704, 94750, 94717, 94769, 94818.

Dimensions. CRL: 21.2-24.6 mm, forelimb length: 3.4 mm.

Discussion. At this stage the embryo is beginning to assume some of the external features typical of dolphins. The left nostril is reduced to a narrow slit and the rostrum is pointed but not elongated. The external auditory meatus is disappearing. The hand has lost its symmetry and second and third fingers are similar in length. Internally the digits of the hand are developed as mesenchymal condensations. The intestines are still herniated through the umbilicus. There is no remnant of a hind limb, and fluke development is now distinct. Stage 6 of Štěrba *et al.* (2000) is defined on the basis of internal features only and combines Carnegie 18 and 19. Štěrba *et al.* (2000) proposed that their Stage 6 occurred at 41-52 days of development.

11.3.12 Carnegie Stage 19

Diagnostic criteria. Second finger longer than third, beak distinct (Figs. 11.4I, 11.5B).

Material. LACM 94624, 94663, 94682, 94685, 94817.

Dimensions. TL: 70-74 mm, forelimb length: 6.9 mm, fluke width: 1.9 mm

Discussion. The snout has grown into a pronounced beak. Eyelids are present, but are not fused, and the external ear is visible as a slight surface elevation. Phalanges of the hand are distinct and can be seen through the skin in a complete embryo. The hand is hyperphalangeous. The trunk is elongating at this stage and the umbilical hernia not retracted.

For these embryos, CRL can be determined, allowing calibration of TL and CRL. For LACM 95817, CRL is 31 mm and TL is 62 mm. For LACM 94696, CRL is 30.8 and TL is 70 mm.

11.3.13 Fetal Stage 20

Diagnostic criteria. Eyelids fused, umbilical hernia retracted (Fig. 11.4J).

Material. LACM 94292, 94547, 94565, 94577, 94583, 94592, 94607, 94625, 94640, 94646, 94660, 94671, 94677, 94679, 94683, 94689, 94696.

Dimensions. TL: 71-161 mm.

Discussion. For the later embryonic stages, the Carnegie System relies mainly on features of the hands and feet. Dolphin development deviates strongly from

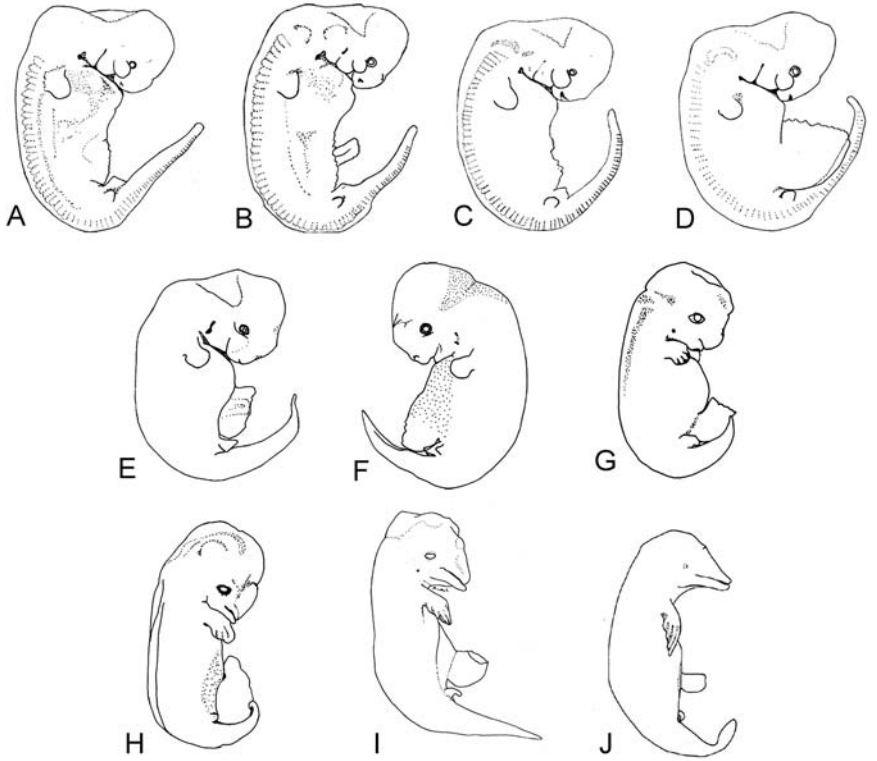

Fig. 11.4 Late embryos and an early fetus of *Stenella attenuata*. **A**. Carnegie Stage 14 (LACM 94701) **B**. Carnegie Stage 14 (LACM 94594) **C**. Carnegie Stage 15 (LACM 94746). **D**. Carnegie Stage 15 (LACM 94613). **E**. Carnegie Stage 16 (LACM 94651). **F**. Carnegie Stage 16 (LACM 94770), left lateral view to show asymmetry in nasal opening. **G**. Carnegie Stage 17 (LACM 94715). **H**. Carnegie Stage 18 (LACM 94634) **I**. Carnegie Stage 19 (LACM 94817). **J**. Fetal Stage 20 (LACM 94671). Not to scale. Original.

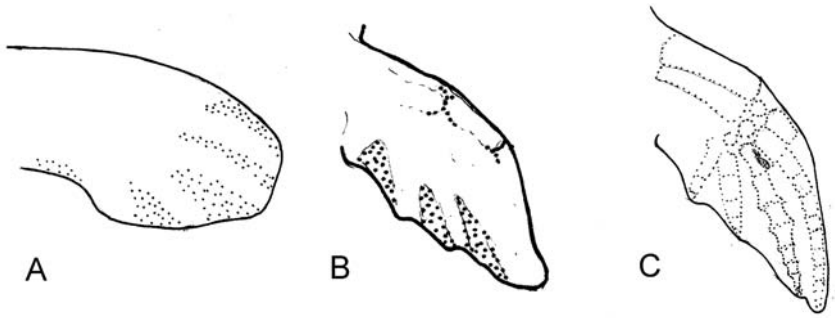

Fig. 11.5 Forelimb development in *Stenella attenuata*. **A**. Right forelimb of Carnegie Stage 17 (LACM 94650). **B**. Right forelimb of Carnegie Stage 19 (LACM 94817). **C**. Right forelimb of Fetal Stage 20, with bone anlagen indicated (LACM 94683). Original.

human development and the Carnegie system therefore does not capture development well. We use features identified by Štěrba *et al.* (2000) to diagnose the fetal stages.

Our Stage 20 matches Stage 7 and 8 of Štěrba *et al.* (2000). Their Stage 8 is characterized by features not present in *Stenella* (skin folded, hairfollicles present), as indicated by these authors (p. 41), making it difficult to distinguish these stages. Hence, we combine Štěrba *et al.*'s Stages 7 and 8.

Fetuses of Stage 20 lack hair entirely, there are no tactile hairs on the face. The fluke changes considerably during Fetal Stage 20. In early fetuses of this stage, the fluke is longer than wide (measured from pedicle to tip), but it is wider than long in later fetuses. For the younger specimens of Fetal Stage 20, both CRL and TL can be determined. These specimens are LACM 94607 (CRL: 42; TL:82), LACM 94683 (CRL: 38; TL: 96), and LACM 94679 (CRL: 44; TL: 97).

11.3.14 Fetal Stage 21

Diagnostic criteria. Tactile hairs present.

Material. LACM 94358, 94385.

Dimensions. TL: 160-170 mm.

Discussion. Tactile hairs are located in a row on the lateral side of the beak (Fig. 11.3F). There are usually 6-8 hairs on each side of the beak. The upper and lower eyelids are fused in these fetuses. Pigmentation in these embryos is present on the head, as a U-shaped crest separating forehead from snout. Fetal Stage 21 follows the diagnosis of Štěrba Stage 9 embryos ages from 78-100 days. The paucity of embryos of Fetal Stages 21 and 22 in our collection suggests that these are very short in developmental time, and their overlap in size suggests variability in the origin of the diagnostic features. When studied in more detail, it may be necessary to combine Stages 21 and 22. However, at this time, we follow the Štěrba *et al.* (2000) in considering them distinct stages.

11.3.15 Fetal Stage 22

Diagnostic criteria. Eyelids separated (Fig. 11.3E).

Material. LACM 94298, 94310, 94393.

Dimensions. TL: 182-221 mm.

Discussion. The epidermal seal of the eyelids is ruptured exposing the cornea (Fig. 11.3F). Determination of the completeness of the epidermal seal on the eyes is difficult in an entire fetus, as this seal can be so thin that it is barely visible with a dissection microscope. Fetal Stage 22 matches Štěrba *et al.*'s (2000) Stage 12, which was placed by these authors between their Stages 9 and 10. Štěrba *et al.* (2000) proposed that these embryos ranged in age from 102-108 days.

11.3.16 Fetal Stage 23

Diagnostic criteria. Pigmentation throughout the skin on head and dorsum.

Material. LACM 94277, 94285, 94290, 94298, 94301, 94302, 94304, 94309, 94358, 94369, 94378, 94382, 94384, 94385, 94386, 94387, 94393, 94397, 94400, 94401, 94406, 94407 (Fig. 11.3G).

Dimensions. Minimum TL: 160 mm. *Stenella* newborns are between 80-85 cm in length (Perrin 2002).

Discussion. Although some pigmentation is already present on the head in Stage 21, it is not until Stage 23 that broad areas of the skin show pigment macroscopically. In preserved specimens, pigmentation is often not easy to determine because the epidermis may have sloughed.

11.4 CONCLUSIONS

Stenella attenuata is the most common species of cetacean represented in embryological collections. As such, an ontogenetic series for it can be the reference series for the study of the development of any other cetacean. Cetacean ontogeny has been studied by a variety of authors and Table 11.1 summarizes the more important works. Comparing our staging system to that of individual publications, we were able to assign tentative stages to these published specimens. This table lays the groundwork for comparative studies by future workers.

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CHAPTER 12

Placental Structure and Comments on Gestational Ultrasonographic Examination

Debra L. Miller[1], Eloise L. Styer[1] and Maya Menchaca[2]

12.1 INTRODUCTION

The placental structure of cetaceans is epitheliochorial and implantation is diffuse (Zhemkova 1967). Mossman (1987) described the fetal membranes of cetaceans as similar to those of Tragulidae (mouse deers) and Camelidae (camels), primarily within the later stages of development and noted that Stump *et al*. (1960) also illustrated early embryos as similar to artiodactyls. Mossman (1987) further noted that there is an early but temporary yolk sac or choriovitelline placenta in cetaceans that is replaced later by the chorioallantoic placenta. The epitheliochorial placenta is considered a secondary specialization and is found in the superorder Laurasiatheria, which was the last mammalian superorder to arise. This superorder includes many types of placentation, but most species with epitheliochorial placentation are found here, including cetaceans, camels, ruminants, pigs, peccaries, hippopotamuses, horses, and pangolins (Carter and Enders 2004).

Placentas of relatively few cetacean species have been described (Benirschke and Cornell 1987; Mossman 1987; Stump *et al*. 1960; Turner 1872; Wislocki and Enders 1941; Zhemkova 1967). Both Turner (1872) and Benirschke and Cornell (1987) described the Killer whale (*Orcinus orca*) placenta, with the latter account detailing observations on delivered placentas from captive animals. Here, we compare our findings on placentas from captive Atlantic bottlenose dolphins (*Tursiops truncatus*) and Pacific white-sided dolphins (*Lagenorhynchus obliquidens*) to previous reports on cetacean placentas by the above authors.

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12.2 MACROSCOPIC DESCRIPTION

12.2.1 General

The placentas of *T. truncatus* and *L. obliquidens* are similar grossly, with no species-defining characteristics apparent. The placentas are shaped like a funnel or cornucopia, reflecting the form of the uterus in which they reside (Fig. 12.1). This shape is due in part to the placement of the fetus, which according to Turner (1872) and Wislocki and Enders (1941) is primarily in the left uterine horn with a chorionic extension into the right horn, although Wislocki and Enders (1941) consider it a chorioallantoic extension. Wislocki and Enders (1941) state that ovulation generally takes place on the left ovary. Interestingly, they also note that the chorioallantoic extension varies markedly in Phocaena. Specifically, the amnion and allantois may fill both horns, the allantoic sac may not extend all the way to the tip of the gravid horn, or the allantoic sac may be so long that it actually doubles over within the horn. Because the placentas we examined had been placed immediately in formalin upon collection, weights of fresh specimens were not available. This was not the case for Benirshke and Cornell (1987), who found weights of 17 and 26.5 kg for two of three delivered placentas from captive orcas with the third being too fragmented to ensure that the entire placenta had been obtained.

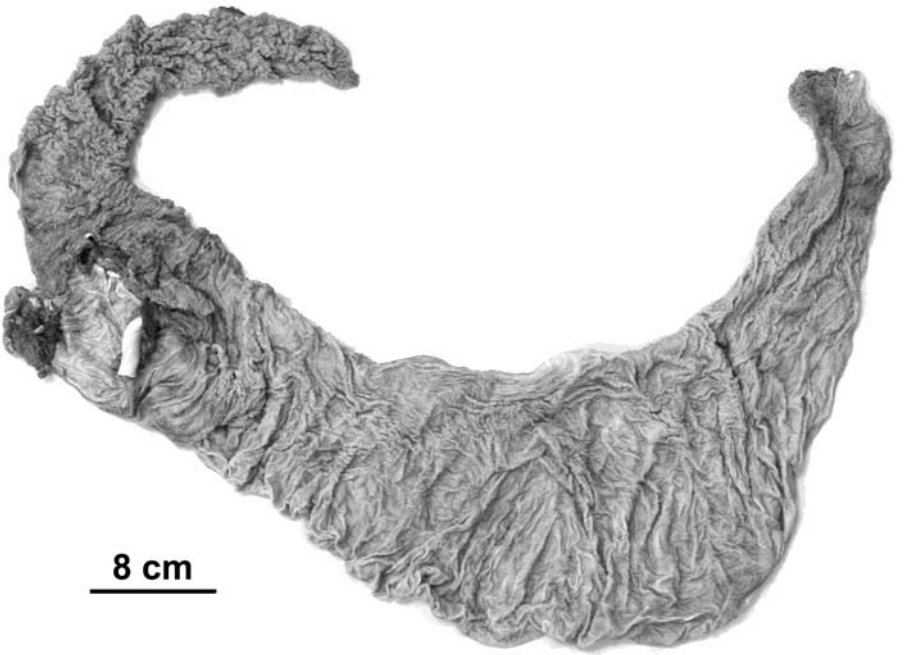

Fig. 12.1 Gross view from the uterine side of a formalin-fixed term placenta collected from a Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) at the Miami Seaquarium, Miami, Florida, USA. The placenta has a funnel or cornucopia shape, presumably reflecting the shape of the uterus. Original.

12.2.2 Umbilical Cord

The umbilical cord of all species has the same general composition, *i.e.,* four vessels (two arteries and two veins) surrounding a centrally located allantoic duct (Fig. 12.2). According to Mossman (1987), the mesometrial insertion of the cord on the chorioallantois (similar to hoofed mammals) implies that the yolk sac of the implanted blastocyst extends mesometrially and the embryonic disc faces antimesometrially. The umbilicus spirals loosely along its axis (Fig. 12.3), although Benirshke and Cornell (1987) documented an exception in *Orcinus orca*. This spiraling is generally attributed to fetal movement.

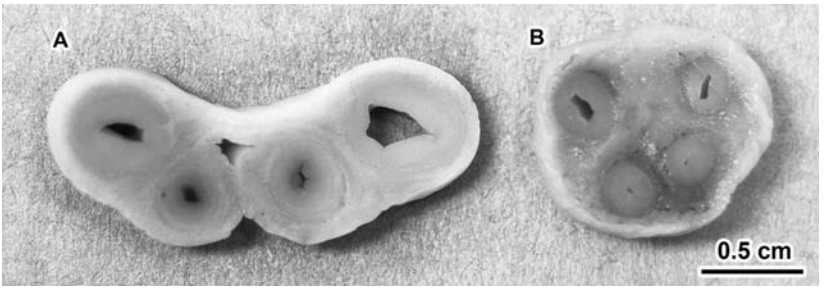

Fig. 12.2 Cross-sectional gross views of formalin-fixed term umbilical cords from placentas collected from a Atlantic bottlenose dolphin (*Tursiops truncatus*) (**A**) and a Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) (**B**) at the Miami Seaquarium, Miami, Florida, USA. The umbilical cord of all species has the same general composition of 4 vessels (2 arteries and 2 veins) surrounding a centrally located allantoic duct. This arrangement is maintained along the cord (**B**) to the point of bifurcation (**A**). Original.

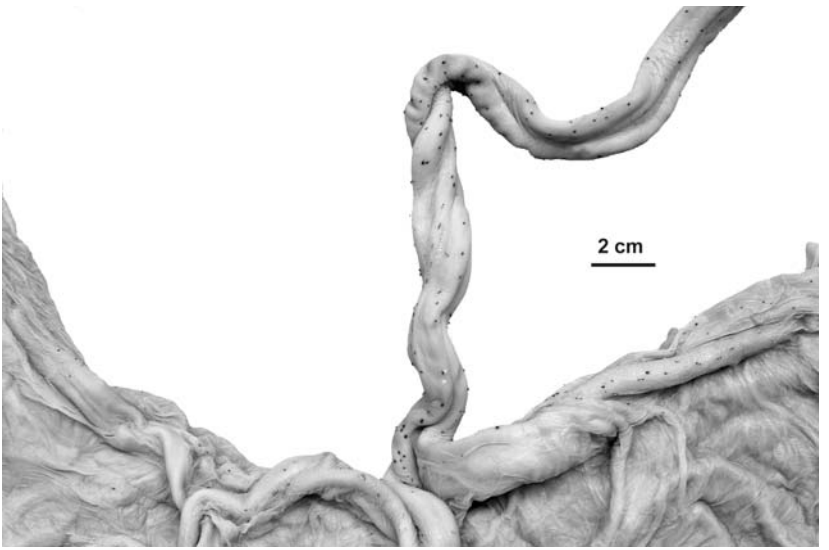

Fig. 12.3 Gross view of the formalin-fixed term umbilical cord of Atlantic bottlenose dolphin (*Tursiops truncatus*) showing loose spiraling along its axis. Original.

The umbilicus bifurcates along the placental surface at the ductal insertion (Fig. 12.4). Paired umbilical vessels (one artery and one vein) then extend along the allantoic sac toward the two placental poles. There is prominent secondary and tertiary branching of the vessels along the placental surface with extensive arborization and eventual apposition of the distal branches on the other side of the sac (Fig. 12.5).

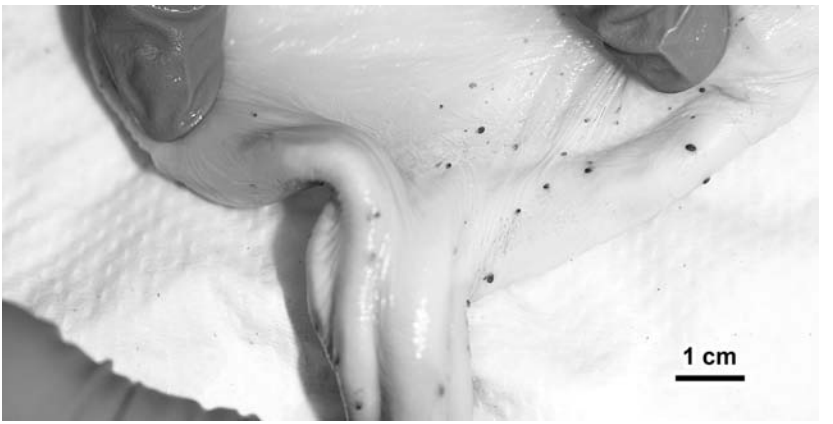

Fig. 12.4 Gross view of the formalin-fixed term umbilical cord of Atlantic bottlenose dolphin (*Tursiops truncatus*) showing the bifurcation at the insertion along the placental surface. Original.

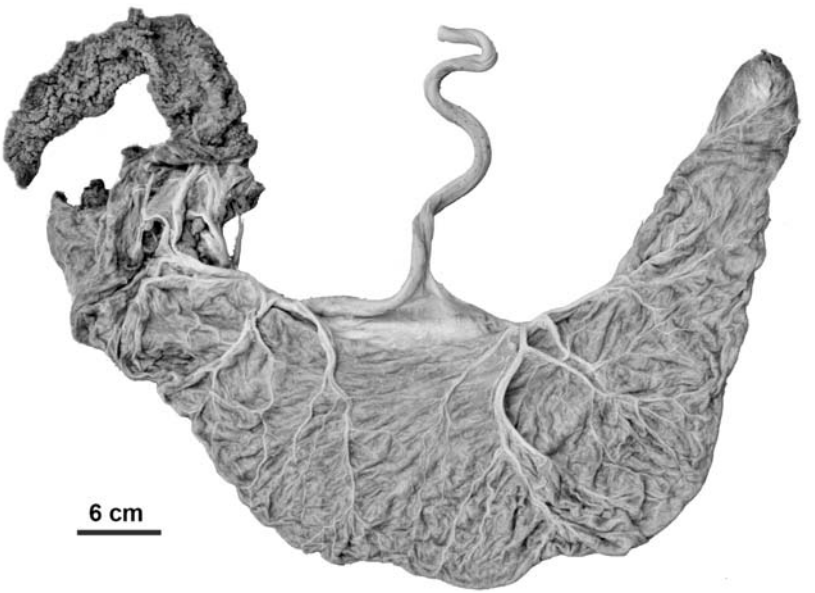

Fig. 12.5 Gross view of the formalin-fixed term placenta (fetal side) collected from a Atlantic bottlenose dolphin (*Tursiops truncatus*) at the Miami Seaquarium, Miami, Florida, USA, showing extensive secondary and tertiary branching of the vessels along the placental surface. Original.

The umbilicus is covered with slightly raised to rarely flush, firm, variably-sized but generally pinpoint, dark brown/black and/or white plaques that are known also as caruncles or callusoids (Fig. 12.6). They are distributed randomly and sparsely over the amniotic surface. These plaques, which

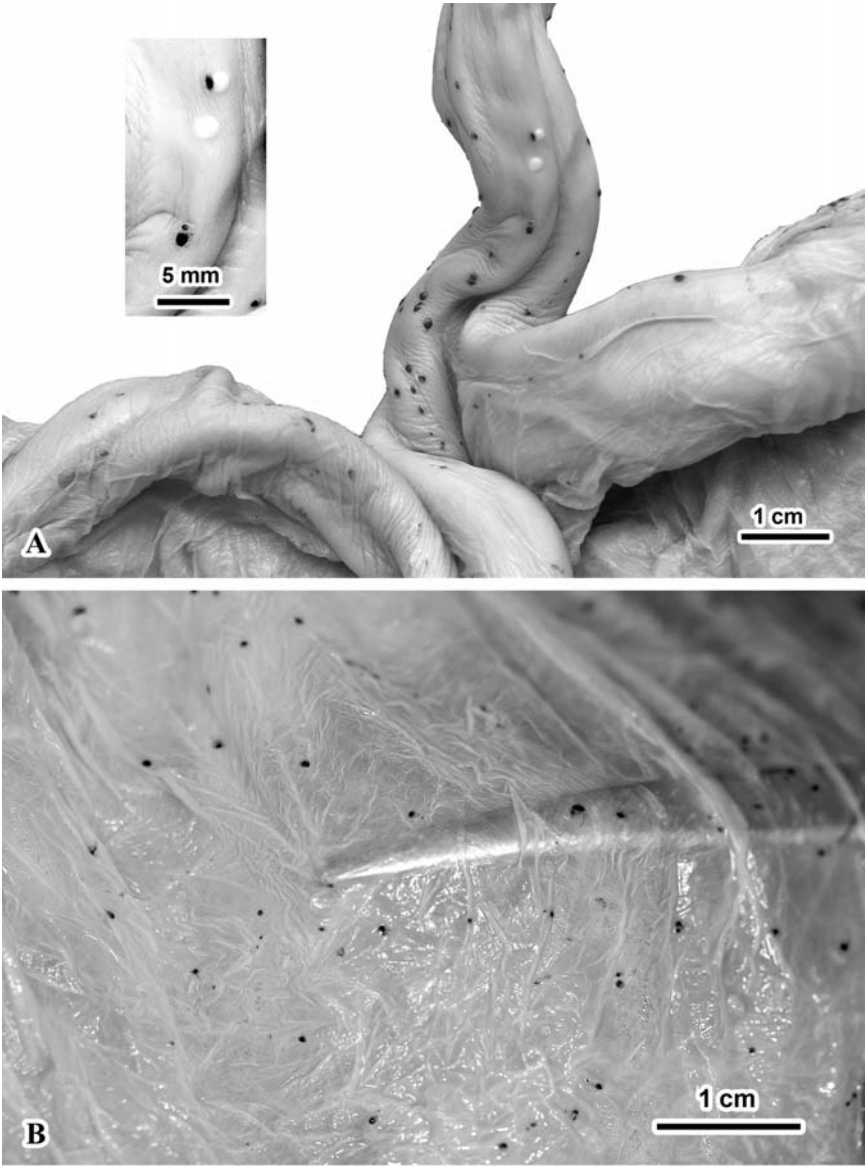

Fig. 12.6 A Gross view of the formalin-fixed term umbilical cord of a Atlantic bottlenose dolphin (*Tursiops truncatus*) showing the numerous variably-sized dark brown/black and/or white plaques. Inset shows the plaques at a higher magnification. **B**. These plaques are present over the amniotic surface. Original.

roughen the umbilical surface, are areas of squamous metaplasia, reflective of their embryonic ectodermal origin. Special immunohistochemical and histochemical stains performed on the umbilical tissue collected from *Tursiops truncatus* and *Lagenorhynchus obliquidens* revealed positive staining for cytokeratin and melanin, respectively (Fig. 12.7). Mossman (1987) and Benirshke and Cornell (1987) noted that the umbilical cords and amnions of Balaenopteridae and *Orcinus orca*, respectively, are similarly studded and likened these plaques to those seen in Artiodactyla and Proboscidea.

12.2.3 Placental Surfaces

As already stated, the shape of the delivered placenta is like a cornucopia or funnel (Fig. 12.1). After examination of an orca at necropsy, Turner (1872) surmised that this shape reflected that of the gravid uterus. Specifically, Turner (1872) found the fetus in the horn contralateral to the corpus luteum, but with extension of the chorion into the ipsilateral horn. On the mesometrial side, the placenta is formed by amniochorion with large vessels arborizing the chorionic sac (Benirshke and Cornell 1987). Among gravid uteri from *Tursiops* and *Phocoena*, Wislocki and Enders (1941) found that the chorion separated easily from the uterine mucosa everywhere except at the poles, where the thick endometrium interlocked intimately with the chorionic villi.

The surface of the placenta is loosely folded or "crinkled" at its widest aspect, presumably due to stretching from the space-occupying fetus. At either pole, the surface is contracted, resulting in tighter folds and an almost compressed appearance (Fig. 12.1). Zhemkova (1967) described placentas from free-ranging odontocetes (Beluga, *Delphinapterus leucas* and Sperm whale, *Physeter catodon*) and mysticetes (*Balaenoptera physalus,* Fin whale, and *Balaeonoptera acutorostrata*, Minke whale), in which he observed diverticula or "cone-shaped hollow formations" on the placental surface, located bilaterally to the base of the umbilical cord. The surface of the placenta was smooth at the peripheral ends of the chorion and along the surface of the diverticula, but otherwise folded (Zhemkova 1967). Similar diverticula were not seen in *Tursiops truncatus* or *Lagenorhynchus obliquidens*.

The chorionic surface is blanketed with villi, consistent with diffuse placentation, as occurs in the Suiformes, camel, pangolin and horse (Banks 1986; Carter and Enders 2004). Zhemkova (1967) noted that villi appeared smaller where the surface was smooth and described variation in villus morphology among cetacean species. Specifically, villi were large and arborescent in the folded regions of the placenta of *Delphinapterus leucas* and *Balaenoptera acutorostrata*, but short and flat in these regions in *Physeter catodon* and *Balaenoptera physalus* (Zhemkova 1967). Similarly, at first glance the villi of *Tursiops truncatus* and *Lagenorhynchus obliquidens* appeared shorter in the segment of chorion overlying the fetus than elsewhere, but upon closer examination we inferred that this is likely artifactual due to stretching of the placenta (Fig. 12.8). Regardless of species, all villi appear to have some level of arborization; this was clearly noted in both *T. truncatus* and *L. obliquidens*.

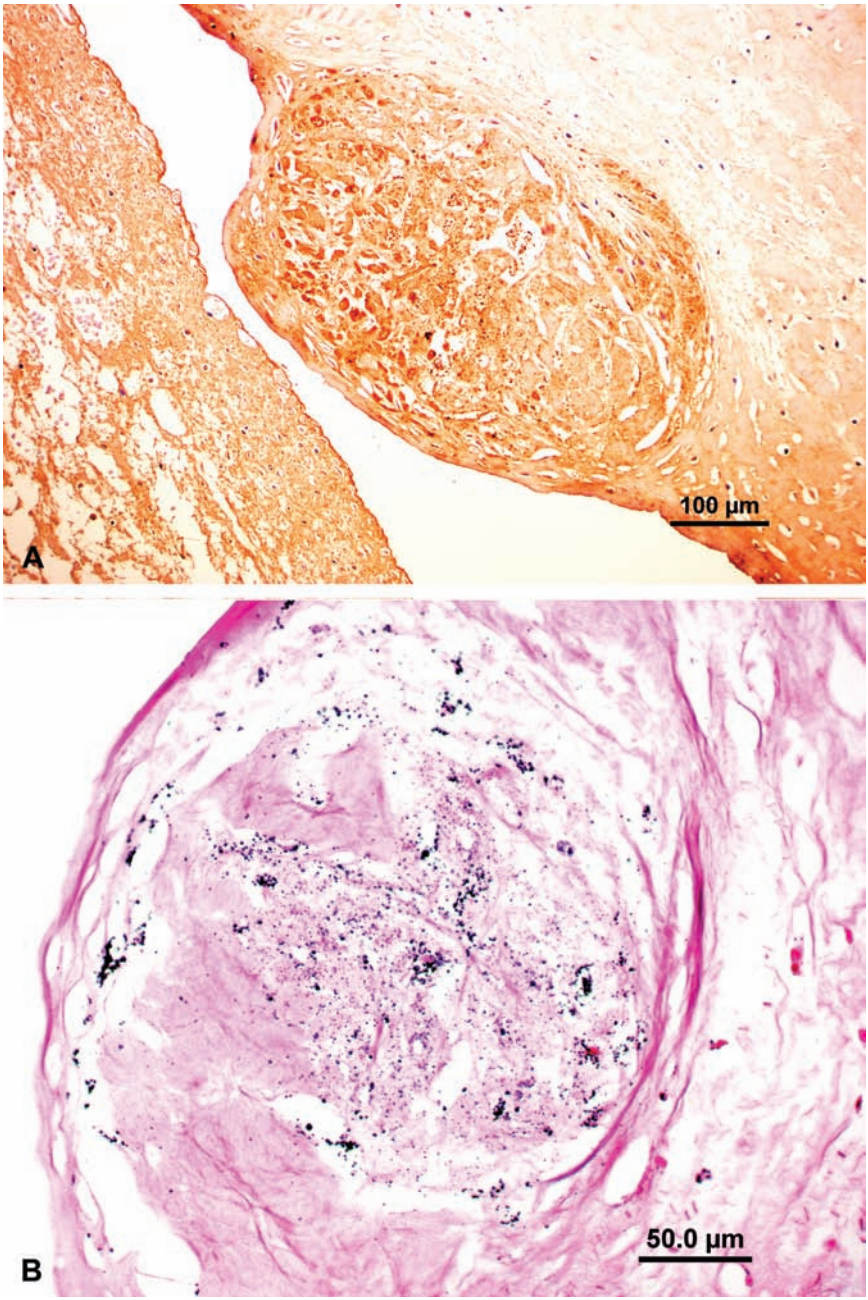

Fig. 12.7 Light microscopic view of the plaques that roughen the surface of the umbilicus and the amnion. These plaques appear as areas of squamous metaplasia and pigment deposition and stain positively by immunohistochemistry for cytokeratin (**A**) and histochemically for melanin with Fontana Mason (**B**). Original.

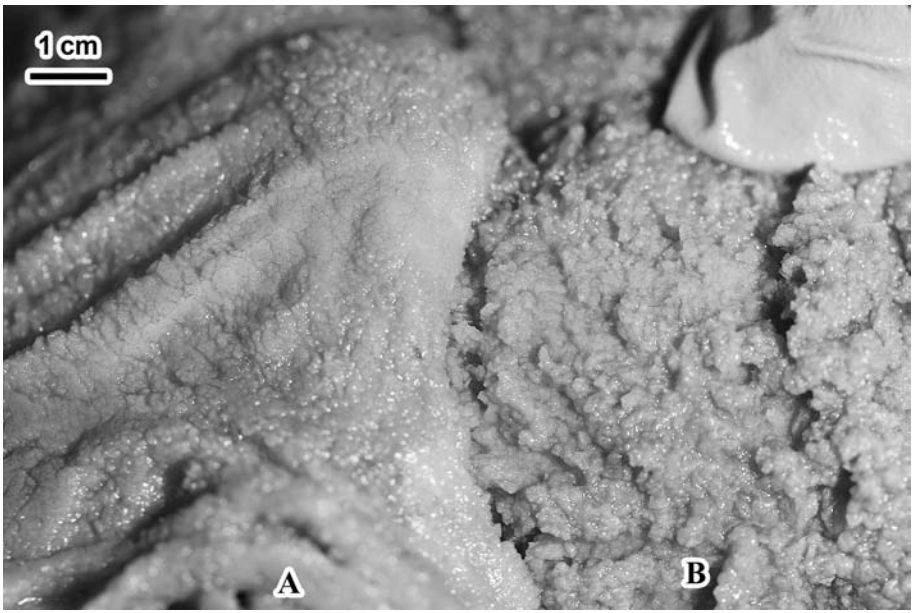

Fig. 12.8 Gross view of a formalin-fixed term placenta of Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) collected at the Miami Seaquarium, Miami, Florida, USA, showing stretched (**A**) vs non-stretched (**B**) regions resulting in perceived variations in chorionic villous morphology. Original.

Chorionic villi of the pig and camel display a comparable arborescent architecture, in contrast to the microcoteledonary presentation of the chorionic villi in the mare. In examination of a gravid uterus, Wislocki and Enders (1941) discovered that these branched chorionic villi interdigitate with the uterine mucosa via minute pits (areolae) which are the grossly visible openings of the uterine glands.

Bare or non-villous areas of the chorion were described in *Orcinus orca* by Turner (1872) but not by Benirshke and Cornell (1987). The closest report to such a finding in other species was by Wislocki and Enders (1941). They remarked on 3 areas with minimal villi in *Tursiops truncatus*, one of which was at the point where the fetus' beak pressed against and likely stretched the chorion. The cause of the other two areas was unclear but also may have been due to pressure from the fetus. Another possibility is that these "less villous" areas are due to postmortem change or perhaps reflect a handling artifact, although both of these causes should be distinguishable histologically.

The allantoic sac is distinct from and considerably smaller than the lumen of the chorion and is fused with the amniochorion on the mesometrial surface, but only with the amnion on the antimesometrial surface (Fig. 12.9). Wislocki and Enders (1941) described the allantois in *Tursiops truncatus* as a bilobed sac, of which the larger portion was fused to the chorion (chorio-allantois) and the smaller portion to the amnion (allanto-amnion). The allanto-amnion

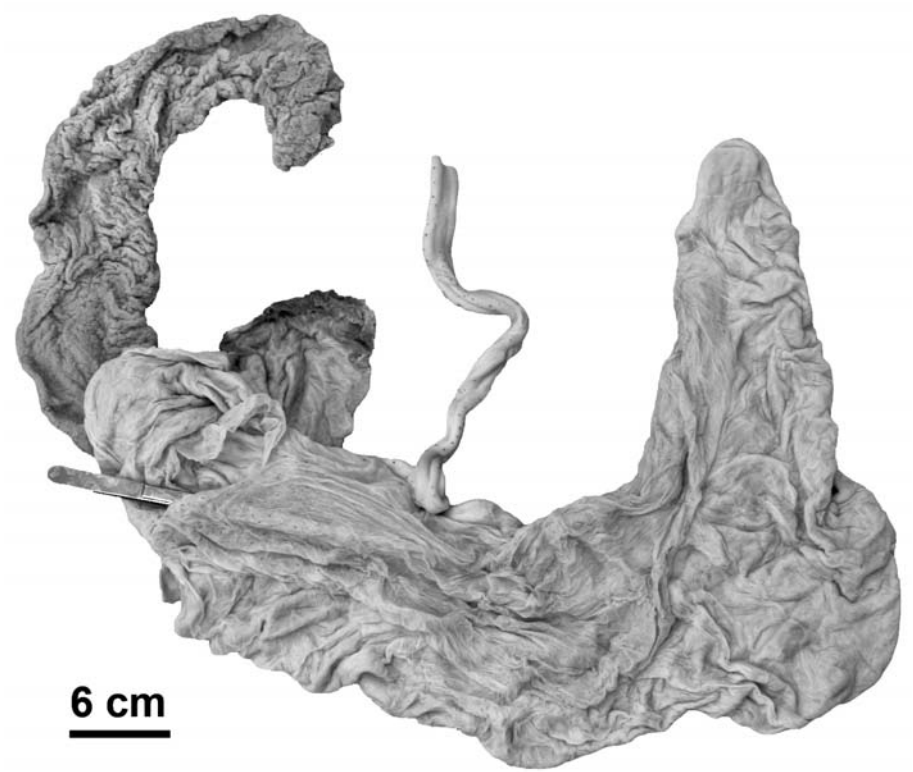

Fig. 12.9 Gross view of a formalin-fixed term placenta (fetal side) of Atlantic bottlenose dolphin (*Tursiops truncatus*) collected at the Miami Seaquarium, Miami, Florida, USA, showing the allantoic sac (inverted for fetal-side view). The sac is fused with the amniochorion on the mesometrial surface and the amnion on the antimesometrial surface (forceps are lifting antimesometrial surface). Original.

of both the *Lagenorhynchus obliquidens* and *T. truncatus* is smooth, glistening, clear to slightly opaque, and smaller than the chorionic sac with sparse small blood vessels but numerous amniotic plaques, similar to those seen on the umbilicus described above (Fig. 12.6B).

12.3 MICROSCOPIC DESCRIPTION

12.3.1 Umbilical Cord

The umbilicus has a microvascular system communicating between the four major vessels, but a function for this rather variable microvasculature remains unclear. The four major vessels (two arteries and two veins) surround an irregularly-shaped allantoic duct (Fig. 12.10). The allantoic duct is lined by a sparsely cellular, occasionally double, row of flattened cuboidal epithelium (Fig. 12.11). Scattered foci of small, variably-sized bundles of smooth muscle are distributed throughout the loose connective tissue stroma of the umbilicus

Fig. 12.10 Histological section of an umbilical cord from Atlantic bottlenose dolphin (*Tursiops truncatus*) showing two arteries and two veins surrounding the allantoic duct. Original.

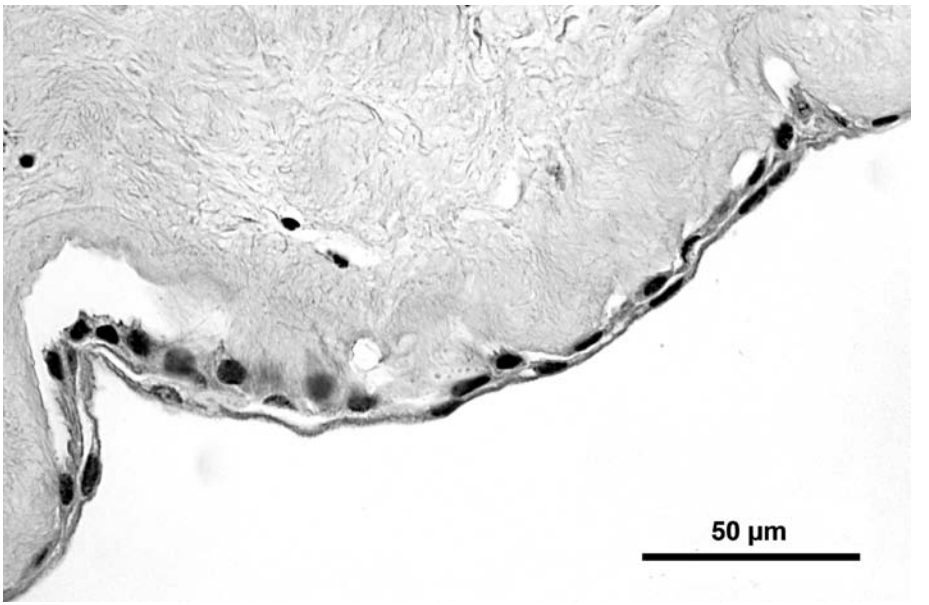

Fig. 12.11 Light microscopic view of an umbilical cord from Atlantic bottlenose dolphin (*Tursiops truncatus*) showing the double row of flattened cuboidal cells lining the allantoic duct. Original.

(Fig. 12.12). While the arrangement of these bundles is somewhat haphazard, they surround the duct in a fairly uniform way. The walls of the major umbilical arteries are themselves vascularized, similar to the aorta, but these small vessels are confined to the outer 1/4-1/3 of the arterial wall (Fig. 12.13). It is presumed that these small vessels communicate with the microvasculature mentioned above that is in the loose connective tissue stroma of the umbilicus.

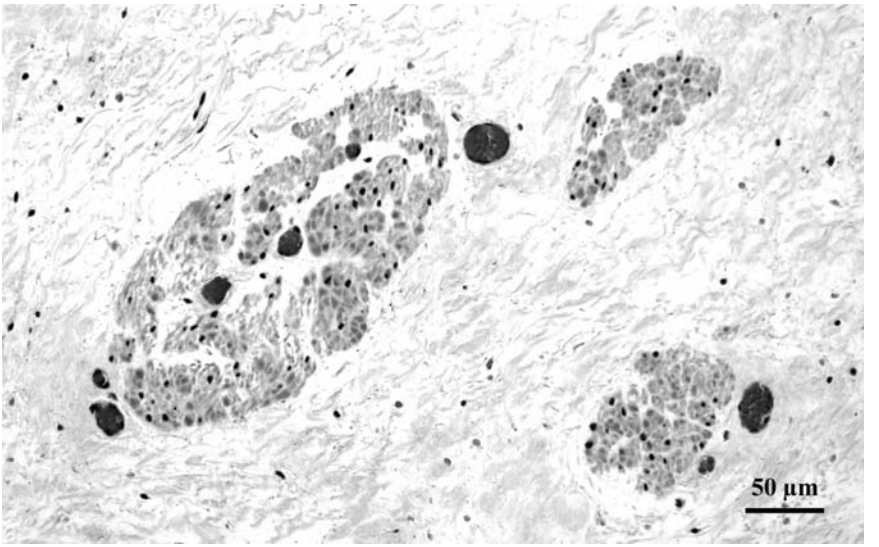

Fig. 12.12 Light microscopic view of an umbilical cord from Atlantic bottlenose dolphin (*Tursiops truncatus*) showing the smooth muscle bundles that are scattered throughout the stroma. Original.

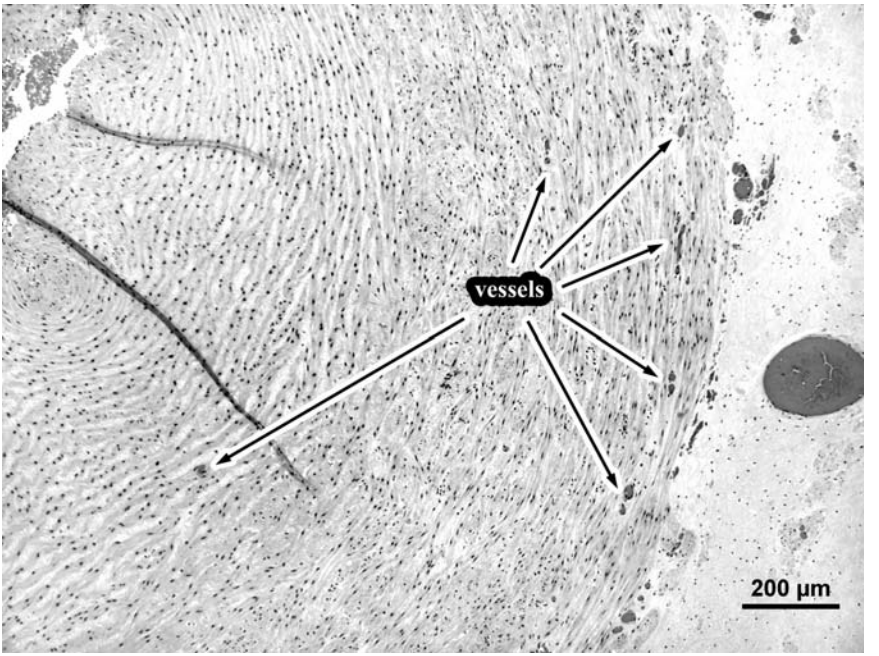

Fig. 12.13 Light microscopic view of an umbilical cord from Atlantic bottlenose dolphin (*Tursiops truncatus*) showing the vascularization of the major vessels. Note that this vascularization is primarily seen in the outermost region of the vessel wall. Original.

12.3.2 Placental Surfaces

Histologically, the chorionic villi are covered by a single row of cuboidal trophoblasts, subjacent to which is a fine collagenous stroma sparsely populated with (fetal) fibroblasts and Hofbauer cells (presumed to be a type of fetal macrophage) and vascularized by small vessels that are in close proximity to the trophoblasts' basement membranes (Fig. 12.14). These small vessels likely arborize from medium-sized vessels at the base of the villi (in the chorionic plate), which in turn probably branch from larger vessels along the luminal surface (Fig. 12.15). Occasionally, perivascular edema is noted around this network of chorionic vessels.

As indicated in the macroscopic description, villi have been observed as varying in size. However, in *T. truncatus* or *L. obliquidens* the difference is not necessarily in villous height but rather in degree of villous arborization, which as already mentioned, may appear diminished by placental "stretching" in response to fetal growth. This reduced arborization was evident in histological examination of "thin" or stretched areas of the placenta associated with the fetus as opposed to "thick" or non-stretched areas near

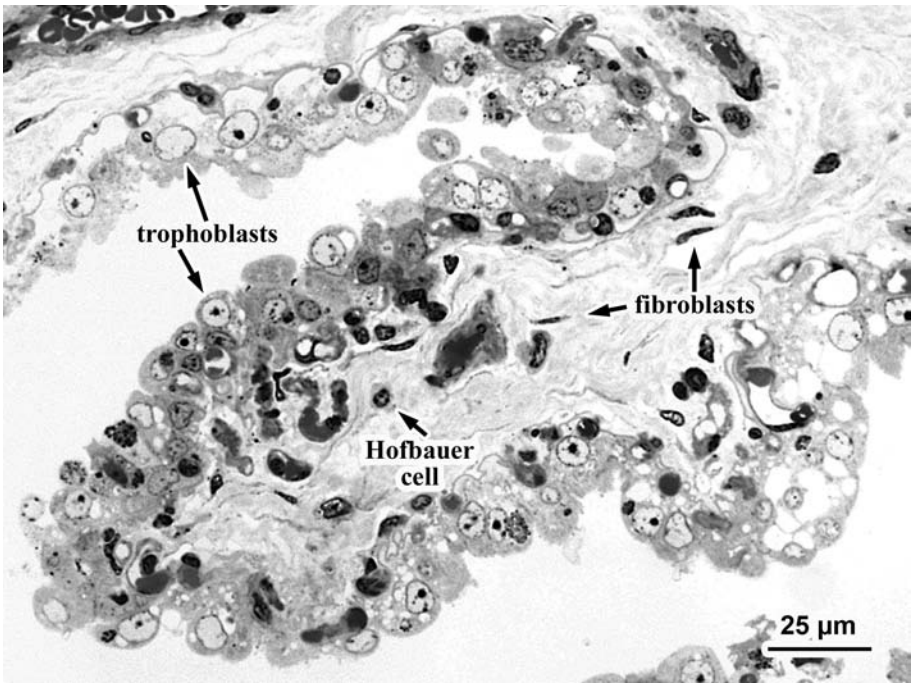

Fig. 12.14 Light microscopic view of the placenta of Atlantic bottlenose dolphin (*Tursiops truncatus*) showing the single row of cuboidal trophoblasts. Within the fine collagenous stroma are fetal fibroblasts and scattered Hofbauer cells. Small blood vessels are often in close proximity to the trophoblasts. Original.

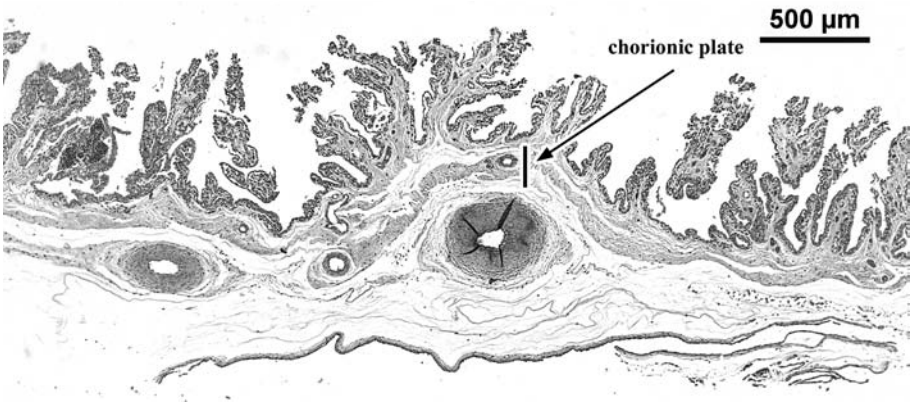

Fig. 12.15 Light microscopic view of the placenta of Atlantic bottlenose dolphin (*Tursiops truncatus*) showing the presumed transition in vascular arborization from the small vessels near the trophoblast, to the medium-sized vessels at the base of villi (in the chorionic plate), and ultimately to the larger vessels along the luminal surface. Original.

the poles (Fig. 12.16). Similarly, "elevated patches" of villi described by Wislocki and Enders (1941) may actually connote the greater arborization manifest in placental zones not subjected to stretching. Wislocki and Enders (1941) also stated that within such "elevated patches," the villi appeared to be more vascular than in "non-elevated" areas. In our examination of *T. truncatus* and *L. obliquidens*, we concluded this to be an artifact related to degree of arborization.

12.4 ULTRASTRUCTURAL DESCRIPTION

Fixation of an umbilical plaque of *T. truncatus* examined by electron microscopy was extremely poor (i.e., plasma membranes were non-existent and cell organelles, such as mitochondria, were unidentifiable). The stroma consisted of loosely- to relatively densely-packed collagen with occasional small vesicles, fine filaments (possibly keratin), cell debris, and melanosomes (cellular organelles containing melanin). Small groups of cells were scattered throughout the stroma (Fig. 12.17A). These cells lacked basement membranes and contained numerous mature melanosomes with electron lucent areas near their margins (Fig. 12.17B).

Transmission electron microscopic examination of the chorionic villi of the placenta from *T. truncatus* showed a single layer of trophoblasts subtended by a prominent basement membrane that was closely associated with numerous capillaries (Fig. 12.18). The trophoblasts had swollen, convex luminal surfaces that were usually devoid of microvilli. Long, sinuous, interwoven microvilli were present in the narrow clefts between neighboring trophoblasts as well as over the luminal surface of that subset of trophoblasts occupying the narrow troughs between the bases of the smallest placental arborizations. Numerous

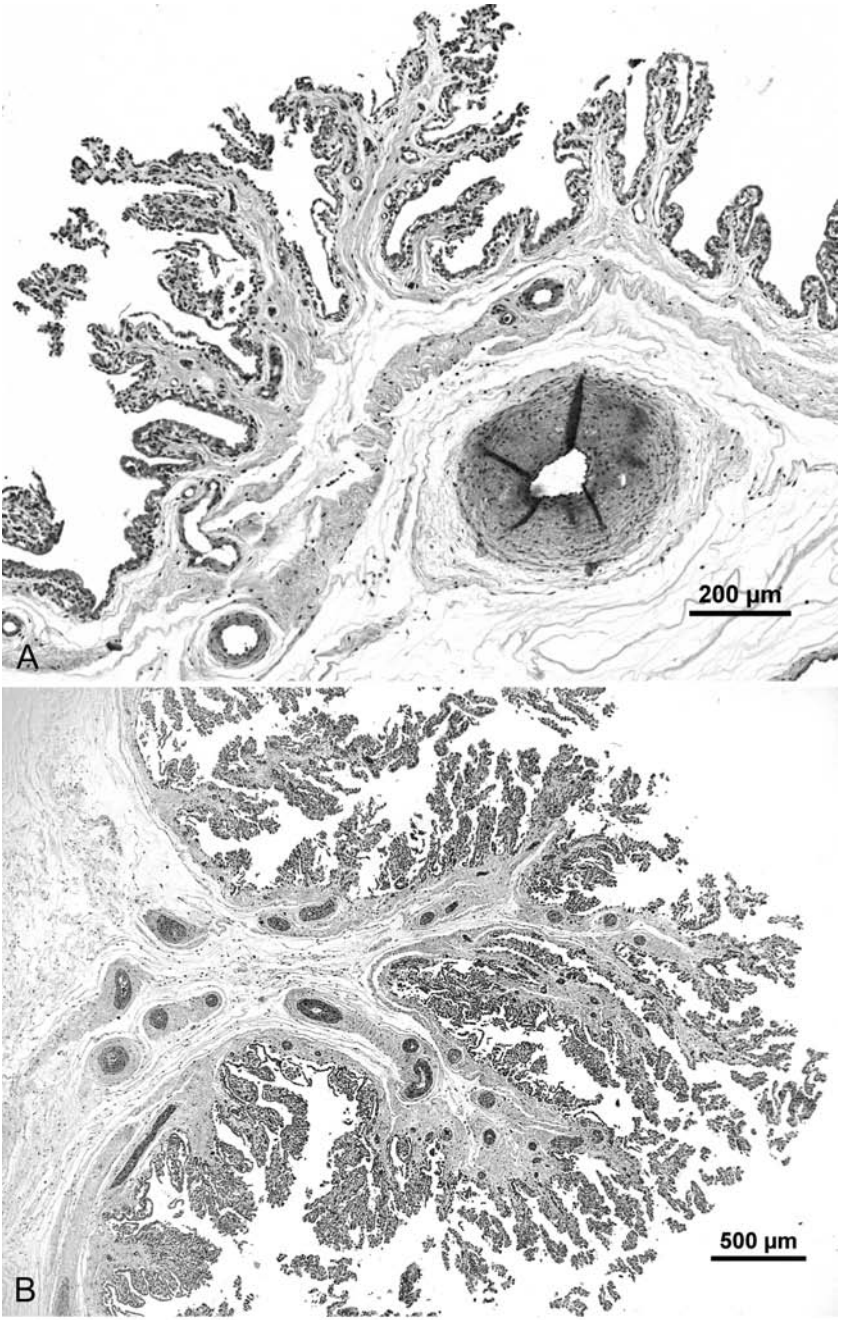

Fig. 12.16 Light microscopic view of the placenta of Atlantic bottlenose dolphin (*Tursiops truncatus*) showing the variation in degree of arborization of the villi in "thin" or stretched areas (*i.e.*, associated with the fetus) (**A**) of the placenta vs "thick" or non-stretched areas (*i.e.*, near poles) (**B**). Original.

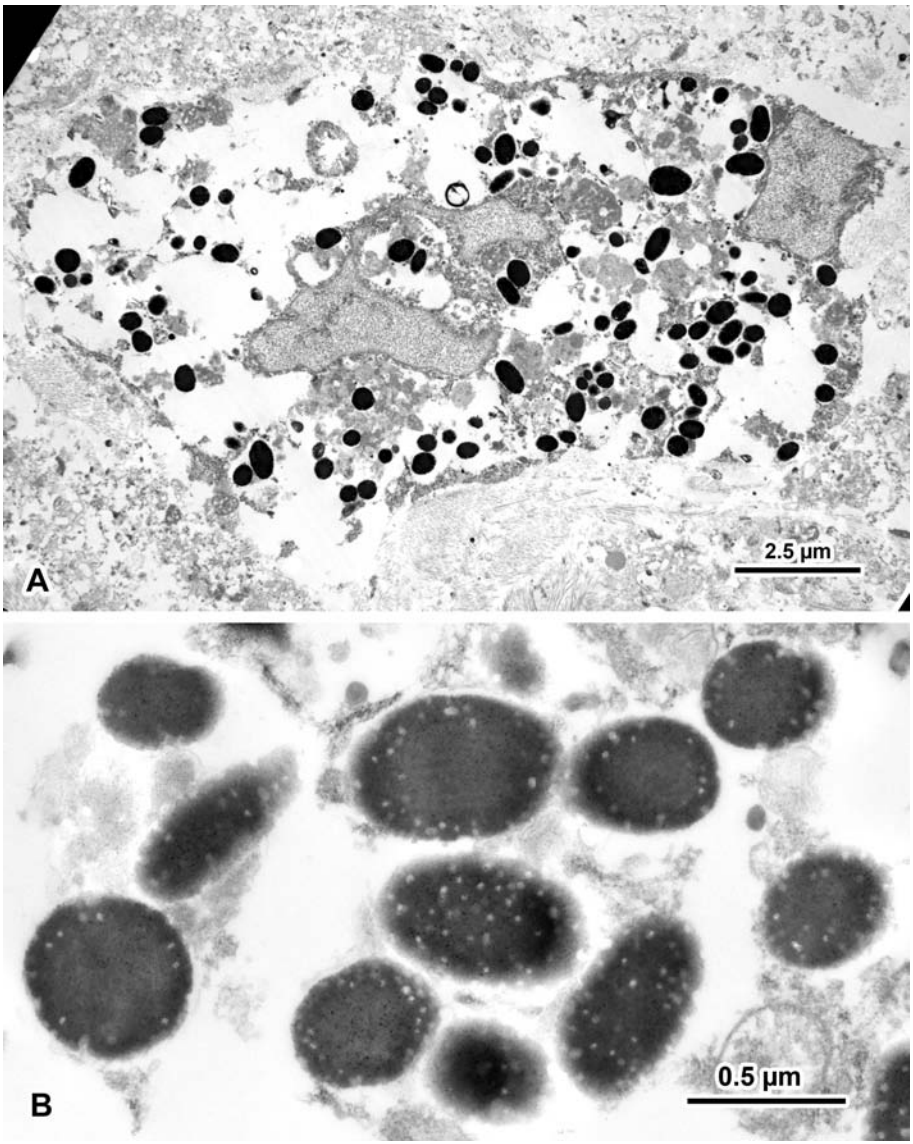

Fig. 12.17 Transmission electron microscopic view of an umbilical plaque on the umbilicus of Atlantic bottlenose dolphin (*Tursiops truncatus*) showing a group of melanocytes (**A**), each with multiple mature melanin-containing cellular organelles or melanosomes (**B**). Original.

cell junctions joined the lateral plasma membranes of adjacent trophoblasts from the lumen to the basement membrane, and occasional trophoblasts contained cytoplamic aggregates of small lipid droplets and membranous material. The collagenous stroma contained poorly preserved, scattered fibroblasts, macrophages (Hofbauer cells) and small blood vessels.

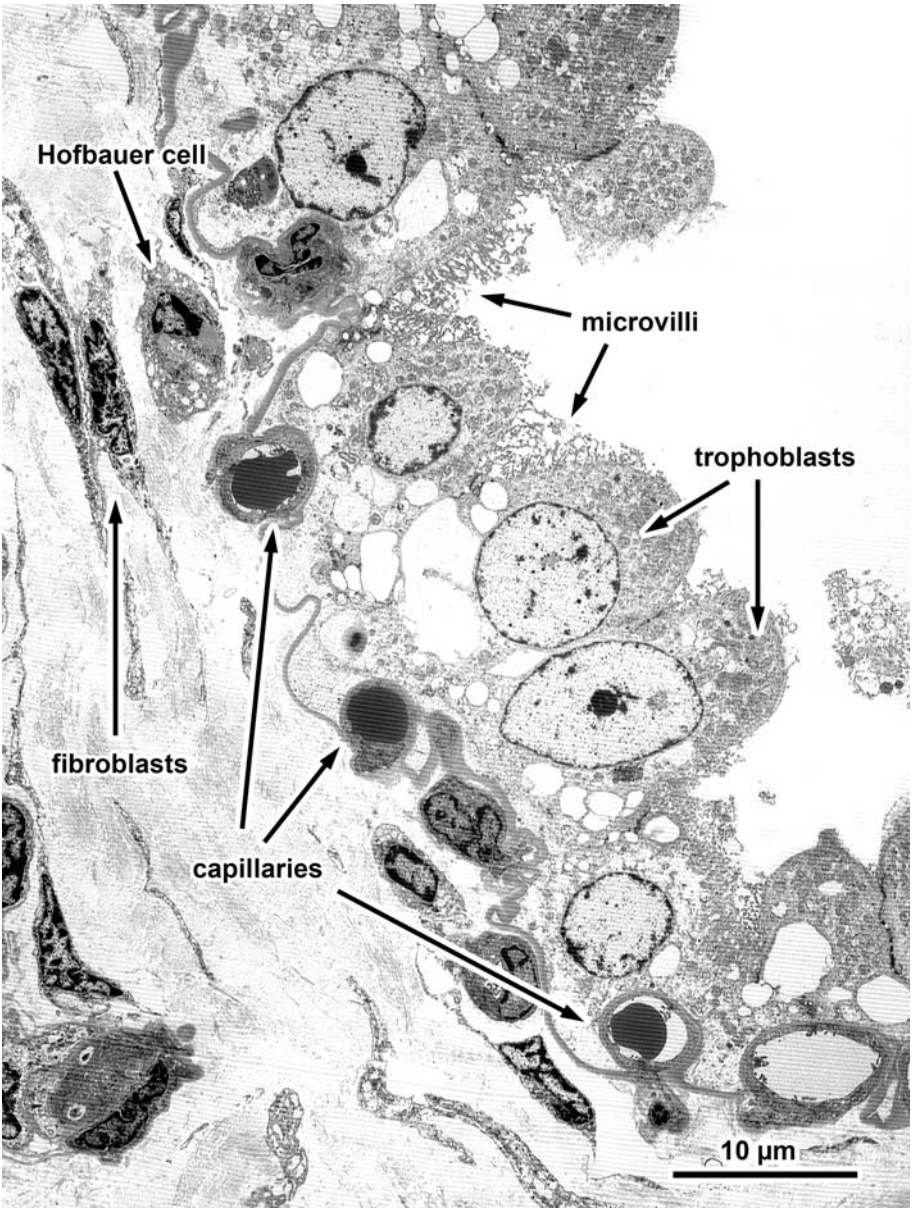

Fig. 12.18 Transmission electron microscopic view of the chorionic villi of a Atlantic bottlenose dolphin (*Tursiops truncatus*) placenta. Shown are the trophoblast (with microvilli) layer covering the stroma and fibroblasts, capillaries, and a Hofbauer cell within the stroma. Original.

12.5 NOTES ON GESTATIONAL ULTRASONOGRAPHIC EXAMINATION

Much about fetal development can be learned from studying aborted fetuses of known gestational age; however, this is not feasible for free-ranging animals. Such studies may even prove difficult in captive situations for species such as dolphins, that may breed year-round. Medical behavior training coupled with ultrasonography are proving beneficial in obtaining pertinent information regarding reproductive activity and fetal development in cetaceans. Recently, colossal advances have been gained by researchers in examing ovaries, targeting ovulation, implementing artificial insemination, and estimating gestational age and delivery date (Brook 2001; Lacave *et al*. 2004; Robeck *et al*. 1998; Robeck *et al*. 2005). Gestation period among Delphinidae varies and in *Tursiops* spp. has been reported as one year with ranges from 360 days to 381 days post-ovulation (Robeck *et al*. 2001). In the context of following multiple pregnancies using ultrasound and beginning soon after conception in 12 *Tursiops* spp., Lacave *et al*. (2004) greatly advanced the use of biparietal (defined by Stone *et al*., 1999, as the maximum external diameter of the skull perpendicular to the skull midline) and thoracic diameters for estimating birth date. The conceptus can be visualized as early as four weeks with the embryo being ca 1 cm and appearing as a soft tissue density within the developing allantois, while the fetal thorax and skull can be identified and measured by eight weeks (Stone *et al*. 1999). Stone *et al*. (1999) noted that although cardiac activity is observed early in gestation, rhythmic cardiac activity is not appreciated until 6-8 weeks. Fetal movement may be observed late in the first trimester but fetal activity becomes truly recognizable during the second trimester (weeks 17-32) (Stone *et al*. 1999). As in humans, gestational aging by morphological measurements remains tentative in the third trimester, when fetal growth is individualized (Stone *et al*. 1999). Although in its infancy, the use of ultrasonography for monitoring and staging gestation is already generating important data that will help us to better understand cetacean reproductive physiology.

12.6 ACKNOWLEDGMENTS

We wish to thank the staff of the Miami Seaquarium and The University of Georgia Tifton Veterinary Diagnostic and Investigational Laboratory for their tireless effort in collecting and photographing placentas and processing tissue sections. We are indebted to Dr. Victoria Woshner for her time and valued editorial analysis of this chapter.

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CHAPTER 13

Courtship and Mating Behavior

Catherine M. Schaeff

13.1 INTRODUCTION

It is surprising how much we know about cetacean reproductive behavior given the difficulties associated with studying these animals. Some information comes from whaling data (length of gestation, timing of breeding activities, and age of sexual maturity), much is provided by long-term behavioral observations (association patterns, agonistic behaviors, acoustic displays, etc.), and, recently, important details have begun to be provided by genetic studies (kinship, paternity). It is important to note that for many species mating is rarely if ever observed, the sex of most individuals remains unknown, and, since males do not provide parental care, it is unclear whether observed courtship and mating behaviors are associated with achieved reproductive success. As a result, although we have fairly detailed information about courtship and mating for a small number of species and a general understanding of patterns for many others, much of what we have pieced together comes from limited, often opportunistic, cetacean data, and from our understanding of other species (e.g. ungulates, elephants, primates).

Mating systems reflect individuals' efforts to maximize their lifetime reproductive success. Strategies vary among species and between sexes within a given species. Cetacean mating systems, like those of other species, reflect the distribution of mates and resources through time and space, and the amount of parental care needed to successfully rear offspring (Trivers 1972).

Mating systems can be defined in terms of pair bond formation (number and duration) and by the number of mates individuals typically have. Depending upon the species and its mating system, pair bonded individuals can remain together for a single breeding attempt or throughout individuals' adult lives. Observed primarily in species where both parents invest heavily

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in parental care, there is little evidence of pair bond formation in cetaceans. Males act as temporary consorts or escorts in some species (e.g., *Physeter macrocephalus*, Sperm whale; *Megaptera novaeangliae,* Humpback whale; and *Tursiops truncatus*, Bottlenose dolphin) but long term associations between breeding males and females are rarely observed (but see Danilewicz *et al.* 2004; Valsecchi and Zanelatto 2003).

The number of mates a given individual has can vary for a number of reasons (age, breeding status, health, etc.). However, in general, in monogamy systems, each individual typically mates with one other individual per breeding attempt or season and in polygamous systems, individuals of one sex tend to mate with multiple individuals of the other sex (polygyny, one male mates with many females; polyandry, one female mates with many males) (Alcock 1998). Monogamy generally evolves when biparental care is required to rear offspring successfully (obligate monogamy). Cetacean offspring require prolonged parental care but females tend to be the providers (internal gestation, prolonged lactation) while males' reproductive contribution is typically limited to their genes. Hence, the evolution of monogamy mating systems among cetaceans is unlikely (but see Kasuya *et al.* 1997). Instead, polygamous systems without pair bonds (individuals of both sexes mating with multiple individuals) are common.

Individuals can benefit from mating with multiple individuals in a variety of ways (Clutton-Block 1989). Males' reproductive fitness (number of copies of genes passed on to the next generation) increases with each successful mating and so male cetaceans' reproductive output depends upon their ability to access receptive females (Trivers 1972). Additional mates can increase the number of offspring produced by females in a given breeding attempt in some species, particularly if males provide energy-rich nuptial gifts (e.g., many insect species). The potential benefits from this mechanism are limited for cetaceans because energy constraints associated with internal gestation and prolonged lactation typically limit females to a single offspring per breeding season. Still, female whales and dolphins could benefit in situations where multiple males vie for access to a female simultaneously because such competition should result in higher quality males being more likely to win the competition to fertilize the egg (e.g., Kraus and Hatch 2001). Similarly, if females are able to exert cryptic (i.e., post-copulatory) choice, then they might be able to avoid using sperm from genetically incompatible males or invest less in the offspring of such males (Tregenza and Wedell 2002). In polyestrous species (e.g., *Tursiops* spp.) where loss of an offspring could provide an additional mating opportunity, females might benefit indirectly from mating with multiple males if paternity confusion reduces aggression directed towards the offspring. Finally, multi-male matings could reduce sexual harassment of the females themselves in seasonal breeders where males do not guard females (for review see Wolff and Macdonald 2004).

13.2 MALE MATING STRATEGIES

Unlike their terrestrial counterparts, cetacean males are unable to access receptive females by monopolizing the resources that they require (e.g., resource-defense territoriality). Among odontocetes and some mysticetes (i.e., rorquals), resources include prey and breeding sites. For other mysticetes (e.g., *Eubalaena* spp., Right whales, *Eschrichtius robustus,* Gray whale and *Megaptera novaeangliae*), resources are restricted to breeding sites (generally protected coastal areas) because females fast during the breeding season. In both cases, the unpredictable and widespread distribution of these resources makes them uneconomical to monopolize.

Males' ability to monopolize groups of females directly (e.g. harem-defense territoriality) also is limited (except possibly for river dolphins) because females are too widely dispersed or too costly to defend. Female distribution typically reflects predation pressures and resource distribution. In species with relatively little predation and/or food that does not lend itself to sharing, females tend to be solitary (mysticetes and a few odontocetes, see Boness *et al.* 2002 for review). Like many cetaceans, females from these species often exhibit seasonality in their breeding due to the timing of food availability (e.g., *Eubalaena* spp., *Physeter macrocephalus*) and climate patterns (e.g., warmer water for newborn calves; *Eubalaena* spp.; *Eschrichtius robustus*; *Monodon monoceros,* Narwhal). Nonetheless, the distribution of solitary females is still too widespread and unpredictable for males to monopolize multiple females simultaneously. Instead, males tend to associate with a given female for a relatively short period (i.e., act as a consort or primary escort as observed in *Megaptera novaeangliae*, *P. macrocephalus*, and *Tursiops* spp.) and then depart to find another receptive female (sequential polygyny). In some species, males remain for a period of time after mating to try and ensure that they are the only one to mate with a given female (mate guarding, e.g., *Phocoena dalli dalli*, Dall's porpoise).

Multiple males can act cooperatively to obtain access to a receptive female and to keep other males away (e.g., male alliances in *Tursiops truncatus*, Wells *et al.* 1987; and *T. aduncus*, Connor *et al.* 1992a,b; Moller *et al.* 2001). This tactic also may be used by other species but data are sparse (e.g., other dolphin species, such as *Stenella frontalis*, Spotted dolphin, *S. frontalis,* Atlantic spotted dolphin, *S. longirostris*, Spinner dolphin, reviewed by Connor *et al.* 2000b; groups of small or immature male *Physeter macrocephalus*, Whitehead and Weilgart 2000; and some Baleen whales, such as *Megaptera novaeangliae*, Clapham *et al.* 1992; Brown and Corkeron 1995; and *Eubalaena glacialis*, North Atlantic Right whale, reviewed in Kraus and Hatch 2001). Given that females generally produce only a single offspring per breeding attempt (and hence males cannot share paternity), male alliances should be favored as a reproductive strategy if they increase access to receptive females. Increased reproductive success could be achieved *via* shared matings with many females over time or through experience gained (Wells 1991; Krutzen *et al.* 2003). Alliances should be more common among less competitive males and in

situations where the likelihood of interacting with other males is relatively high. For example, in large bodied species, especially those with strong sexual dimorphism (e.g., *P. macrocephalus*), groups of small or immature males may cooperate (Whitehead and Weilgart 2000) but large, mature males seem to actively avoid such associations (Whitehead 1993). Additionally, alliances should be more common among species with longer calving intervals, extended periods of female availability, and relatively high female densities, except in cases where male breeding success is strongly influenced by female mate choice (e.g., *P. macrocephalus*) or sperm competition (Whitehead and Connor 2005). A final situation in which males may benefit from male-male alliances is in a population with limited male dispersal, where kin selection could increase the benefits of being in multi-male groups (Connor *et al.* 1992a,b; Krutzen *et al.* 2003). The importance of kinship as a basis for alliance formation appears to vary among *T. aduncus* populations [e.g., absent in Sarasota Bay, Florida (Connor *et al.* 2000b) and in Port Stephens, southeastern Australia (Moller *et al.* 2001) but present in Little Bahama Bank, northern Bahama (Parsons *et al.* 2003a; Parsons 2004) and Shark Bay, Australia (Krutzen *et al.* 2003)].

Females in species that experience relatively high levels of predation (generally from sharks and *Orcinus orca*, Killer whale) often adopt social or gregarious lifestyles to benefit from increased anti-predator vigilance, predator dilution, cooperative foraging, and/or allomaternal care (Alcock 1998). When females form relatively stable, multigenerational groups (natal group philopatry, e.g., *O. orca*, *Globicephala* spp., Pilot whales, *Physeter macrocephalus*, and possibly *Fersa attenuata,* Pygmy killer, and *Pseudorca crassidens*, False killer whales; reviewed by Connor 2000), males tend to pursue three main strategies to access receptive females. If there are benefits to being part of a group, males may remain with their natal group and access potential mates from other groups when two groups meet (*O. orca,* Bigg *et al.* 1987, 1990; and *Globicephala melas*, Long-finned pilot whale, Amos *et al.* 1993, cited in Connor 2000). They also may leave their natal group, attach themselves to another group, and mate with those females as they become receptive (no known examples among whales and dolphins). Finally, if the potential costs of group living outweigh the benefits, then males will rove among groups of females looking for receptive individuals (e.g., *P. macrocephalus,* Best 1979; Connor 2000; Whitehead and Weilgart 2000). The latter pattern is observed in the majority of species with gregarious females that do not form long-term, multigenerational groups.

There are two examples of cetaceans that exhibit male (and female) natal group philopatry; resident *Orcinus orca* (Bigg *et al.* 1990) and *Globicephala melas* (Amos *et al.* 1993 as cited in Connor 2000). Although these males' access to receptive females is likely to be limited to some extent, there are several reasons why it might be beneficial for males to remain with their natal group rather than attach themselves to a group of unrelated females. For instance, group foraging can increase prey encounter rate and, when well coordinated,

should also decrease prey-handling time and increase the success rate with larger or more dangerous prey (Zeh and Zeh 1990). Examples of highly coordinated cooperative foraging often involve species that form related social groups (e.g., *Panthera leo*, lions, Packer *et al.* 1990; and *Canis lupus*, wolves, Smith *et al.* 1997). Multigenerational groups may be more efficient at coordinating their hunting behaviors due to increased group stability and/or better transmission of learned behaviors. Stomach content analyses indicate that individual populations of *Orcinus orca* specialize in particular types of prey (Felleman *et al.* 1991; Jefferson *et al.* 1991; Baird *et al.* 1992) and there is evidence from behavioral observations that adult *O. orca* teach hunting skills to their young (Lopez and Lopez 1985; Guinet 1991; Hoelzel 1991). If individual pods utilize different hunting methods, then resident orcas, which exhibit natal group philopatry, may forage more efficiently with relatives who share a common culture. There are no data to support this hypothesis among resident populations; however, transient *O. orca* do exhibit pod-specific foraging tactics (Baird *et al.* 1992) and association patterns among these animals are consistent with the idea that individuals forage preferentially with relatives. Females that leave their natal pods tend to establish their own pods as soon as they have enough mature offspring to assist with foraging and group defense, whereas males generally hunt and travel alone despite the benefit associated with cooperative foraging (i.e., unrelated males do not form pairs or groups, Baird 1994).

Remaining with a natal group also could be beneficial if males' presence enhanced the survival of related offspring (kin selection, Hamilton 1964). Association patterns suggest that direct assistance with young may not be common (e.g., among resident *Orcinus orca*, adult males often associate with post-reproductive mothers or mothers without juvenile offspring, Bigg *et al.* 1990). However, males could still enhance relatives' success, and hence their own fitness, through group defense and cooperative foraging (Baird 2000) especially in sexually dimorphic species, in which males are substantially larger than females. They also may benefit from the opportunity for breeding coalitions with their brothers or mothers (another possible expression of kin selection, Connor 2000).

When the potential gain in reproductive success from better access to receptive females outweighs that from associating with relatives, males should leave their natal group and join another, unrelated group of females (e.g., *Panthera leo*, *Prebytis entellus*, *Hanuman langur*). Thus far, there are no known examples of this among cetaceans (Connor *et al.* 2000a). Instead, in species where females exhibit natal group philopatry and males do not, males tend to rove between groups of females (e.g., *Physeter macrocephalus*, Best 1979).

Group defense remains important for *P. macrocephalus* but the benefits from cooperative foraging do not seem to be as critical (Whitehead 1996; Whitehead and Weilgart 2000; Coakes and Whitehead 2004). Hence, although there may be some advantages to foraging in groups, they do not appear to offset the extra burden experienced by maturing males from competing with females for food (Whitehead and Weilgart 2000). Instead, male *P. macrocephalus* leave their

natal groups as juveniles and travel to high latitudes where less competition for food and larger prey items enhance males' ability to achieve a competitive adult body size (Best 1979). Males' failure to reunite with their natal group or to join another, unrelated, group of females upon their return from the higher latitudes suggests that the benefits of group living do not outweigh the costs even after adult size is achieved. Adult males do not appear to engage in social feeding, nor do they require the benefits of group defense (Whitehead and Weilgart 2000).

The amount of time a roving male spends with a particular group of females varies. The decision whether to stay with a given group or to move on to the next should depend on which strategy provides males with the best access to receptive females and the highest reproductive success. Whitehead (1990) suggested that males should tend to rove when the duration of female estrous is longer than the traveling time between groups. Field observations suggest that among *Physeter macrocephalus* off the Galapagos Islands, males tend to spend only a few hours or less with any given group of females (Whitehead 1993).

13.2.1 Male Intrasexual Competition

The level of aggression in male-male competitions typically reflects the variation in male reproductive success or the cost of 'losing' contests for access to females. Variation increases when some males mate successfully with multiple females, leaving others without mates (polygyny). The operational sex ratio, the sex ratio among individuals that breed in a given season, is further skewed if females produce offspring in multiyear intervals (e.g., *Physeter macrocephalus* 3-6 yr, *Monodon monoceros* 2-3 yr, *Orcinus orca* 2-14 yr, *Eubalaena* spp. 2-5 yr; see Boness *et al.* 2002 for review). The more skewed the operational sex ratio, the more intense the competition among males for access to the available females. The level of sexual selection acting on populations can vary within as well as between species. For example, among *S. longirostris*, degree of polygyny is higher in the eastern versus the whitebelly form (Perrin and Mesnick 2003). The difference in mating systems was deduced from observed morphological differences – the eastern form has more sexual dimorphism in male dorsal fin shape and more within-population variability in testis size, indicating that a smaller proportion of males appear to engage in mating (Perrin and Mesnick 2003). The more intense intrasexual selection reflects a higher operational sex ratio among the eastern animals apparently due to more limited prey availability and longer calving intervals (Perrin and Mesnick 2003).

13.2.2 Contest Competition

Male-male competition for access to females occurs in four main ways; contest competition, female mate choice competition, sperm competition and scramble competition. Contest competition occurs when males interact aggressively to gain access to females or to prevent other males from gaining access (Fig. 13.1

Fig. 13.1 A-C Male Humpback whales (*Megaptera novaeangliae*) interacting aggressively; escort male breaching over a challenger. Photo: Jeff K. Jacobsen. **D**: Sexual socializing in Bottlenose dolphins (*Tursiops aduncus*) in Northern New South Wales, Australia. Photo: Christine Fury.

A-C). Many cetaceans exhibit this type of competition, especially among the odontocetes (e.g., *Monodon monoceros*; *Delphinapterus leucas*, Beluga; *Physeter macrocephalus*; *Orcinus orca*; *Hyperoodon* spp., bottlenose whales) Male-male aggressive interactions include head butting (review by Lusseau 2002), biting (reviewed by MacLeod 1998) and striking each other with various parts of the body (peduncle, flukes, etc.). The degree of sexual dimorphism present in body size and other traits used as weapons provides insight into the intensity of the competition (Alcock 1998). Sexual size dimorphism (SSD) is present in a number of odontocete families (e.g., Delphinidae, Monodontidae and Physeteridae, and among beaked whales in the genera *Ziphius* and *Hyperoodon larger*; see Connor *et al.* 2000b and Boness *et al.* 2002 for review). *Physeter macrocephalus*, the largest odontocete, displays extreme SSD with males one and a half time longer and three time heavier than females (Lockyer 1981; Rice 1989). These males also experience delayed maturation compared to females, both physically (Best *et al.* 1984) and socially (i.e., males delay competing for mates for up to a decade after they reach sexual maturity; Rice 1989), another indication that competition for females is intense. Despite the apparent intensity of the competition, male-male fights appear to be relatively rare (Whitehead and Weilgart 2000), possibly because the potential costs of such a fight are great (MacLeod 1998) and/or because mating abilities vary greatly among males and are relatively self-evident. A similar explanation has been proposed for the lack of observations of escalated violence between male *M. monoceros* (MacLeod 1998; Connor *et al.* 2000a), another species with relatively strong SSD (Hay 1980), delayed sexual maturity by males, and multiyear calving intervals (reviewed in Boness *et al.* 2002).

Sexual size dimorphism is either absent among mysticetes or, in many species, females are slightly larger than males (i.e., reverse size dimorphism, Brownell and Ralls 1986). Nonetheless, in species that aggregate on seasonal breeding grounds, males do appear to compete directly for access to females (e.g., *Megaptera novaeangliae*, *Eubalaena glacialis* and *Eschrichtius robustus,* see Boness *et al.* 2002 for a review). Body size relative to other males remains important among *M. novaeangliae* since larger males are more likely to hold dominant positions (e.g., principal escorts of receptive females, Spitz *et al.* 2002; Fig. 13.1 A-C). This trend may also hold true for *E. glacialis* and *E. robustus* (see discussion below).

Male *Megaptera novaeangliae* antagnostic interactions include the production of bubble streams, inflation of the ventral pouch, vocalizations, and various types of physical contact as challengers try to displace males escorting females (Tyack and Whitehead 1983). The intensity and importance of these interactions is reflected in the frequency with which males on the breeding grounds display superficial wounds (Clapham 2000) and the relatively high degree of dorsal fin scarring on primary escorts or challengers compared to other males (Chu and Nieukirk 1988).

Male *Eschrichtius robustus* and *Eubalaena* spp. also compete directly for females but less aggressively. In *Eubalaena* spp., numerous males compete physically for a position beside a single adult female using their callosities

(rough horny material on their heads) to help displace competitors (Payne and Dorsey 1983; Kraus and Hatch 2001). *E. robustus* mating also involves females lying inverted on the surface surrounded by competing males, but males from this species do not possess callosities (nor do any cetaceans outside the *Eubalaena* spp.) and appear to interact less aggressively than do *Eubalaena* spp. (Swartz 1986). This reduced level of intrasexual competition could be due to the shorter mean calving interval in *E. robustus* (i.e., operational sex ratio is about 1:2 for *E. robustus* compared to 1:4 for *Eubalaena* sp.; Kraus and Hatch 2001) and shorter breeding season (less opportunity for individual males to monopolize multiple females). On the other hand, the lower level of competition observed among *E. robustus* and *Eubalaena* spp. compared to *Megaptera novaeangliae*, which has an operational sex ratio of about 1:2.5 and a longer mating season than *E. robustus*, could be linked to the relative importance of sperm versus contest competition among the former species. Hence, although a relatively large body size may yield greater success in achieving alpha positions beside *Eubalaena* and *E. robustus* females, other traits, such as endurance and agility, likely are important (see 13.2.4 Sperm Competition below).

In species where contest competition is important, sexual dimorphism is also common in morphological traits used as weapons. For example, the thickness of forehead bones is a sexually dimorphic trait observed in species whose males butt heads, such as *Hyperoodon ampullatus,* the Northern bottlenose whale, and *Mesoplodon densirostris,* Blainville's beaked whale (Gowans and Rendell 1999; MacLeod 2002). Carrier *et al.* (2002) suggested that the greatly enlarged melon of male *Physeter macrocephalus*, the spermaceti organ, may be used as a battering ram in male-male interactions, in addition to its primary role in sound production (e.g., Norris and Harvey 1972).

Callosities on *Eubalaena* spp. tend to be more numerous on males than females, as do the scars that result from their use as weapons (Payne and Dorsey 1983; Kraus *et al.* 1986). Males from other species also display extensive scarring compared to females, most associated with interactions involving teeth. Sexually dimorphic and specialized teeth (e.g., size, number or shape) are present in numerous odontocetes. In *Monodon monoceros*, only males have the characteristic long tusk which functions as a weapon in male-male encounters (Gerson and Hickie 1985; Connor *et al.* 2000a). Similarly, most beaked whales (excluding *Berardius* spp.) have lost all but one or two pairs of mandibular teeth and the remaining pairs, tusk-like protrusions called battle teeth, are sexually dimorphic and functional only in males (e.g., *Mesoplodon biden,* Sowerby's beaked whale, MacLeod and Herman 2004; *M. densirostris,* Blainville's beaked whale, MacLeod 2002). This pattern of extensive tooth reduction is found primarily in squid-eating (teuthophagous) species where teeth are no longer required for feeding (an exception is *M. monoceros*, MacLeod 1998). Females from these species typically retain few if any functional teeth (*Physeter macrocephalus* and *Berardius* spp. are exceptions) and scarring on males is thought to be an indicator of male quality (MacLeod 1998). Other whale and dolphin species clearly use their teeth in antagonistic

encounters but show no specialization (e.g., *Orcinus orca, Tursiops* spp., Scott *et al.* 2005).

The importance of teeth in male-male aggression is evident from the extensive scarring observed in many species including representatives from four odontocetes families, Delphindae, Platanistidae, Physteridae and Ziphiidae (McCann 1974). In fact, MacLeod (1998) suggested that there has been selection against the repigmentation of wounds in some species to maximize the usefulness of scarring as an indicator of male quality (i.e., results in permanent scars that accumulate over time). The level of scarring can be a useful indicator of intrasexual competition (e.g., Scott *et al.* 2005), but its signal must be interpreted cautiously since wounds from many of the other weapons used in contest competition (e.g., heads, fins, flippers and peduncles) may not result in obvious external wounds (e.g., Parsons *et al.* 2003b).

Males in a given species may not follow the same strategy for accessing receptive females, especially when sexual selection is intense. For example, males less likely to succeed at contest competition, e.g. relatively small or inexperienced animals, may opt for roving among females (scramble competition) or displaying (mate choice competition) rather than participating in multi-male groups.

13.2.3 Female Mate Choice Competition

Sexual selection can lead to the evolution of ornaments as well as weapons. Ornaments are used in mate choice competition, where one sex, typically males, competes to be chosen by the other sex. There is little evidence of ornaments in whales and dolphins (except perhaps for the scarring patterns and the narwhal tusk, Connor *et al.* 2000a). However, there are numerous traits that could provide females (and other males) with information about a male's mating status and quality (reviewed in MacLeod 1998; Boness *et al.* 2002; Ralls and Mesnick 2002). These include morphological traits such as the dorsal fin (e.g., *Orcinus orca* and *Globicephala melas*) and postanal hump (e.g., *Phocoenoides dalli dalli,* Dall's porpoise; *Lagenodelphis hosei*, Fraser's dolphin; *Stenella longirostris*; *Delphinus delphis*, Short-beaked common dolphin, *Neumann et al. 2002*) caudal peduncle (*Tursiops truncatus*, *P. dalli dalli*), and flippers (*O. orca*; *Peponocephala electra,* Melon-headed whale; *Cephalorhynchus heavisidii*, Heaviside's dolphin; and *Delphinaptera leucas*). Cues for mate assessment could also be associated with dimorphic acoustic and behavioral displays. In *M. novaeangliae*, singing seems to be linked to male-male competition but there is evidence that it may also be important in female choice (Tyack 2000). Similarly, although 'slow clicks' are only produced by mature male *Physeter macrocephalus*, it is not known whether they are used by males to assess or avoid competitors and/or by females to assess potential mates (Whitehead 1993; Whitehead and Weilgart 2000). Other species that may use vocal acoustic displays include *Balaenoptera physalus,* Fin whale (Croll *et al.* 2002) *Balaena mysticetus,* Bowhead (Tyack 2000) and *Eubalaena* spp.

(Clark 1983; Kraus and Hatch 2001; Parks *et al.* 2005). Non-vocal acoustic displays (e.g., bubble production in *Tursiops truncatus* and *Megaptera novaeangliae,* Caldwell and Caldwell 1972; Tyack and Whitehead 1993) and various non-acoustic behavioral displays (breaching, tail slapping, etc.) could be informative (Figs. 13.1, 13.2). Finally, in addition to competing to be chosen by females as the 'best' male based on ornaments or other displays, males' performance in male-male interactions (e.g., high speed chases after a female or jockeying for position beside a receptive female) could be used by females to assess the relative quality of males (i.e., interaction with contest competition) (Figs. 13.1, 13.2C).

Male mate choice can occur if males are limited in the number of matings they can achieve in a given breeding season and females differ in quality. As discussed above, male cetaceans' ability to mate with multiple females is often limited by the distribution of receptive females through space and time (female dispersal patterns, degree of breeding synchrony and length of breeding season) and the degree of competition for access to these females (operational sex ratio). Additionally, delayed maturation and multiyear calving intervals mean that females will differ in the likelihood of becoming pregnancy in a given breeding season. Evidence supporting male mate choice in cetaceans comes from the baleen whales (monoestrous, seasonal breeders). These data suggest that males differentiate among available females and associate preferentially with those with higher reproductive potential (e.g., associate with adult versus immature females, as in *Eubalaena glacialis*, Kraus and Hatch 2001; and lone females rather than those with a calf, such as in *Megaptera novaeangliae*, Craig *et al.* 2002; and *E. robustus*, Perez-Cortes *et al.* 2004). More data are required to determine whether males differentiate beyond these very broad categories. Unfortunately, our understanding of mate choice in cetaceans is limited by our inability to link specific traits or behaviors with realized reproductive success. Mating is rarely observed and paternity is generally unknown (i.e., males do not provide parental care and few paternity studies have been able to identify individual fathers, Amos *et al.* 1993 as referenced in Connor 2000; Schaeff 1993; Clapham and Palsboll 1997; but see Krutzen *et al.* 2004 and Section 13.3, Female Strategies, below).

13.2.4 Sperm Competition

Another type of competition, sperm competition, occurs when multiple males have the opportunity to mate with single females. Competitive success is generally achieved by producing great quantities of sperm (i.e., higher relative representation among sperm present or better displacement of rivals' sperm; see Wedell *et al.* 2002; Snook 2005, for review). This selects for large testes relative to body size since increased testis size correlates with greater numbers of spermatozoa per ejaculate (e.g., Brownell and Ralls 1986). Multi-male mating groups are common in a number of mysticetes but relatively large testes are present in only some (e.g., *Eubalaena* spp., *Eschrichius robustus*, *Balaena mysticetes* but not *Megaptera novaeangliae*; Brownell and Ralls 1986).

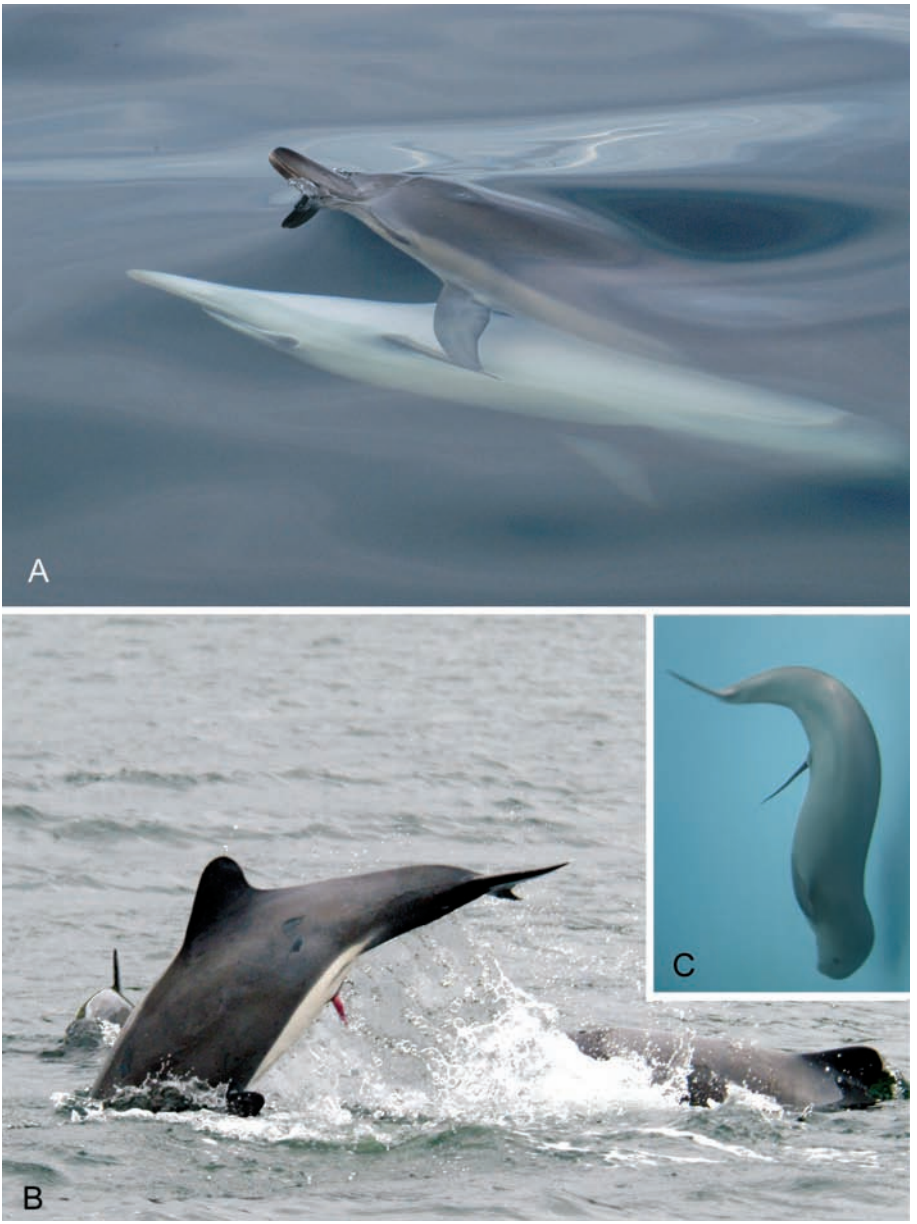

Fig. 13.2 A. Copulation behavior by Common dolphins (*Tursiops truncatus*). Photo: Karen A. Stockin. **B**. Young male Chilean dolphins (*Cephalorhynchus eutropia*) involved in sexual socializing. Photo: Sonja Heinrich. **C**. Finless porpoise (*Neophocaena phocaenoides* subspecies *sunameri*), [Japanese form]. Arching behavior may be used to elicit behavior related to mating or courtship. Photo: Grant Abel.

The importance of sperm competition, inferred from relative testis size, also varies among odontocetes (e.g., *Tursiops* spp. with relatively large testes versus *Pontoporia blainvillei*, La Plata dolphin—locally known as Franciscana, and *Physeter macrocephalus,* both of whom have testes less than 0.05% of their body mass Danilewicz *et al.* 2004; Brownell 1989).

Numerous studies have shown that fertilizing efficiency is associated with sperm traits such as sperm size, longevity, viability, and mobility (see Snook 2005 for review) and hence males increase their competitiveness by producing higher quality sperm. Size and morphology of spermatozoa vary among cetaceans with interspecific variations corresponding to taxonomic classifications (Kita *et al.* 2001; Meisner *et al.* 2004). In some species longer penises could also be selected for if they increase the chances that sperm will be deposited closer to the ova (e.g., *Eubalaena* spp., Brownell and Ralls 1986).

Sperm competition is associated with multiple males competing for access to a single female (i.e., alpha positions beside the female, Kraus and Hatch 2001; Fig. 13.1D). Some traits associated with contest competition may therefore be present in species that engage in sperm competition although a number of authors have suggested that endurance and agility, rather than power, may be important. Female mate choice competition can also influence sperm competition. Females can affect individual males' chances of fathering their offspring by vocalizing or otherwise signaling their receptivity non-randomly, *via* biased participation in courtship groups and through cryptic (post-copulatory) female choice. As a result, traits signaling male quality (female mate choice, section 13.2.3) as well as those associated with locating or catching females (scramble competition, section 13.2.5) may be important for species that engage in sperm competition.

13.2.5 Scramble Competition

The fourth main type of intrasexual competition is scramble competition where males disperse to find sexually receptive females. It is common when females are widely or unpredictably dispersed (e.g., territories not maintained) and the breeding season is relatively short. Scramble competition appears to be a basic strategy in whales and dolphins, particularly among odontocetes (Wells *et al.* 1999; Mann *et al.* 2000; Boness *et al.* 2002). Its importance is lower among social than solitary species [e.g., species exhibiting natal group philopatry (*Orcinus orca* and *Globiocephala melas*) versus mysticetes] but is used by roving males engaged in sperm or contest competition (e.g., *Eubalaena* spp. and *Physeter macrocephalus*, respectively). Scramble competition has resulted in sexual dimorphism in traits associated with locomotion in some terrestrial species (e.g., Salamandridae, newts, Able 1999, and Oniscidea, terrestrial crustaceans, Lefebvre *et al.* 2000) but data are not yet available to test this among cetaceans.

13.3 FEMALE STRATEGIES

Female cetaceans invest heavily in their offspring. Given that males contribute only their genes, it is advantageous for females to maximize their opportunity to mate with high quality males. For female cetaceans, this opportunity is maximized via female mate choice competition and/or by breeding with the winner in male-male interactions (contest, sperm or scramble competition). Females' behavior suggests that they exert some control over whether particular males are able to mate with them (e.g., rolling belly up, suddenly leaving social groups or avoiding male song playbacks; Tyack 1981; Swartz 1986; Clapham *et al.* 1992; Connor *et al.* 1992a,b; Kraus and Hatch 2001). Further, females may pursue various strategies to enhance competition among their potential mates and hence improve the chances that high quality males will fertilize their eggs. For example, some females lead males on high-speed chases prior to mating (*Eubalaena robustus*, Swartz 1986; *Megaptera novaeangliae*, Tyack and Whitehead 1983). Other females may vocalize (e.g., *Eubalaena glacialis*, Kraus and Hatch 2001; Parks and Tyack 2005) or pursue additional strategies to make themselves more visible (e.g., flippering and lobtailing, *M. novaeangliae*, Clapham 2000). The occurrence of cooperative herding (e.g., *Tursiops truncatus* in Shark Bay, Australia, Wells *et al.* 1987) and other coercive tactics used by males to control females supports the idea that females do not always passively accept winning males as their mates.

13.4 COURTSHIP AND HYBRIDIZATION

In addition to providing individuals with the opportunity to assess potential mates' reproductive status and relative fitness, courtship rituals frequently include features that enhance species recognition. Species-specific courtship displays are an example of pre-fertilization reproduction isolating mechanisms (RIMs) and they evolve when hybridizations with sympatric species produce less fit offspring. Pre-fertilization RIMs include behavioral patterns that reduce the likelihood that breeding individuals from two species will overlap in time and space (e.g., different breeding seasons or breeding locations) or, if they do overlap, that they will select each other as mates (e.g., species-specific behavioral cues including vocalizations; *Physeter macrocephalus*, *Megaptera novaeangliae*). Isolating mechanisms also include physical differences that prevent mating from occurring (size, shape of genitalia; less likely among cetaceans) and biochemical incompatibility at the level of the gametes (e.g., chemical characteristics of the environment or the egg prevents fertilization; influence for cetaceans not known). Post-fertilization RIMs include situations where the fertilized egg fails to thrive and develop or the resulting hybrid offspring are unable to reproduce, either because they do not survive to adulthood or because they are sterile.

Observations of mating behavior by cetaceans are fairly rare. Hence, evidence of cross-species mating is generally limited to the identification of hybrid offspring. Most hybrids identified to date involve relatively small

bodied odontocetes including *Phocoenodies dalli dalli* (Dall's porpoise) with *Phocoena phocoena* (Harbour porpoise) (Willis *et al.* 2004) and *Tursiops truncatus* with *Delphinus capensis* (Long-beaked common dolphin; Zometzer and Duffield 2003) or *Stenella frontalis* (Atlantic spotted dolphin) (Herzing *et al.* 2003). Another possible hybrid is between *Lagenorhynchus obscurus* (Dusky dolphin) and *Lissodelphis peronii* (Southern right whale dolphin) (Yazdi 2002). The only hybrid offspring observed among the mysticetes are *Balaenoptera musculus* (Blue whale) and *B. physalus* (Fin whale) crosses (e.g., Arnason *et al.* 1991; Spilliaert *et al.* 1991; Berube and Aguilar 1998; Cipriano and Palumbi 1999).

The parentage of hybrids can be determined by comparing the hybrid's maternally inherited mtDNA with that of the two parent species. Thus far, all hybrids arising from *Phocoenoides dalli dalli-Phocoena phocoena* crosses have involved male *P. phocoena,* suggesting that the cross-species mating is driven by these promiscuous males rather than the less polygynous *P. dalli dalli* (Willis *et al.* 2004). *Balaenoptera musculus-B. physalus* crosses include males from either species (e.g., Arnason *et al.* 1991). Both balaenopterids are dispersed rather than congregated during the breeding season and emit low frequency, long range vocalizations which may be involved with reproduction (e.g., Watkins *et al.* 1987).

It is unclear whether the occurrence of hybrids is a relatively recent phenomenon, driven by anthropomorphic factors such as population depletion due to whaling or modified habitat-use patterns due to habitat modification. Since many hybrid offspring, particularly females, appear to be able to reproduce (Spilliaert *et al.* 1991; Zometzer and Duffield 2003; Willis *et al.* 2004) there may be limited selection against hybridization, at least for these species.

13.5 CONCLUSION

Cetacean mating and courtship is highly varied. Although all share the ocean habitat, the diverse ecological conditions under which species live have resulted in a myriad of mating strategies. Research indicates that individuals' mating behavior varies not only at the species level but also among individuals within a population and for a given individual throughout their life. For example, although highly competitive (i.e., older and/or larger males) males engage in contest competition, others (younger and/or smaller) may focus on trying to find receptive females first (i.e., scramble competition).

Our knowledge of whales and dolphins has increased dramatically over the past decade but is still limited owing to difficulties associated with identifying and tracking individuals and the paucity of direct observations of mating behavior. Genetic work has revealed that habitat-use patterns are often influenced by female-directed philopatry (e.g., *Tursiops aduncus*, Scott *et al.* 1990; *Eubalaena glacialis*, Schaeff *et al.* 1993; *D. leucas*, Brown Gladden *et al.* 1997; *Eschrichtius robustus*, Steeves *et al.* 2001; Goerlitz *et al.* 2004) but the location of many whales during the breeding season remains unknown,

particularly among the mysticetes (e.g., *E. australis,* Best *et al*. 2003). Since paternity data are usually unavailable, we are also unable to investigate the relationship between various reproductive strategies and individuals' reproductive success.

Cetacean mating and courtship behavior are an exciting and interesting field of study. It is also an important one. As our knowledge in the area increases so too does our ability to calculate effective population sizes and assess population vulnerability to various anthropomorphic factors including inbreeding (Schaeff *et al*. 1997). This in turn enhances our ability to develop effective management and conservation plans.

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CHAPTER 14

Reproduction in Relation to Conservation and Commercial Exploitation

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14.1 INTRODUCTION

Life history and demographic parameters of a species are critical factors in understanding population vulnerability, predicting the impact of exploitation and decline, and predicting the potential for recovery (Lewison *et al*. 2004). As large mammals, cetaceans have long life spans, lengthy generation times, high survival rates, and extended parental investment (Evans and Stirling 2001). These traits buffer the populations against short-term impacts but also limit their ability to recover when population levels are low (Stearns 1992). The primary cause of reductions in cetacean populations has been directed fisheries or incidental bycatch. Other anthropogenic factors may contribute to reductions in population size that lead to instability and sub-optimal recovery. These include the chronic and still unknown effects of chemical and physical pollutants. For cetaceans, data on sub-clinical or sub-lethal effects on reproductive parameters are limited. Nonetheless, there is information that population density and other factors affect reproductive parameters in ways that contribute to or inhibit potential conservation and recovery of populations.

14.2 LIFE HISTORY AND DENSITY DEPENDENCE

Density dependence in life-history parameters involves changes coincident with changes in population density. The changes reflect increasing

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population growth rates at low density and decreasing growth rates at high density (Fowler 1981a). Density-dependent changes can occur in parameters such as calving interval, ovulation rates, age and size at sexual maturation, survivorship, and disease transmission (Fowler 1981a).

In long-lived mammals, such as most cetaceans, the parameters most commonly showing density-dependent changes are juvenile and adult survival, fecundity, inter-birth interval, and age at first reproduction (Fowler 1984, 1987). Density-compensatory adjustments to life-history parameters in large mammals generally have been attributed to changes in resource levels (Fowler 1981a). Fowler (1984) documented that, in addition to numerous terrestrial mammals, at least sixteen species of marine mammals (cetaceans, seals, sea lions, and dugongs) show evidence of reproductive changes related to changes in density of the marine mammal or prey populations.

The long history of commercial exploitation and incidental mortalities have reduced some cetacean population levels and provided data that lend insight into cetacean density-compensatory responses. The earliest documented density-dependent changes were described in populations of whales that had been commercially exploited. For example, a correlation between prey abundance and reproductive parameters was found in *Balaenoptera physalis* (Fin whale) taken during Icelandic whaling operations (summarized in Lockyer 1987). Results of samples collected from 1976 through 1985 revealed a significant positive correlation between prey abundance (euphausiids) and *B. physalis* body condition (Lockyer 1986, 1987). In 1977 and 1978, both potential prey abundance and the proportion of mature females ovulating were low. Over the 10-yr sampling period, the proportion of females with a corpus luteum followed the same pattern, *i.e.*, in years with abundant prey more females were ovulating. Conversely, reduced prey abundance resulted in relatively fewer females ovulating. While acknowledging that the patterns were correlations rather than a direct measure of cause and effect, Lockyer (1987) hypothesized that increased prey availability resulted in enhanced body condition, which in turn resulted in increased fecundity as measured by the proportion of females ovulating. Similarly, Straley *et al*. (1994) concluded that sufficient prey resources and female body condition were the primary factors related to *Megaptera novaeangliae*'s (humpback whale) ability to ovulate annually and successfully carry a fetus to term in Alaskan waters.

Interestingly, Lockyer (1987) concurrently reported what initially seemed to be a counterintuitive result. Both 1980 and 1982 were years with high prey abundance, good body condition of *Balaenoptera physalis*, and unusually high ovulatory rates. For *B. physalis*, one result was an immediate postpartum return to estrus for many females during the winter of 1981/82. This is in sharp contrast to the usual pattern of ovulation suppression until weaning (ca 1yr). Thus, the normal 2-yr reproductive cycle was compressed to a 1-yr reproductive cycle. Furthermore, although prey abundance in 1983 was average, ovulation rates were very low. Lockyer (1987) speculated that the whales that had calved in the two previous years did not ovulate the third

year (1983) due to energetic constraints. These results suggest that sustained increases in calving rates are possible when resource levels are high but that there are limits to further increases due to energetic needs or other limitations. Lockyer (1986) offered yet another possible explanation for the low 1983 ovulation rates. She suggested that measurements of krill (or prey) abundance may not reflect the actual prey available to the whales. It is possible that prey availability is masked by whale selectivity for prey size, whale flexibility in switching prey species to optimize foraging, and other various climactic and oceanographic factors that may affect prey productivity.

In southern hemisphere balaenopterids, there have been changes in other reproductive parameters following exploitation, including a decrease in age at sexual maturation and increased pregnancy rates (Lockyer 1979; Boyd *et al*. 1999). Kato (1987) reported a decline in age at sexual maturation from 14 yr in the 1940's to 6 yr in the late 1960's for *Balaenoptea acutorostrata* (Minke whale) from the Antarctic. He found, however, that size at sexual maturation remained constant during that time period, indicating a higher somatic growth rate in the latter period. He suggested that the changes were due to a reduction in the number of whale populations in the Antarctic, thereby reducing competition for prey and increasing the carrying capacity for *B. acutorostrata*. Alternatively, these changes may have occurred because of reductions in the size of *B. acutorostrata* populations themselves.

Density-dependent changes in life-history parameters also have been documented in odontocetes. *Stenella longirostris* (Spinner dolphin) and *Stenella attenuata* (Spotted dolphin) mortalities incidental to the Yellowfin tuna purse seine fishery in the eastern Tropical Pacific Ocean were in the hundreds of thousands in the 1960's and 1970's, decreased to 133,000 in 1986, and then to 1,877 in 1998 (Hall *et al*. 2000). Changes in reproductive parameters, including age at sexual maturation and increased reproductive rate, were found when comparing heavily exploited and less exploited populations of both spinner and spotted dolphins (Perrin *et al*. 1976, 1977; Smith 1983).

Kasuya (1985) compared the age composition and reproductive status of *Stenella attenuata* and *S. coeruleoalba* (Striped dolphin) taken in drive fisheries in Japan. Catches of *S. attenuata* had been relatively small compared to those of *S. coeruleoalba,* whose fishery had occurred for a longer period of time. He found that average age at sexual maturation declined in *S. coeruleoalba* from 9.7 yr to 7.4 yr between the cohort born in 1957 and that born in 1969, while there was no concomitant change in *S. attenuata*. Both species showed a decrease in age of the youngest sexually mature animals. An increase in the proportion of females pregnancy and lactating also was observed for *S. coeruleoalba*. Furthermore, there was an indication that they may have experienced a decrease in average calving interval from 4.0 yr in 1955 to 2.76 yr in 1977; there was no downward trend in mean lactation time and mean resting period. If survival remained constant, these changes would lead to increased reproductive rates by decreasing age at maturation and calving interval. In contrast, there were no changes in these parameters in the less exploited *S. attenuata* population.

Phocoena phocoena (Harbor porpoise) have been subjected to significant bycatch mortality from commercial gillnet fisheries throughout their range (Gaskin 1984). For porpoise from the Bay of Fundy, the life-history parameters from samples collected from 1969-1973 were compared to those collected from 1985-1988 (Read and Gaskin 1990) and a number of changes were found. The age at sexual maturation declined from 3.97 yr to 3.44 yr, with a concomitant decline in average length at maturation from 147 cm to 143 cm. The average length of calves (not average length at birth) also increased from 92 cm to 108 cm. The authors speculated that these changes resulted from a decrease in porpoise density.

Phocoena sinus (Vaquita) populations in the Gulf of California are highly endangered, having been reduced to very low abundance and restricted to a small geographic range in the northern Gulf of California (Vidal *et al.* 1999). On average, *P. phocoena*, a species very closely related to *P. sinus*, mature at 3-4 yr of age and then calve annually (Read and Hohn 1995). Few ovaries have been available from *P. sinus* to evaluate their reproductive parameters; however, Hohn *et al.* (1996) found that the apparent calving interval for *P. sinus* is biennial rather than annual as would be expected given its phylogeny and population status. It is possible that carrying capacity in the northern Gulf of California has changed for *P. sinus* and resources are insufficient to accommodate the increased energetic demands of annual calving and lactation, preventing a density-compensatory response. A similar situation exists for *P. phocoena* in California and Maine. *Phocoena phocoena* in the Gulf of Maine give birth annually while those in California have a two-year calving cycle (Read and Hohn 1995). Read and Hohn (1995) hypothesize that this difference may be due to disparate food resources in these locations.

Reproductive changes also have been documented in large odontocetes. *Orcinus orca*, off British Columbia and Washington, endured a small live-capture fishery between 1962 and 1977. Bigg (1982) showed average birth rates of 6.94 % and 9.77% for unexploited and exploited *O. orca* populations. Net recruitment of calves was greater in exploited pods (5.3% calves/total number whales versus 4.08% in non-exploited pods) as was the overall annual rate of increase (3.01% versus 1.67% in exploited versus non-exploited pods, respectively). Calving intervals also were shown to decrease from 12.47 yr to 8.59 yr (unexploited and exploited pods, respectively). In *Physeter macrocephalus,* Best (1980) and Best *et al*. (1984) noted an increased pregnancy rate and a decreased calving interval (from 6 yr to 5.2 yr) from intensely exploited years, 1962-1967, relative to a period of reduced exploitation, 1973-1975, respectively. Interestingly, the increased pregnancy and birth rates were more evident in the older females of these populations.

Population monitoring must include analyses of the proportion and number of adult females in an exploited population. In North Atlantic *Eubalaena glacialis* (Right whale), slow population recovery appears to be due in part to a decrease in the proportion of parous females (38%) versus populations in the South Atlantic (54%) (Brown *et al*. 1994). This decrease in

reproductive females is likely linked to the decreased calving rate in the northern versus southern populations (2.5% versus 7.6%, respectively). The differential proportion of females and severity of depletion is attributed to preferential hunting of mature females and their offspring in North Atlantic populations (Oldfield 1988).

Monitoring reproductive as well as other vital parameters may provide rough indicators of the status of the populations. Models predict that in large mammals most density-dependent changes will occur while the population is still at high levels relative to carrying capacity (Fowler 1981b, 1984). For marine mammals, models suggest that density-dependent changes will occur at population sizes between 50 and 85%, with insufficient data for most species to estimate a more precise range (Taylor and DeMaster 1993). For development of more precise models, baseline data would need to be collected either prior to changes in abundance or through recovery (*i.e.,* until the populations recover to relatively high levels) and under natural conditions (Fowler 1981a). The protracted generation times of large, long-lived mammals require that population monitoring, data collection, and analyses occur over long periods of time. In addition, other factors, such as chemical pollutants, biotoxins, disease outbreaks, or habitat loss, may act synergistically to accelerate or inhibit density-dependent changes in reproductive parameters.

14.3 REPRODUCTIVE SENESCENCE

Reproductive senescence is a decline in age-specific fecundity with age (Promislow 1991). For cetaceans, data on reproductive senescence are available for few species. Postreproductive *Stenella attenuata* (Perrin *et al*.1976) and *S. longirostris* (Perrin *et al.* 1977) were identified in samples collected from dolphins taken incidentally in the tuna fishery in the eastern Pacific. These animals were described as having atrophic ("regressed" or "withered") ovaries. For both species, the incidence was less than 1% of the sample of mature females. Myrick *et al.* (1986) examined a larger sample of ovaries collected from *S. attenuata* taken in the tuna fishery. Of 542 mature females, nine had atrophic ovaries. It was determined that an additional 26 females had decreased fertility (all of these females were old or had many corpora albicantia) and had few follicles. They concluded that the reduction in fertility was not strictly age related; that the number of corpora (including corpora atretica) present in the ovaries was a more important factor. The same conclusion was reached for *S. attenuata* in the western Pacific (Kasuya *et al.* 1974) and for *Physeter macrocephalus* (Best 1967). For the most part, studies on ovarian structure in cetaceans rarely mention ovaries lacking follicles or with pathological changes that would result in senescence. For most cetacean species, senescence is rare and, when observed, often attributed to some pathological change.

A notable exception is *Globicephala macrorhynchus* (Short-finned pilot whale). Marsh and Kasuya (1984) reported 63% (31 of 49 females examined)

of females over 40 yr of age (5% of all mature females) had ovaries that lacked macroscopic follicles. In addition, there was a general decrease in follicle number, depleted oocyte populations, few recent corpora albicantia, and more atretic (degenerative) follicles present with increasing age of the female. Other changes observed in older females that were characteristic of ovarian aging observed in humans included thinning of the ovarian cortex, increased fibrosis, and thickening and sclerosis of blood vessels (Marsh and Kasuya 1984).

Another likely exception is *Orcinus orca*. Long-term observation of *O. orca* in the Pacific Northwest has allowed for monitoring of individuals as they age. Olesiuk *et al.* (1990) noted that a number of older females had not calved for over 10 yr. Although the calving interval for *O. orca* is long, 7.7 yr on average (Olesiuk *et al.* 1990), calving intervals rarely exceed 10 yr. Therefore, for females known to have reproduced sometime in the past, Olesiuk *et al*. (1990) suggested that females were senescent if they had not calved for at least a consecutive 10 yr period.

With the exception of *Globicephala melaena* and *Orcinus orca*, reproductive senescence is not considered a normal part of the life cycle of female cetaceans. Nevertheless, estimates of population growth rates must take into account the proportion of females reproductively senescent or growth potential will be overestimated. Additionally, an increase in the number of females with pathological changes that would result in cessation of successful reproduction may reflect increases in disease or contaminant effects.

14.4 EFFECTS OF DISEASE

Emerging and resurging diseases that affect reproduction can contribute to population instability and failure of recovery after exploitation. Infectious disease, neoplasia, and toxins are known to directly affect the reproductive tract, fetal viability, and reproductive success. Infectious diseases include those caused by bacterial, viral, protozoal, and fungal agents. For example, genital papillomavirus lesions may result in physical impedance of copulation, as well as result in vertical transmission of virus and congenital papillomatosis in stillborn calves (Bossart *et al*. 2002). Papilloma lesions in *Phocoena spinipinnis* (Burmeister's porpoise) and *Lagenorhynchus obscurus* (Dusky dolphin) off the central coast of Peru were documented to be severe enough to possibly impede copulation (Van Bressem *et al*. 1996). Genital papillomatous lesions have been reported in both males and females of various odontocete species, including *Orcinus orca, Physeter macrocephalus, Phocoena phocoena,* and *Tursiops truncatus*, suggesting an infectious etiology and venereal transmission (Landy 1980; Lambertsen *et al*. 1987; Bossart *et al*. 2002).

Similar to other mammals, herpesvirus has been shown to cause neonatal mortality in *Phoca vitulina* (Harbor seals) (Borst *et al*. 1986); however, whether this phocid herpesvirus causes abortions (as do other alpha-herpesviruses) is

still undetermined. Fatal disseminated cases of alphaherpesvirus have been reported in stranded, free-ranging *T. truncatus* (Blanchard *et al.* 2001), although there have been no reported cases of perinatal mortality associated with herpesviruses in cetaceans. Lipscomb *et al.* (2000) identified a gammaherpesvirus in association with metastatic carcinoma of genital origin in stranded free-ranging *Zalophus californianus* (California sea lions). This neoplasm has been previously reported to occur with a high prevalence in California (Gulland *et al*. 1996). The mass of the neoplasm itself can potentially obstruct the reproductive tract precluding copulation or compromising delivery. In cetaceans, recently characterized novel gammaherpesviruses have been detected in genital mucosal lesions of *T. truncatus*, *Kogia sima* (dwarf sperm whale), *Grampus griseus* (Risso's dolphin), and *Mesoplodon densirostris* (Blainville's beaked whale) (Smolarek Benson et al. 2006, Saliki et al. 2006).

Herpesvirus virions have been observed by transmission electronmicroscopy in papillomatous urogenital lesions of free-ranging Atlantic *Tursiops truncatus* (Bossart *et al*. 2005). Renner *et al*. (2004) showed by PCR and sequencing of amplicons that a captive *T. truncatus* with persistent penile lesions was transiently infected with a poxvirus and persistently infected with unique herpes and papilloma viruses. The authors speculated that one of the two latter viruses or their simultaneous action on the penile mucosal tissue may have been responsible for the clinical persistence of these lesions for over 3 yr.

Among the bacterial agents that are known to result in reproductive dysfunction is *Brucella* species. *Brucella* spp. have been isolated from several cetacean species and are associated with abortions and increased probability of reproductive disorders (Miller *et al*. 1999). Reproductive problems, particularly abortions in females and orchitis/epididymitis in males, are the primary manifestations of *Brucella* infection in terrestrial domesticated and wild mammals. *Brucella* has been isolated from the epididymis and uterus or associated with epididymitis and orchitis in *Phocoena phocoena* from Scottish coastal waters (Foster *et al*. 2002). The high level of *Brucella* seropositivity in many marine mammal species suggests that brucellosis could have a significant role in reproduction and population dynamics (Foster *et al*. 2002).

Reproductive failure characterized by abortion and premature parturition also has been noted in *Zalophus californianus* infected with caliciviruses; however, whether the virus was causative or in a synergistic relationship with endemic leptospirosis (*Leptospira interogans* serovar *pomona*) remains undetermined (Gilmartin *et al*. 1976; Gulland *et al*. 1996). Although calicivirus has been isolated from *Tursiops truncatus* and antibodies to marine caliciviruses have been documented in *Physeter macrocephalus, B. physalus*, *B. borealis* (Sei whale), *Balaena mysticetus* (Bowhead whale), and *Eschrichtius robustus* (Gray whale), the reproductive impact of this virus in cetaceans is unknown (Kennedy-Stoskopf 2001).

Various bacteria or fungi have been isolated from the uteri of pregnancy small odontocetes with suppurative endometritis (Robeck and Dalton 2002;

R. Y. E., unpublished data). Although these females died as the result of other causes, the likelihood of carrying and delivering a viable calf to term or caring for the neonate shortly thereafter would be questionable. Isolates observed include pure culture of the fungi *Fusarium* sp., *Saksenaea vasiformis*, and mixed cultures of *Enterobacter cloacae*, *Enterococcus* sp., and *Acinetobacter baumannii* (Robeck and Dalton 2002; R. Y. E., unpublished data).

Urolithiasis (*i.e.*, calculi in the urogenital tract) resulting in either penile urethral or vaginal obstruction can result in compromised reproductive success; however, the prevalence and significance of urolithiasis on reproduction in free-ranging marine mammal populations is unknown (Harms *et al*. 2004; McFee and Osborne 2004). Vaginal calculi have been reported in small cetaceans, including *Delphinus delphis, L. obliquidens, S. attenuata, L. obscurus*, and *T. truncatus* (Woodhouse and Rennie 1991; Van Bressem *et al*. 2000; McFee and Osborne 2004). This condition has been reported in sexually mature and immature females. The etiopathogenesis has been attributed to lower urinary tract infections caused by urease positive bacteria, calcified semen or mucus plugs, and/or sequella to fetal death including fetal maceration and incomplete abortion (Van Bressem *et al*. 2000). The presence of large vaginal calculi could certainly impair successful parturition.

Protozoal infections have previously been documented as potential threats to reproduction and calf survival. These infections have been shown to be transmitted by land-based pollution run-off of infectious and environmentally resistant oocysts that are shed in the feces of felids and transported via freshwater runoff into the marine ecosystem (Conrad *et al*. 2005). Inskeep *et al*. (1990) first reported a case of disseminated *Toxoplasma gondii* infection in a mother and calf *T. truncatus*. Subsequently, *Toxoplasma* has been shown to be transmitted to the fetus transplacentally in a *Grampus griseus* and congenital toxoplasmosis has been documented in a calf born to an infected captive *Tursiops aduncus* (Indo-Pacific bottlenose dolphin) (Jardine and Dubey 2002; Resendes *et al*. 2002). It also has been shown that wild populations of dolphins in Sarasota Bay, FL, can be up to 100% seropositive for *Toxoplasma gondii* (Dubey *et al*. 2003). Given the increased human population and potential for pollution, the impact of protozoal infections therefore may be high. In addition, immunosuppression and other conditions of physiologic stress, including pregnancy and lactation, can stimulate recrudescence of latent protozoal infection (Dubey *et al*. 2003).

Neoplasia, although relatively rare amongst cetaceans, has been reported in the genital and reproductive tracts of various captive and free-ranging cetaceans. Fibro- and leiomyomas are not uncommon benign neoplasms found in older female cetaceans including *Globicephala melaena* (Long-finned pilot whales) and *G. macrorhynchus*, *Tursiops truncatus*, *Steno bredanensis* (Rough-toothed dolphin), *Balaenoptera musculus* (Blue whale), *Physeter catodon*, and *Pseudorca crassidens* (False killer whale) (Cowan 1966; Landy 1980; Bossart *et al*. 2002; R. Y. E., unpublished data). Granulosa cell tumors of the ovary have

been reported in *B. musculus* and *B. physalus* in addition to other ovarian cystadenoma and carcinoma (Landy 1980). Uterine adenocarcinoma also has been reported in a *T. truncatus* from Argentina (Sanchez *et al*. 2002) and has been observed in a stranded *S. bredanensis* (R. Y. E., unpublished data). Other genital diseases reported in odontocetes have consisted of various ovarian cysts (*i.e.*, Graffian follicle cyst and luteinized cyst), ovarian dysgerminoma, and uterine tumors, as well as inflammatory changes in *Lagenorhynchus obscurus* from Peru (Van Bressem *et al*. 2000). Genital neoplasia in male odontocetes is a relatively uncommon occurrence not frequently reported. Tumors described in male odontocetes include a Leydig cell tumor in a *D. delphis*, metastatic seminoma in a *T. truncatus*, and both a Sertoli cell tumor and metastatic seminoma in a *Stenella frontalis* (Atlantic spotted dolphin) (Cowan *et al*. 1986; Estep *et al*. 2005).

In addition to primary neoplasia, there are increasing reports of neoplasms in connection with viral and toxin agents. For example, the incidence of neoplasia has been increasing, especially in environments shown to have high chemical pollutant (*e.g.*, organochlorine) levels. *Delphinapterus leucas* (Beluga) from the St. Lawrence estuary in Canada have been extensively studied for many years in regards to general population health and estuarine contaminant levels. Martineau *et al*. (2002) described a greater incidence of tumors in these animals, including several mammary and uterine adenocarcinomas. Given the increasing prevalence of contaminated waters and the documented potential for many of these contaminants to increase the risk of neoplasia, a conservation concern is that the incidence of neoplastic disease may increase and affect population growth in many cetacean species.

A metabolic cause of reproductive failure in captive *Tursiops truncatus* attributed to congenital diffuse hyperplastic goiter is likewise suspected in free-ranging *T. truncatus* (Garner *et al*. 2002). Congenital diffuse hyperplastic goiter can be associated with nutritional disorder (*e.g.*, decreased maternal dietary iodine levels), chemical disruption of thyroid hormone synthesis and secretion, goitorgenic compound exposure (*e.g.*, xenobiotics), or the result of a heritable disorder (Capen 1993). Xenobiotics, like chlorinated hydrocarbons (*e.g.*, DDT) and polyhalogenated biphenyls (*e.g.*, PCBs), are known to exert direct affects on the biosynthesis, secretion, and/or metabolism of thyroid hormones.

In domestic species, hypothyroidism in reproducing animals can result in reproductive complications, including decreased libido, reduced sperm count, abnormal estrus cycles, reduced conception rates, retained fetal membranes, significantly prolonged gestation with subsequent larger fetus size, and possible dystocia due to the enlarged thyroid gland. Additionally, various domestic species demonstrate low neonate survivability with congenital goiter (Capen 1993, 1997).

During the Mediterranean morbillivirus epizootic of 1990-1992, luteinized ovarian cysts were observed on several morbillivirus infected *Stenella coeruleoalba* which also had high levels of PCBs (Munson *et al*. 1998). Munson

et al. (1998) propose that either the morbillivirus infection, the PCBs effect on the hypothalamic-pituitary axis, or the PCBs' effect on ovarian response may have impeded ovulation resulting in ovarian cyst formation and that these cysts may impede population recovery. The findings of Munson *et al.* (1998) regarding multiple etiologies for reproductive dysfunction (*e.g.*, ovarian cysts) and population dynamics are intriguing and could potentially be the basis for reproductive decline in other populations.

In summary, diseases that affect reproduction in cetaceans are varied with numerous etiologies. Many of the infectious agents can maintain latent infections which can be stimulated to recrudesce in times of immunosuppression and physiologic stress. In an increasingly polluted ocean environment with potentially toxic chemicals and land-based pathogens, physiologic stress and emerging diseases can provoke underlying immune compromise and, therefore, increased susceptibility to physical and biological threats. There are complex interactions among contaminants, biotoxins, climate, and pathogens that, coupled with decreased populations as a result of exploitation, can affect reproduction and population recovery.

14.5 EFFECTS OF CONTAMINANTS AND TOXINS

The potential hazards and risks posed by chemical pollutants and biotoxins add an entirely new level of complexity to the conservation of cetacean populations as chemical and biologic contaminants in the waters are shown to have significant negative impacts on male and female reproduction, fertility, or fecundity (see for example Schwacke *et al*. 2002). Experimentally, the marine biotoxin, domoic acid, produced by the diatom *Pseudonitzschia,* has been found to be fetotoxic in rats, causing hippocampal damage, lasting subtle neurobehavioral impairment, cognitive deficits, and altered locomotor activity in rats exposed *in utero* to low doses that were not found to be clinically significant in adult animals (Dakshinamurti *et al*. 1993; Levin *et al*. 2005). During recent *Pseudonitzschia* blooms along southern California in 1998 and 2000, increased mortality and morbidity was observed in various species, especially *Zalophus californianus* (Gulland *et al*. 2002). In addition to some clinical signs of intoxication (*i.e.,* seizures, ataxia, and head weaving) that were observed in males and females, the veterinarians rehabilitating the animals observed that pregnancy animals were more intractable to palliative therapy and subsequently improved only after the pregnancy was terminated. The majority of pregnancies generally ended in reproductive failure that was characterized by cases of late term abortion, still births, and premature parturition (Gulland *et al*. 2002). Although cetaceans are exposed to *Pseudonitzschia* blooms, similar reproductive problems have not been documented.

Early studies in rodents on the teratoxicity of the red tide toxin (brevetoxin, *Karenia brevis*) also show toxin transport across the placenta, into milk of lactating females, and into fetal tissues by 30 minutes after dosing (J. Z. *et al.*,

unpublished data). In the Gulf of Mexico, *Tursiops truncatus* and *Trichechus manatus* (Manatee) are frequently exposed to brevetoxin. One effect is mortality (Bossart *et al.* 1998). Sublethal effects on adult females or fetal effects have not been documented, but might be anticipated.

In addition to data on terrestrial vertebrates, there are several documented examples of adverse reproductive effects and diminished fetal viability induced by chemicals and xenobiotics including, *e.g.*, decreased survivorship, decreased fecundity, implantation failure, and sterility in many marine mammals. Organochlorine levels were shown to be 2-8 times higher in tissues of *Zalophus californianus* that had premature births than in females with full term births (Delong *et al*. 1973). Reijnders (1986) documented polycholorinated biphenyls (PCBs) as the cause of decreased reproductive rate in *Phoca vitulina* (Harbor seal) In this experimental study, seals were fed diets differing in amounts of PCBs by selecting naturally occurring prey from different locations throughout the range of the seals. Those animals fed the diet with the highest level of pollutants had significantly decreased reproductive success that was apparently due to post-ovulation and/or implantation disruption. No comparable studies have been conducted on cetaceans.

Delphinapterus leucas in the St. Lawrence estuary have tissue levels of contaminants known to induce severe reproductive dysfunction in other animals at similar or lower levels (Martineau *et al*. 1987). Specifically, levels of contaminants in adipose tissue in *D. leucas* were up to five times greater than seen in sea lions or seals that had documented reproductive failures (*e.g.*, abortions). Thus, there is a possible link between contaminants and low recruitment possibly due to hormonal interference during pregnancy (Martineau *et al*. 1987).

A number of studies have documented detrimental effects of high contaminant loads of primiparous females on their offspring. First-born calves are the initial recipients of lipophilic contaminants that have accumulated in the female and are transferred during lactation. Higher levels of organochlorine compounds in first offspring relative to subsequent offspring have been documented, *e.g.*, in *Orcinus orca* from British Columbia, Canada (Ross *et al*. 2000), *O. orca* from Prince William Sound, Alaska (Ylitalo *et al.* 2001), and a *Balaenoptera physalus* (Aguilar and Borrell 1994). Similarly in captive *Tursiops truncatus*, Ridgway and Reddy (1995) found a higher organochlorine burden in milk from younger females, *i.e.*, those with fewer prior offspring.

Complementary studies have shown that first offspring survivorship is lower than that of subsequent offspring, *e.g.*, in *Tursiops truncatus* (*e.g.*, Schwacke *et al.* 2002) and *Globicephala malaena* (Borell *et al.* 1995). In *T. truncatus* from South Africa, Cockcroft *et al.* (1989) found that primiparous females transfer most of their contaminant burden within seven weeks of lactation. In captive *T. truncatus*, Reddy *et al.* (2001) report on the effects of maternal organochlorine exposure on pregnancy outcome. They found that the mean concentration of ∑DDT was more than three times as high among dolphins whose calves died as among dolphins whose calves survived

beyond 6 mo. The mean ∑PCB was more than 2.5 times higher in females whose calves did not survive. It remains to be determined whether differences in calf survival are the result of differences in maternal care, level of experience between primiparous and multiparous females, maternal contaminant levels, or a combination of these factors. Reddy *et al.* (2001) propose that this captive population could facilitate future studies to assess reproductive and transgenerational effects of contaminants and potential biomarker development.

What is the relevance of these observations to the conservation of cetacean populations? Schwacke *et al.* (2002) conducted an assessment of the risk of detrimental reproductive effects due to high PCB levels in *Tursiops truncatus* from three sites along the Atlantic and Gulf of Mexico coasts of the U.S. They found that the PCB-related risk of stillbirths or neonatal mortality from primiparous females ranged from 60-79% among the three sites. Females that had calved previously had off-loaded most of their PCBs, so the risk of reproductive failure for subsequent calves ranged from 2-10%. They noted that loss of the first-born calf effectively increases the age at first birth by delaying the first viable calf, thereby reducing population growth rates and the ability of a population to recover from disease outbreaks or other causes of population decline.

By analogy, other populations of cetaceans would be predicted to suffer effects from high contaminant loads. Ross *et al.* (2000) found high PCB concentrations in *Orcinus orca* from the waters of British Columbia, Canada. They indicate that these whales can be considered among the most contaminated cetaceans in the world and, therefore, suggest that these animals are at risk for toxic effects.

In addition to the direct toxic effects of these agents, there are increasing data across all taxa for sub-clinical and sub-lethal effects on reproductive parameters. These effects include gamete fertility, reproductive failures (*e.g.*, abortions), and increased susceptibility to infection that could compromise not only adult survival but also uterine and placental health, and consequently fetal health and survival. The mechanisms of actions and types of effects can vary but data exist to clearly suggest the negative impact of sub-clinical exposure to contaminants especially those mimicking hormones (*e.g.*, xenoestrogens) (Kavlock *et al*. 1996). For example, exposure to an endocrine disrupter during a sensitive stage in development or differentiation may result in non-reversible and usually latent sexual dysfunction (altered sexual behavior) or physical abnormalities (feminization and male infertility) (Kavlock *et al*. 1996). Given the relative phylogentic conservation of hormones and hormone receptor binding, studies in other species and taxa may be relevant in predicting potential outcomes in marine mammals exposed to many chemical pollutants. Translating subtle effects on individuals into specific population-level effects is a challenge and requires more field and experimental data.

14.6 DISCUSSION AND RECOMMENDATIONS

Across the many species of cetaceans, there is a paucity of reproductive data that evaluates the combined effects of exploitation, density-dependence, and contaminants and biotoxins. Reproductive data and information can be obtained in a multitude of ways. In addition to data from free ranging populations (via temporary capture/release studies or long-term monitoring studies of known individuals), data may be acquired from necropsy of carcasses and histologic study of tissues. Gross and histologic analyses of carcasses obtained from areas of direct exploitation and fisheries' activities may be examined to document sex, age, body length, and reproductive status (Lockyer and Smellie 1985). For example, histology of the ovaries, uterus, and mammary gland can be used to assess sexual maturity, pregnancy status, and lactation, as well as ovulation rate and potential reproductive rate (Lockyer and Brown 1979; Lockyer and Smellie 1985). Collectively over time, these data may reveal subtle changes in reproductive or fertility patterns which may reflect acute or chronic responses to overexploitation, habitat degradation, and/or recovery. Further, it is valuable to examine populations of nonthreatened species (particularly from tenuous coastal areas) because these data contribute comparative data and provide a large and valuable database to use if future activities threaten these populations or species. Sophisticated molecular techniques (for biotoxin analysis and genetic analysis) are now available to help evaluate the effects of stressors on reproduction and fertility. New technology has resulted in the need for less invasive sampling methods from live populations, often because small samples are required. It is beyond the scope of this chapter to discuss these in detail; however, these techniques are increasingly available and affordable. Furthermore, these techniques have the potential to greatly increase our knowledge base as they can be applied to future tissue/sample collections as well as archived specimens.

Can we predict relative risk to certain populations? Data from other disciplines (*e.g.*, toxicologic pathology) can aid in clarifying reproductive effects of certain chemicals or toxins. These data, in combination with population surveys and other environmental and oceanographic information can be used to model the minimal sustainable population sizes and critical time frames. Much uncertainty still remains in these models because extreme climatic changes and weather events are only two of the many factors that can overshadow any well devised model. For example, the emergence and resurgence of infectious diseases are critically important issues that can drastically affect risk assessment and conservation efforts (CIESM 2004). Despite the inherent uncertainty, marine mammal scientists, biologists, and veterinarians have an increasingly large pool of information from which to draw to help make better management and conservation decisions and assessments for the conservation of cetaceans.

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CHAPTER 15

Population Genetics of Marine Mammals

Greg O'Corry-Crowe

15.1 INTRODUCTION

Population genetic investigations of marine mammals date back several decades. The earliest studies examined patterns of phenotypic variation in blood proteins and enzymes to estimate the level of gene flow among spatially discrete groupings of animals (e.g., Shaughnessy 1969; McClenaghan and O'Shea 1988; Gales *et al*. 1989; Daníelsdóttir *et al*. 1992), to assess the genetic consequences of population bottlenecks and founder events (Bonnell and Selander 1974), and to test theories about the relationship between life history strategies and genetic diversity (Allendorf *et al*. 1979). These studies launched a new field of inquiry into the evolution, ecology and behavior of marine mammals that quickly developed from surveys of phenotypic variation in gene products to assessments of variation within the genetic material, the DNA, itself. Over the next 30 years, genetic investigation of marine mammal populations was revolutionized by the advent of cloning technology and the development of the Polymerase Chain Reaction, by remote biopsy methods of sample collection and more efficient methods of sample preservation, and by the development of new approaches to analyzing and interpreting molecular genetic data.

This chapter reviews population genetic studies on marine mammals, with particular emphasis on the molecular genetic analysis of selectively neutral markers. The chapter begins with a brief history of population genetics as a scientific discipline, and a summary of the evolutionary forces that determine patterns of variation within genetic loci. The following sections review population genetic investigation in marine mammals, and center on four main subjects: (1) genetic relatedness among individuals, (2) gene flow and dispersal on contemporary timescales (3) patterns of genetic diversity within populations over time, and (4) gene flow and dispersal over evolutionary timescales. I conclude with a brief summary of current limitations and future

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challenges. Space limitations prevented a review of a number of aspects of marine mammal population genetic studies, including a summary of genetic markers and molecular methods, and an assessment of the role of population genetic study in marine mammal conservation and management. Fortunately, these subjects have recently been dealt with at length elsewhere and the reader is directed to the following volumes and relevant chapters therein (Dizon *et al.* 1997; Hoelzel 2002; Perrin *et al.* 2002).

15.1.1 Population Genetics: Principles and Definitions

Population genetics is a long established field that traces its origins to Darwin's (1859) theory of evolution by natural selection, and Mendel's (1866) elegant breeding experiments on the garden pea that demonstrated the predictable patterns of inheritance of dominant and recessive traits, and to the modern evolutionary synthesis and mathematical models of Wright, Haldane, Fisher and others in the early 20th century that bridged these two traditions. Population genetics is the study of inheritance and the patterns of genetic variation within and between populations, and of the evolutionary forces that determine these patterns: mutation, genetic drift, gene flow, and selection (Wright 1931; Nei 1987; Maynard Smith 1989; Hartl and Clark 1990). A thorough understanding of how these forces interact to shape patterns of genetic variation provides insight into the mechanisms of evolution, and enables inference on the demographic histories of natural populations and the behavior of individuals within and among these populations over time. Thus, before I proceed to an assessment of marine mammal population genetic investigation, I feel it is important to review them briefly here. Box 1 provides a simple schematic representation of how these forces might shape genetic patterns within and between two populations.

Box 1 Heredity and the evolutionary forces that shape genetic differentiation within and among populations**.**

The schematic representation in Fig. 15.1 demonstrates heredity and three major influences on variation in a genetic marker within two populations over time: mutation, genetic drift, and natural selection. For clarity, gene flow, perhaps the easiest to conceptualize, is not included. A haploid marker (*e.g.*, mtDNA) is used for simplicity and both populations pass through six phases in their history prior to being studied.

Phase 1, the point of population divergence, the pattern of genetic variation is similar for both populations. Here, both possess the same variant or haplotype, Hap A, a probable scenario if the ancestral population or the founding group was small.

In Phases 2 and 3 both populations experience a period of growth, as, for example, in species (re)colonizing new habitat following an ice age, and the genetic variant is passed from one generation to the next through reproduction. In Population 1, a mutation occurs in one individual, giving rise to a new haplotype, Hap B. Likewise, a mutation also occurs in an individual in Population 2, resulting in a second unique haplotype, Hap C. Through variation in reproductive success and survival among individuals within populations both new haplotypes become established by chance in their populations of origin.

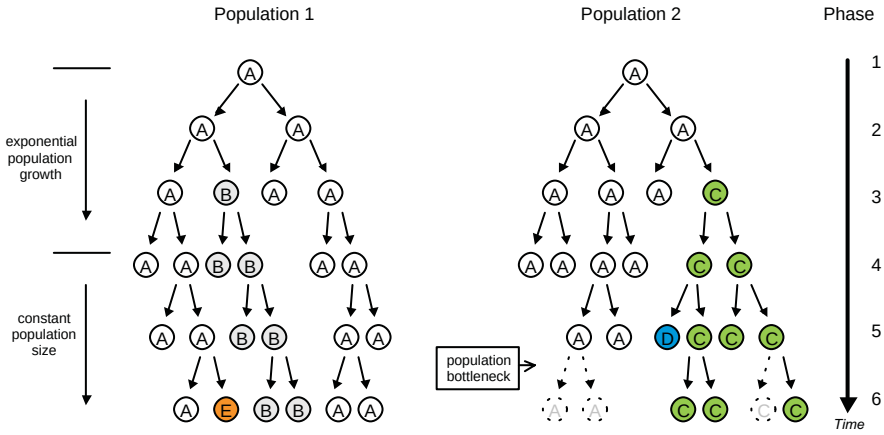


Fig. 15.1 Heredity and the mechanisms that influence genetic variation within populations. Circles denote individuals, letters denote unique haplotypes, and reproductive success is denoted by arrows from parents to offspring. Individuals lost due to a population bottleneck are indicated by dotted circles. Original.

By Phase 4, the genetic composition of Populations 1 and 2 differ from their ancestral state and from each other substantially, due to the combined effects of genetic drift and mutation. As time goes by these two isolated populations continue to diverge (Phase 5), Hap C becomes the dominant haplotype in Population 2 and gives rise to a new variant, Hap D. However, this haplotype drifts to extinction by chance. The emergence of Hap C in Population 2 may simply be the cumulative result of random lineage sorting over many generations. Alternatively, if we view our schematic as a model for a locus under some selective pressure, the relatively rapid rise of Hap C may indicate that this variant confers some selective advantage to those individuals who possess it. Similarly, the rapid loss of Hap D may reflect a deleterious mutation that compromised the fitness of its host.

In Phase 6, Population 1 has reached equilibrium between the diversifying process of mutation and homogenizing effects of drift. By contrast, Population 2 has just witnessed a severe population bottleneck that resulted in the loss of much genetic variation. By chance, the most common haplotype pre-bottleneck, Hap C, is the only one that survived. Under our alternative model of a marker under selection, Hap C may have conferred a selective advantage on its hosts that increased their probability of surviving the bottleneck.

It is easy to see what would happen if gene flow was included in this scheme. If it occurred early on in the history of the two populations, say during Phase 2, it may have little discernible effect. If it occurred later, the rate of genetic divergence through drift and mutation would be slowed, to what extent depends on the level of genetic exchange.

It is at this point that we have decided to study these two populations, and observe that they differ in population size, they share no haplotypes in common and the diversity is higher in Population 1 than in Population 2. The challenge for the population geneticist is to establish the contemporary relationship among these populations and to reconstruct their respective histories from these data.

Mutation is the process by which new variation is produced. It occurs predominantly through errors in the replication of the genome during meiosis. Rates of mutation differ among regions of the genome and are influenced by the primary sequence of the genetic code itself, generation time, and by intrinsic and extrinsic mutagens. The establishment of a new mutant allele within a population depends in large part on the reproductive success of the carrier relative to others within the population (genetic drift), the breeding and dispersal behavior of the carrier (gene flow) and whether the site is under selection or not. Mutations are usually rare events that typically give rise to a new variant or allele. An understanding of the mode and rate of mutation at a particular genetic marker facilitates the reconstruction of phylogenetic relationships among alleles and the timing of lineage divergences, thus providing unique insights into the evolutionary history of taxa (see below).

Gene flow is the exchange of variants among groups of organisms via dispersal and interbreeding, such that close relatives share more alleles by descent than unrelated animals, and populations experiencing high levels of gene flow have fewer differences in genetic composition than do isolated populations. Genetic exchange is the primary force limiting differentiation among populations (Slatkin 1987) and its influence on the genetic landscape depends on who successfully disperses, when and how. Sex-biased versus non-biased dispersal, the emigration of close kin versus random dispersal, periodic versus continuous dispersal, all leave distinctive signatures in patterns of variation at genetic markers. Understanding gene flow can thus provide insight into mating systems, dispersal, social structure and population subdivision.

Genetic drift is the loss of variation over time due primarily to differences in survival and reproductive success among individuals within a population. In the absence of the introduction of new variants via gene flow or mutation, discrete populations will ultimately drift to fixation for alternative alleles such that among-population heterogeneity is maximized and within-population heterozygosity is minimized. The rate of drift is determined by the effective population size, N_e, and the generation time (Frankham 1995). In a random mating population with equal sex ratio, where all mature individuals have an equal likelihood of breeding successfully, $N_e \approx N$, the number of mature individuals. In species with kin-based social groupings and in species with highly skewed reproductive success, as for example in polygynous species, N_e can be much smaller than N and genetic drift can be a potent force in shaping patterns of genetic variation. Changes in N_e over time can have lasting effects on the pattern of genetic diversity within populations. Substantial reductions in abundance and population bottlenecks can result in severe losses of genetic diversity through drift (Nei *et al*. 1975; O'Brien *et al*. 1987), with potentially dramatic consequences for population viability. Because of the slow rate of mutation, these effects can be detectable long after the event. Thus, levels of genetic diversity can reveal much about mating systems and the evolutionary history of populations.

The final factor influencing patterns of genetic variation is selection. Natural selection is the underpinning of Darwin's theory on evolution and is a deterministic relationship between how freely a genetic locus is allowed to vary and how essential its function is to an individual's fitness. The direction and extent of selective pressure is often difficult to quantify, and may change with changing environmental conditions, such that loci under weak or no selection are often preferred in population genetic studies of behavior, demography and population history. The rapid loss of diversity at genetic loci through drift limits the variation upon which selection can act, and thus may compromise the evolutionary potential of a population.

15.2 POPULATION GENETIC STUDIES OF MARINE MAMMALS

Direct assessment of the mechanisms of evolution is particularly challenging in long-lived, relatively inaccessible species such as marine mammals. While some studies have assessed patterns of variation in markers under selection in wild populations of marine mammals (e.g., Slade 1992; Murray and White 1998; Hoelzel *et al.* 1999a), the investigation of the direction and extent of selection acting on a genetic locus or suite of loci requires detailed pedigrees and life histories, as well as a sound understanding of the link between phenotype and genotype, a tall order as yet for most marine mammal species. Conversely, the past few decades have seen a dramatic increase in the collection of samples from several species that have facilitated surveys of variation within selectively neutral markers that reflect the accumulated effects of mutation, genetic drift and gene flow across time. These investigations span the gamut of population genetic study and have provided unique perspectives on the evolution, ecology and behavior of marine mammal populations. The following two sections elaborate on four areas of enquiry.

15.2.1 Population Subdivision and Gene Flow in Marine Mammals

Two of the most fundamental questions in population biology are: what defines a population or smaller grouping of individuals, and how do these groupings relate to one another (Mayr 1970)? The key to answering these questions is an understanding of the level and form of dispersal and interbreeding within and between them (Shields 1987). While population dynamics models are typically developed for closed populations (Turchin 2003) more realistic models must take dispersal, termed migration in genetic parlance, into account. Further, management and conservation are often concerned with resolving the demographic and reproductive connectedness among groups of organisms. Something so fundamental as dispersal, however, is difficult to estimate directly in natural populations, especially in marine mammals. This is where population genetic analysis comes in.

The theoretical relationships between the forces shaping patterns of genetic diversity within and between populations were established by Wright (1931, 1943, 1951), Kimura and Weiss (1964) and others (see Box 2), and subsequently confirmed through captive breeding and simulation studies (e.g., Slatkin and Barton 1989). This enabled the indirect estimation of various population parameters, including the rate of dispersal (m) and the number of dispersers per generation ($N_e m$) among populations, from genetic data. Alternatively, a statistical approach can be used to assess dispersal and genetic exchange. For example, allele frequencies can be used to test a specific hypothesis, eg. random mating (diploid markers) or mixing (haploid markers) among groups of animals, where the resultant estimate of statistical significance of the measure of genetic differentiation (e.g., F_{st}, χ^2) tells you something about the degree of population subdivision. A third approach to resolving patterns of dispersal and gene flow is to assign individuals to populations of origin based on the likelihood of their genotype or haplotype occurring in each sampled population, high levels of 'missassignment' indicating extensive mixing (e.g., Paetkau *et al.* 1995).

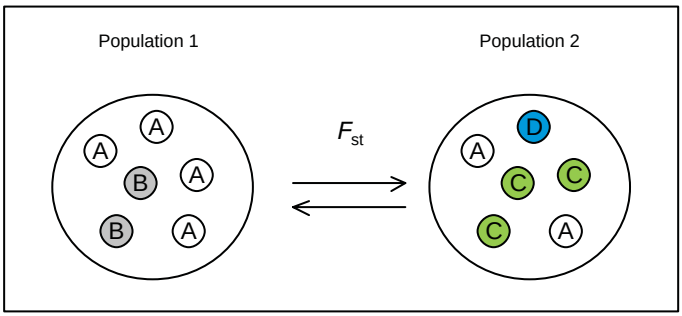
15.2.1.1 Kinship, mating systems and social organization

To fully understand dispersal and gene flow among natural populations, and to calculate meaningful estimates of N_e within populations, knowledge of mating systems and social organization within populations is required (Shields 1987; Frankham 1995; Storz 1999). For example, mating systems where reproductive success can vary widely among individuals will reduce N_e, while kin-based societies can also affect N_e as well as violate random mating expectations assumed in most genetic methods of dispersal estimation. Such information, however, is difficult to obtain through direct observation in most marine mammals, particularly cetaceans and aquatic mating pinnipeds (Amos *et al*. 1993; Coltman *et al*. 1998). Fortunately, genetic markers can be used to resolve pedigree relationships thereby providing detailed information on parentage and kinship which, in turn, can be used in studies of mating systems, social structure, sexual selection and kin selection. By using several diploid markers which are polymorphic enough such that closely related individuals have a high likelihood of possessing different alleles, pedigree relationships can be estimated based on the allelic frequencies at these loci within the population (Quellar *et al*. 1993; Goodnight and Queller 1999).

Genetic profiling revealed that *Globicephala melas* (Long finned pilot whales) form stable, kin-based social groups or pods, where both females and males remain within their natal pod but mate with unrelated whales from other pods, most likely when different pods temporarily consort with each other (Amos *et al*. 1993). A similar genetic analysis of cow-calf pairs revealed a promiscuous mating system in female *Megaptera novaeangliae* (Humpback whales), which is consistent with field observations of females associating with several males (Clapham and Palsbøll 1997).

Box 2 Population structure and gene flow.

Observed differences in allele frequencies can be used to estimate average levels of gene flow between natural populations. Figure 15.2 represents two populations at equilibrium where differences in the frequencies of alleles at a selectively neutral haploid marker (e.g., mtDNA control region, see Box 1) reflect the relative strengths of gene flow and genetic drift.



Wrights island model

$$F_{st} = \frac{1}{2N_em + 1}$$

Fig. 15.2 Gene flow and genetic drift among populations in equilibrium. Circles denote individuals, letters denote unique haplotypes. Original.

Wright's island model of population structure uses the extent of genetic differentiation (F_{st}) to indirectly estimate gene flow in terms of the average number of dispersers per generation (N_em). F_{st} ranges from 0, when the two locations are part of a single random mixing population, to 1, when the two locations represent two populations that are fixed for alternate alleles.

Here, Wright's model is modified slightly for a haploid marker.

In pinnipeds, genetic analyses are complementing more traditional field studies of mating behavior and association patterns on breeding colonies. In the highly polygynous *Mirounga angustirostris* (Northern elephant seal), DNA fingerprinting and microsatellite analysis found discrepancies between observed mating success and actual reproductive success in some dominant males (Hoelzel *et al*. 1999b). In the aquatically mating *Phoca vitulina* (harbor seal), microsatellite analysis revealed low variance in male reproductive success (Coltman *et al*. 1998) while a multilocus DNA fingerprinting study found high levels of breeding-colony site fidelity in females but no evidence of kin selection in the evolution of fostering behavior by females (Schaeef *et al*. 1999). In *Halichoerus grypus* (Gray seal), molecular genetic studies documented

polygyny and inter-year mate fidelity simultaneously occurring within the same breeding colony (Amos *et al*. 1995). A subsequent study, confirmed that male-reproductive success is highly skewed but also found that, a high proportion of pups were not fathered by known males, suggesting that aquatic mating involving males that seldom haul out on shore occurs more frequently than previously thought (Worthington Wilmer *et al*. 1999).

Conclusion. These types of studies of relatedness and parentage are revealing that random mating is probably atypical in marine mammals, that N_e is less than the census population size, that there is a strong tendency to remain in your group of birth or return to your site of birth, and that marine mammals employ a variety of strategies to maximize reproductive success and avoid consanguineous matings.

15.2.1.2 Gene flow and dispersal on contemporary timescales

The vast majority of population genetic studies on marine mammals to date have been concerned with elucidating patterns of population subdivision, dispersal and gene flow. In this section genetic exchange on ecological time scales are discussed. Differentiation on evolutionary timescales is dealt with in Section 15.2.2.2 (see below).

The first studies of population genetic structure in cetaceans screened for variation at enzyme loci and detected restricted gene flow across ocean basins in several baleen whales (e.g., Daníelsdóttir *et al*. 1991, 1992; Wada and Numachi 1991) as well as subdivision on smaller spatial scales in smaller species (Anderson 1993). Though informative, many of these electrophoretic studies were limited by sample size and distribution, and by the redundancy of the genetic code.

Subsequent studies of mtDNA variation demonstrated population subdivision in several whale, dolphin and porpoise species (e.g., Pastene *et al*. 1993; Secchi *et al*. 1998; Escorza-Treviño and Dizon 2000; Yoshida *et al*. 2001), and found strong female-directed philopatry to geographically discrete feeding and breeding areas in a number of highly migratory species including *Delphinapterus leucas* (beluga whale), *M. novaeangliae* and *Eubalaena glacialis* (right whale) (Baker *et al*. 1993; Schaeff *et al*. 1993; Palsbøll *et al*. 1995; Brown-Gladden *et al*. 1997; O'Corry-Crowe *et al*. 1997, 2002; Baker and Medrano-González 2002). This tendency to return to the same locations generation after generation is presumably mediated by the cultural transmission of migration destinations from mother to offspring facilitated by the relatively long period of maternal care in these species, and is likely driven by the predictable availability of seasonal resources with the result that these groupings eventually become demographically discrete populations.

Recent investigations have returned to examining bi-parentally inherited markers, this time screening for variation within the DNA itself as opposed to within gene products. Significant heterogeneity in nuclear DNA variation, indicating restricted gene flow, has been documented at global, regional and local scales in several cetacean species (e.g., van Pijlen *et al*. 1995; Andersen

et al. 1997; Chivers *et al*. 2002; LeDuc *et al*. 2005). A number of studies found levels of differentiation within microsatellite markers that were much lower than levels recorded within mtDNA (Larsen *et al*. 1996; Brown-Gladden *et al*. 1999; deMarsh and Postma 2003; G. O'Corry-Crowe, unpublished data). These differing patterns suggest more extensive male-mediated gene flow, which may occur on common breeding areas or via male-biased dispersal, a characteristic of many mammalian species (Greenwood 1980; Melnick and Hoelzer 1992). Mixing on common breeding grounds has been confirmed by genetic mark-recapture studies in the case of *M. novaeangliae* (Palsbøll *et al*. 1997). Caution, however, is required when interpreting these differing levels of differentiation in other species as lower heterogeneity in nuclear markers may also be the result of limited divergence through genetic drift because of the larger effective population size (N_e) of nuclear compared to haploid markers.

Population differentiation has also been documented at a number of spatial scales in pinnipeds. Heterogeneity has been found in nuclear and mtDNA markers in *P. vitulina*, indicating limited dispersal and interbreeding among regional populations as well as among subspecies (Lamont *et al*. 1996; Stanley *et al*. 1996; Goodman 1998; Burg *et al*. 1999; Westlake and O'Corry-Crowe 2002). Genetic studies have also revealed substantial population subdivision in *Eumetopias jubatus* (Steller sea lions) (Bickham *et al*. 1996; Hoffman *et al*. 2006; O'Corry-Crowe *et al*. 2006) and *Mirounga leonina* (Southern elephant seal) (Gales *et al*. 1989), and have determined that dense polar pack ice and behavioral philopatry are strong forces promoting population subdivision in *Odobenus rosmarus* (Walrus) (Cronin *et al*. 1994; Andersen *et al*. 1998; Andersen and Born 2000). Conversely, limited subdivision has been detected in a number of ice breeding seals, including *Phoca largha* (Spotted seal) (O'Corry-Crowe and Westlake 1997; Mizuno *et al*. 2003; G. O'Corry-Crowe, unpublished findings), *Phoca hispida* (Ringed seal) (Davis 2004; Palo *et al*. 2001) and *Pagophilus groenlandicus* (Harp seal) (Perry *et al*. 2000). Gene flow among geographically discrete breeding concentrations in these species is likely facilitated by the seasonal movements of sea-ice. The ability to haul out on a mobile substrate can result in passive movements over long distances.

Subdivision has also been documented in *Trichechus manatus* (Manatee) (McClenaghan and O'Shea 1988; Garcia-Rodriguez *et al*. 1998), *Enhydra lutris* (Sea otter) (Cronin *et al*. 1996) and *Ursus maritimus* (Polar bear) (Patkeau *et al*. 1995, 1999). Despite the extensive movements individual polar bears make across sea ice (Mauritzen *et al*. 2002), microsatellite analysis revealed restricted gene flow among several local populations of this species, particularly in the Canadian high Arctic archipelago, where the insular geography may restrict dispersal (Paetkau *et al*. 1995, 1999).

Conclusion. Genetic studies are revealing that dispersal, breeding behavior and population subdivision in marine mammals are influenced by a variety of factors, including the physical environment, life history and behavior. Global-

scale patterns tend to be shaped by the juxtaposition of continental land masses and the world's oceans, by physical oceanography, including sea ice, water temperature and ocean currents, and by biological oceanography and prey distribution. Life history sets the requirements for survival which results in non-uniform distributions and population structure in heterogeneous environments. The need to haul out on remote islands close to a patchy food source, for example, greatly restricts the distribution of breeding colonies of several pinnipeds. It is the influence of individual behavior, however, that has been the most difficult to elucidate by traditional methods and where genetics is providing the greatest insight. Despite their capacity for long-distance movements, and the paucity of obvious physical barriers to movement within ocean basins, genetic studies are revealing high levels of population subdivision in many species of marine mammal. This often reflects behavioral philopatry to natal site or home range and limited interbreeding among geographically distinct groups.

Genetic investigations are thus not only documenting patterns of population subdivision in marine mammals but are also providing unique perspectives on the factors that shape this subdivision. For these reasons, population genetics has found wide application in the identification of units of management and conservation in marine mammals, and provides insights into population biology that inform managers on how best to attain their management goals.

15.2.2 Reconstructing the Past

Up to now, population genetic studies of marine mammals have typically involved sampling individuals at a number of locations at a single point in time. The observed patterns of genetic variation, however, reflect the recorded history of populations. As in human oral histories, new events are occurring all the time (mutation), some episodes have been lost (drift) but much is still there to be retold (inheritance). Through the predictable process of inheritance, and the somewhat predictable processes of mutation and drift, events in the history of populations, such as bottlenecks and divergences, colonizations and range expansions, can leave distinctive signatures in the patterns of variation within genetic markers that can be detected long afterwards. Thus, the analysis of variation within genetic markers enable us to reconstruct the evolutionary and demographic history of groups of organisms including the reconstruction of phylogenetic relationships (e.g, Swofford *et al.* 1996) and estimation of historical population dynamics (Nei 1987; Drummond *et al.* 2005). In Box 3, we return to our two populations from Box 1 to illustrate how the reconstruction of the phylogenetic relationships among extant haplotypes provides insight into the populations' past. With the recent advent of 'ancient DNA' technologies it is now possible to actually revisit past populations, where direct comparisons with contemporary populations offer incredible opportunities to reconstruct population histories.

15.2.2.1 Genetic diversity, inbreeding and population history

Large populations tend to harbor high diversity. Such patterns are a feature of many seal (Mizuno *et al*. 2003; O'Corry-Crowe and Westlake 1997; Perry *et al*.

Box 3 Phylogeography of a haploid marker in two populations.

Returning to our two Populations from Box 1, here we display the sequences of the 5 unique haplotypes, reconstruct the phylogenetic relationships among the haplotypes and map their geographic distribution at the time of sampling, and thereby attempt to learn about the history of both Populations.

Haplotype	DNA sequence
A	CGTTACGATAGACC
B	CGT**A**ACGATAGACC
C	CGTTAC**C**ATAGACC
D	CGTT**T**C**C**ATAGACC
E	CGTTACGATA **C**ACC

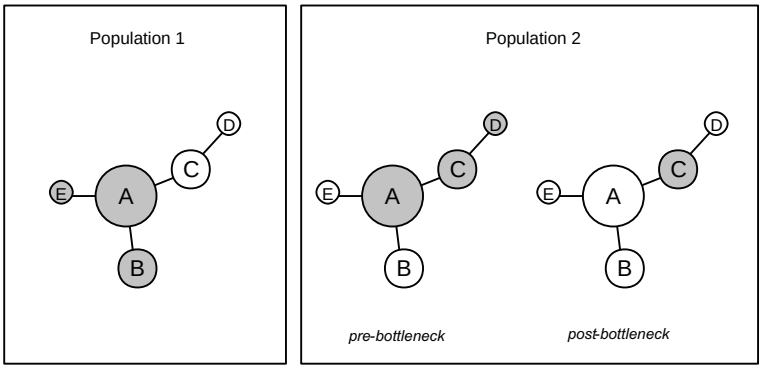


Fig. 15.3 DNA sequence and minimum spanning tree of haplotypes found in Population 1 and Population 2 and their contemporary distribution. Pre- and post-bottleneck trees are provided for Population 2. Original.

The phylogenetic reconstruction is a minimum spanning network that links haplotypes with the smallest number of mutational differences together. Haplotype size reflects the frequency of the haplotype across all populations at the time of sampling, and those haplotypes found in a particular Population are highlighted for that population.

The central position of Hap A in the network and its connection to several other haplotypes, suggests that it is an ancestral haplotype. The fact that it has a high frequency and is found in both Populations also argues for its ancient origins.

Conversely, Hap D is on a branch tip and connected to only one other, internal, haplotype, suggesting that it is a more recently derived haplotype. The fact that it is rare and has been found in only one Population may mean that it arose in that population relatively recently.

Comparing our reconstructions to actual population histories in Box 1 will demonstrate that our inferences are, not surprisingly, accurate.

2000; Palo *et al*. 2001; Westlake and O'Corry-Crowe 2002) and pelagic dolphin species (e.g., Dizon *et al*. 1994), and suggest the maintenance of large population sizes over time. Conversely, small populations typically retain low levels of diversity. This often raises concerns about inbreeding depression and the ability of these populations to deal with changing environmental conditions. Naturally small populations, however, may be uniquely adapted to high levels of inbreeding and low levels of genetic diversity (Lande 1988; Nei *et al*. 1975). The rare *Phocoena sinus* (Gulf of California porpoise, or Vaquita) is characterized by a complete lack of variability within mtDNA (Rosel and Rojas-Bracho 1999). This species may never have been very abundant and through the purging of deleterious recessive alleles may be adapted to high levels of consanguinity and low diversity (Taylor and Rojas-Bracho 1999). Nevertheless, limited genetic variation in combination with small current population size means *P. sinus* still faces an uncertain future in a changing world (Rojas-Bracho and Taylor 1999).

Population expansions and contractions over various time frames leave distinctive signatures or 'footprints' in the pattern of genetic variation within them (Slatkin and Hudson 1991; Rogers and Harpending 1992). Over evolutionary timescales, population expansions will be accompanied by the generation of new diversity, the new variants typically being one or two mutational steps from the original, ancestral ones. Populations that have undergone expansions in the distant past may retain both the ancestral and derived variants, such that their gene phylogenies are more akin to a well filled-out bush than a tree (Fig. 15.4). A number of species of marine mammal possess such star-like mtDNA phylogenies, including *Monodon monoceros* (Narwhal) (Palsbøll *et al*. 1997), *Delphinaperus leucas* (Fig. 15.4, O'Corry-Crowe *et al*. 1997, 2002; Brown-Gladden *et al*. 1997) and *Phocoena phocoena* (Rosel *et al*. 1995), which have been interpreted as evidence of population expansions associated with the (re)colonization of marine habitats following the retreat of the Pleistocene ice sheets.

Just as population expansions can generate new diversity, so population reductions can lead to a loss of diversity (Cornuet and Luikart 1996; Nei *et al*. 1975). In a landmark population genetic study on a marine mammal, Bonnell and Selander (1974) attributed the complete lack of electrophoretic variation observed in 24 protein loci in the Northern elephant seal to a 19th century population "bottleneck" caused by overhunting. Subsequent studies reaffirmed the absence of allozyme variation and detected low variability within mtDNA (Hoelzel *et al*. 1993) and minisatellite loci (Lehman *et al*. 1993), and similarly concluded that these low levels of genetic variation were consistent with a severe population bottleneck (Hoelzel *et al*. 1993). The consequences of the genetic bottleneck on individual fitness, however, appear to have been minimal. Although the species may have been reduced to as few as 20 individuals, it has achieved a spectacular recovery and now numbers in excess of 120,000 animals (Stewart *et al*. 1994). Commercial harvest in the 18th and 19th centuries also resulted in dramatic reductions in genetic diversity in

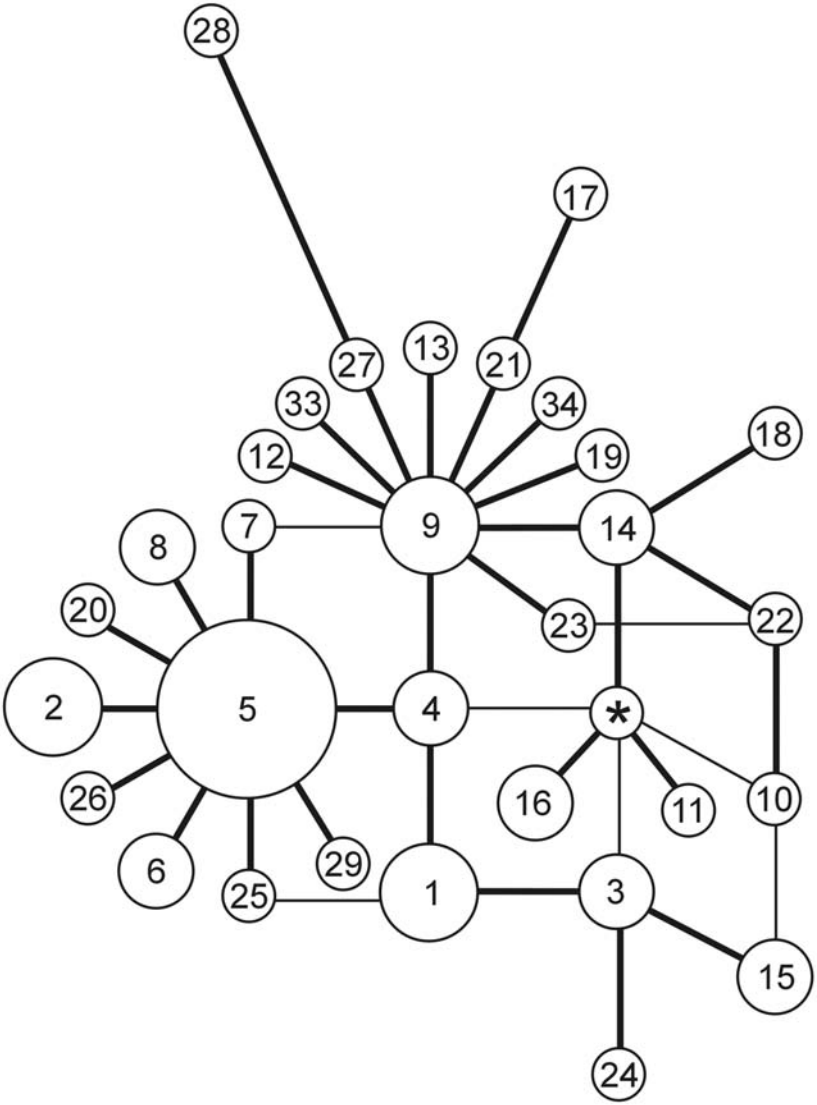


Fig. 15.4 Minimum spanning network of 31 unique mtDNA haplotypes found in *Delphinapaterus leucas* (Beluga whale). The reconstructed consensus haplotype is denoted by an asterisk. Each link represents a unique mutational event, except in the case of the link between haplotypes 27 and 28 which represents two mutations. Haplotype sizes reflect their abundance in the total sample. A single-most parsimonious tree is indicated by the bold lines. (Note that haplotypes nos. 30, 31 and 32 are not represented in the network as they were found in a separate study.) From O'Corry-Crowe, G. M., Dizon, A. E., Suydam, R. S., and Lowry, L. F. 2002. Pp. 53-64 in C. J. Pfeiffer (ed.), *Molecular and Cell Biology of Marine Mammals.* Krieger Publishing Co., Malabar, Florida, USA, Fig. 6.2.

other species. Using ancient DNA techniques, Weber *et al.* (2004) documented much higher levels of mtDNA diversity in pre-exploitation *Arctocephalus townsendi* (Guadalupe fur seal) bones collected at archaeological middens than in samples collected from contemporary populations. Similarly, Larson *et al*. (2002) found higher levels of nuclear DNA variation (microsatellites) in Sea otter bones that pre-date the dramatic population reductions in this species wrought by the fur trade compared to samples from extant populations. In cetaceans, lower levels of nuclear and mtDNA variation have been found in *Eubalaena glacialis* (Southern right whale) compared to *E. australis* (South Atlantic right whale) (Schaeff *et al*. 1991, 1997). The authors interpret the lower diversity in the northern species as a consequence of a population bottleneck resulting from centuries of whaling, and suggest that the reduced fertility, fecundity and survival observed in the northwest Atlantic population may be evidence of inbreeding depression. By contrast, at least some populations of the closely related *Balaena mysticetus* (Bowhead whale), still retain substantial genetic diversity despite the depredations of commercial whaling (Rooney *et al*. 1999, 2001; LeDuc *et al*. 2005). Moderate to high diversity may be the case in most large whale species, where numbers were not reduced low enough for long enough by commercial whaling for substantial diversity to be lost (Amos 1996).

Finally, using a number of simplifying assumptions, it is theoretically possible to estimate population parameters such as long-term effective population size from patterns of genetic variation (Nei 1987). In a recent study, Roman and Palumbi (2003) used contemporary estimates of mtDNA diversity to estimate pre-exploitation population sizes in a number of large whale species in the North Atlantic. Species estimates ranged from 240,000 to 360,000 whales far exceeding previous calculations and questioning the efficacy of current management goals. Caution, however, is required when evaluating these estimates of historic population size as considerable uncertainty remains over some of the assumptions made in Roman and Palumbi's calculations, including the ratio of effective to census population size, the rate of mtDNA substitution in baleen whales, and whether populations were in drift-mutation equilibrium (Clapham *et al*. 2004).

Conclusion. The level and pattern of genetic diversity within marine mammal populations provides insight into the demographic history of populations, the degree of inbreeding and its consequences on individual fitness, and, potentially, the extent and direction of natural selection. Assessments of genetic diversity can thus also give guidance as to the conservation status and evolutionary potential of a population.

15.2.2.2 Phylogeography

As the analysis of mtDNA haplotype and nuclear allele frequencies can reveal much about contemporary levels of gene flow and dispersal, so the reconstruction of the phylogenetic relationships among genetic lineages and the mapping of their present-day geographic distribution can provide unique

insights into the evolutionary and demographic history of populations. Over the past two decades this "phylogeographic" approach (*sensu* Avise *et al*. 1987) has illuminated the species and population histories, migratory and dispersal behavior of many marine mammals (e.g., Rosel *et al*. 1995; Stanley *et al*. 1996; Westlake and O'Corry-Crowe 2002). One marker has found particular application in phylogeographic investigations of marine mammals: mitochondrial DNA (mtDNA). Because of its maternal mode of inheritance and absence of recombination, mtDNA phylogenies represent extended maternal pedigrees or matrilines (Avise 1995; Brown 1983; Wilson *et al*. 1985). If female dispersal among populations is restricted, differences in mtDNA haplotype frequencies will emerge through the action of genetic drift. If dispersal is restricted for long enough, phylogeographic differences among the populations will eventually develop through the combined action of drift and mutation. Mapping the contemporary geographic distribution of these maternal lineages can thus provide a detailed account of the demographic relationships among populations over time (Avise 1995), and thus help guide conservation and management of natural populations (Avise 1995; Dizon *et al*. 1992; Moritz 1994; Vogler and DeSalle 1994).

Substantial phylogeographic partitioning of mtDNA lineages has been found in a number of highly migratory cetacean species indicating that the female-mediated philopatry to traditional migration routes and destinations is a long-established behavior. Extensive phylogeographic sorting of mtDNA haplotypes has been found among *Megaptera novaeangliae* populations in different ocean basins, and among geographically discrete feeding concentrations and similarly discrete wintering concentrations of this species within ocean basins (Baker *et al*. 1993; Baker and Medrano-Gonzáez 2002). Substantial geographic partitioning of mtDNA lineages has also been observed in *Delphinapterus leucas* (Fig. 15.5). These patterns, in combination with the star-like phylogeny of this marker, argue that the origins of separate summering concentrations of *D. leucas* date back to postglacial expansion from refugial populations and indicate limited dispersal among these summering groups for long periods, in some cases over evolutionary time scales (Brown-Gladden *et al*. 1997; O'Corry-Crowe *et al*. 1997, 2002).

In pinnipeds, the phylogeography of mtDNA variation in *Odobenus rosmarus* may indicate an ancient divergence between Atlantic and Pacific subspecies (Cronin *et al*. 1994) and between populations of the Atlantic subspecies to the west and east of Greenland (Andersen *et al*. 1998; Cronin *et al*. 1994), while in *Eumetopias jubatu*s strong phylogeographic partitioning of mtDNA between eastern and western North Pacific Ocean rookeries indicate at least two evolutionarily distinct populations that may have originated in separate glacial refugia (Bickham *et al*. 1996; Harlin-Cognato *et al.* 2006; O'Corry-Crowe *et al.* 2006).

Conclusion. Investigations of mtDNA phylogeography in marine mammals have revealed that present-day patterns of dispersal, philopatry and migration are in many cases long established behaviors such that populations

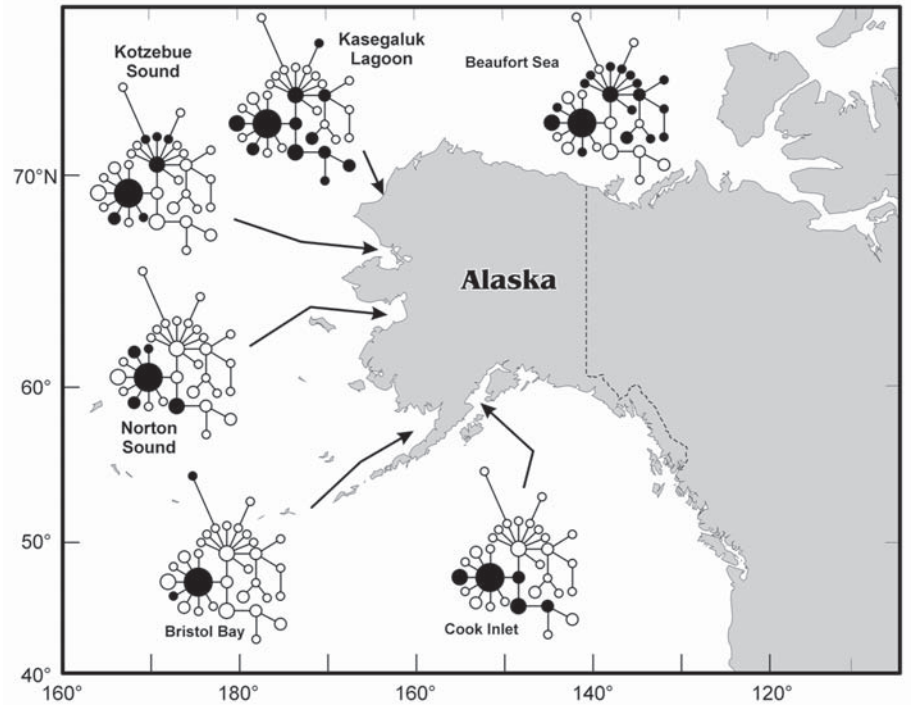


Fig. 15.5 MtDNA haplotype diversity among summering concentrations of *Delphinapterus leucas* (Beluga whale) in Alaska and northwest Canada represented on an optimum minimum spanning tree. (Solid disks indicate the set of haplotypes in the tree that were found in each area.) Haplotype size reflects the overall frequency, not the frequency within each area (see Fig. 15.4 for more details). After O'Corry-Crowe *et al.* (1997, 2002).

are evolutionarily as well as demographically distinct. This approach has facilitated the reconstruction of population histories, including identifying the likely location of refugial populations during previous ice ages and the routes of population expansion following ice retreat. The ability to assess marine mammal populations in a historical as well as contemporary context helps in the assessment of the importance of individual populations to species survival and to prioritize management objectives.

15.3 FUTURE CHALLENGES

Population genetic investigations have provided unique insights into the ultimate and proximate forces that shape subdivision and genetic diversity in marine mammal populations, and have thereby greatly improved our understanding of the demographic and evolutionary processes acting within and among marine mammal populations over time, as well as aided in the conservation and management of these highly mobile, often elusive animals.

Advances in population genetic theory, sampling methods and molecular technology have played major roles in the development of the field. Challenges, however, remain.

Fresh insights into the mechanisms of evolution within marine mammal populations and into the inherent genetic component of individual fitness and population viability will require the examination of markers that code for specific traits (e.g., Mackay 2001) and more interdisciplinary research where genetic studies of reproductive success, mating systems, kinship and dispersal are conducted in conjunction with studies of survival, health, population trend and physiological response. At the conceptual level, it is becoming increasing apparent that many marine mammal populations behave as metapopulations (e.g., York *et al.* 1996) comprising discrete local populations linked via dispersal and gene flow, where elucidating the patterns, causes and consequences of dispersal through genetic investigation can lead to a greater understanding of population and evolutionary dynamics across the metapopulation's range (Hanski and Gaggiotti 2004).

Many population genetic studies on marine mammals to date have suffered from limited statistical power to elucidate the underlying patterns of gene flow and population subdivision. This is often the case in large populations with recent common ancestry where the diversifying power of genetic drift is minimal such that distinct populations may not have diverged much genetically. Failure to detect subdivision may also be a consequence of small sample sizes, uninformative markers or inappropriate statistical methods (Dizon *et al.* 1995; Taylor *et al.* 1997; Ryman *et al.* 2006). Thus, new sampling approaches, new genetic markers and alternative statistical techniques are required to improve statistical power.

On the analytical front, traditional methods of population genetic inference are based on idealized models of populations in equilibrium or undergoing deterministic expansion (e.g., Slatkin and Barton 1989; Beerli and Felsenstein 2001). However, most species of marine mammals are unlikely to comprise of populations that have been demographically stable enough for long enough to have attained equilibrium between the opposing forces of drift and gene flow at selectively neutral loci. Regular climatic oscillations and marine ecosystem regime changes have resulted in histories of population expansions and contractions in several marine mammals, while many species have also suffered dramatic declines (and in some cases spectacular recoveries) over the past few centuries from commercial harvest. The populations shown in Box 2 were represented as two populations that had attained equilibrium such that Wright's idealized model could be used to infer the average level of gene flow. As may have already been noticed, the haplotype frequencies match those of Populations 1 and 2 from Box 1 (Phase 5). The point here is that similar haplotypic distributions could arise in populations that are in equilibrium, expanding or even declining, and that average measures of gene flow estimated in this way are essentially meaningless in populations not in equilibrium. New mathematical models

and methods of data analysis are emerging (e.g., Paetkau *et al.* 1995; Pritchard *et al.* 2000; Gaggiotti *et al.* 2002; Wilson and Rannala 2003) that are now facilitating genetic investigations of marine mammal populations that are not in equilibrium.

Key to successfully facing these and future challenges will be empirical studies that track the fortunes of individuals, and temporal analyses that put the traditional 'snapshot' studies of spatial variation in the proper context of dynamic ecosystems and of the ever evolving populations of marine mammals that inhabit them.

15.4 ACKNOWLEDGMENTS

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