Ovary, Oogenesis, and Ovarian Cycle

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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 cryopreservation studies.

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).
Fig. 7.1 Morphology of *Balaenoptera bonaerensis* (Antarctic minke whale) ovaries. **A.** Mature pregnant 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 (3 to 4 mm in cattle) suppresses the development of neighboring small follicles by secretion of increasing concentrations of estradiol-17b (E2) 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 (P₄, E₂ 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.
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 mm. 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 mm; zona-pellucida, 35.5 ± 2.93 and 32.9 ± 2.9 mm, respectively) were slightly larger than those of bovine immature oocytes (total, 164 ± 4.3 mm and zona-pellucida, 15.5 ± 0.9 mm) (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 mm. As the follicular diameter increases to

**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

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approximately 2 mm and the oocytes increase in diameter from 110 to 120 mm, 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, ³11 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 E2), 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 acutorostrata* (Greenland minke whale). Grade A (³5 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. acutorostrata* 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-supplemented IVM media (FSH + E2 and PMSG + hCG) for *Balaenoptera bonaerensis* fresh oocytes using a portable CO₂ incubator. Asada et al. (2000) previously investigated the effect of FSH + E2 and PMSG + hCG in an IVM medium on pronuclear formation and cleavage of *B. acutorostrata* 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.
COC cultured in media supplemented with either E2 + FSH or PMSG + hCG (Fig.7.7). The proportion of matured oocytes cultured in the medium supplemented with FSH + E2 (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, \(n=23\)) 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 + E₂. 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 + E₂. After Iwayama, H., Ishikawa, H., Ohsumi, S. and Fukui, Y. 2005. Journal of Reproduction and Development 51: 69-75, Fig. 2.
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, MgSO₄ (anhydrous) and CaCl₂ · 2H₂O 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.
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 acutorostrata*. The morphologically viable proportion of post-thawing *B. acutorostrata* 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. acutorostrata* 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 cryoprotectant 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 *in vitro* maturation rates of frozen-thawed porcine and *Balaenoptera acutorostrata* oocytes were markedly lower than those of other species (Didion et al. 1990; Asada et al. 2000). Perhaps one reason why porcine
and *B. acutorostrata* 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 prepupal 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 prepupal 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 (prepupal, 80.8%; adult, 88.4%) rather than OPS (prepupal, 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 prepupal Cryotop group (14.4%), the adult OPS group (14.3%), and the prepupal 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 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 *estadiol-17b* (*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 (P₄)
levels: 1) ovarian active phase, 2) pseudo-pregnant phase, and 3) resting
(anestrous) phase. The ovarian active phase is reflected by high levels (5-15
ng/ml) of P₄ and the pseudo-pregnant phase is the period of maintaining a
high P₄ level for several months. In the resting phase, distinctive P₄ levels
usually are not observed, but secretion of E₂ (around 5 pg/ml) is continued.
This continual secretion of E₂ 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

### 7.3.1 Regulating Hormones

In Mysticeti, little is known about circulating reproductive hormone levels,
including sex steroids and correlation with reproductive activity. Progesterone (P₄)
is known as one of the important sex steroids produced in the ovaries and placenta of many mammalian species. Elevation of P₄ 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 P₄ levels and female reproductive status in 46 *Stenella coeruleoalba* (striped dolphins) and 11 *Globicephala macrorhynchus* taken during October in Taiji, Japan. Progesterone (P₄) 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 P₄ levels. Additionally, Yoshioka and Fujise (1992) measured P₄ 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 P₄ levels lower than 1 ng/ml, while ovulated and pregnant females had much higher levels with averages of 17.0 and 17.6 ng/ml, respectively. These data indicate that P₄ concentrations below 1 ng/ml can be considered as basal circulating levels but not as ovulated or pregnant 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 P₄, E₂ and LH levels in three female *Tursiops truncatus* and observed no cyclic elevation of P₄ 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 P₄ production is observed for approximately 4 wk in the non-conceptive ovarian cycle. During the luteal phase and early pregnancy, when P₄ metabolites are elevated, estrogen metabolite excretion remains low. Atkinson et al. (1999) also examined P₄ profiles to study general reproductive patterns in three captive female *P. crassidens* and found that plasma P₄ 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 acutorostrata* 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. acutorostrata* (Iga *et al.* 1996), concentrations of P₄, E₂ 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 E₂ 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 (P₄, E₂ and T) by EIA in 95 male and 67 female *B. acutorostrata*. 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. acutorostrata* 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. acutorostrata* 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 ultrasonography following hormonal treatment for induction of ovulation and measurement of serum or urine hormone patterns, such as E₂ 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 Mysticeti. 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 P₄ concentrations in CL tissues of early and late pregnant whales were 11.7 and 4.0 mg/wet g, respectively, and indicated that the CL appears to be a major source of P₄ 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 P₄ 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.

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## 7.5 LITERATURE CITED


Ovary, Oogenesis, and Ovarian Cycle 211


