

Estrous cycle characterisation and artificial insemination using frozen–thawed spermatozoa in the bottlenose dolphin (*Tursiops truncatus*)

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Abstract

The reproductive endocrinology of the bottlenose dolphin, *Tursiops truncatus*, was characterized to facilitate the development of artificial insemination using cryopreserved spermatozoa. Specific objectives were: (i) to determine the excretory dynamics of urinary luteinizing hormone (LH) and ovarian steroid metabolites during the estrous cycle; (ii) to evaluate the effect of an exogenously administered synthetic progesterone analog (altrenogest) on reproductive hormone excretion; (iii) to correlate follicular growth and ovulation (as determined by transabdominal ultrasound) to urinary LH and ovarian steroid metabolites; (iv) examine the *in vivo* fertilisation capacity of cryopreserved semen, and (v) to develop an intrauterine insemination technique. Based on urinary endocrine monitoring of natural estrous cycles (2 consecutive cycles) and nine post altrenogest cycles in ten females, estrous cycles were found to be 36 days long and comprised of an 8 day and 19 day follicular and luteal phase, respectively. Peak estrogen conjugates (EC; 5.4 ± 3.8 ng/mg creatinine (Cr)) occurred 8 h prior to the LH surge (70.9 ± 115.7 ng/mg Cr). The time of ovulation, as determined by ultrasonography, occurred 32.1 ± 8.9 h and 24.3 ± 7.0 h after the onset of the LH surge and LH peak, respectively. Mean preovulatory follicular diameter and circumference were 2.1 ± 0.5 cm and 6.5 ± 1.5 cm, respectively. Of the 27 estrous synchronisation attempts, 13 resulted in an ovulatory cycle, with ovulation occurring 21 days post-altrenogest treatment. Intrauterine (4 of 5) and intracornual (1 of 3) inseminations conducted across eight estrous cycles resulted in five pregnancies (63%), one pregnancy resulted from the use of liquid stored semen, whereas four were achieved using cryopreserved semen. These data provide new information on female bottlenose dolphin reproductive physiology, and demonstrate that the combination of endocrine monitoring and serial ultrasonography contributed to successful AI using liquid-stored and cryopreserved semen.

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Introduction

Reproductive success in the bottlenose dolphin (*Tursiops truncatus*), the most common cetacean held in captivity, has reached a point that parallels or exceeds that observed in wild populations (Duffield *et al.* 2000, Wells 2000). Despite a large captive population, most animals are dispersed among numerous genetically isolated facilities. Although movement of animals between facilities can enable genetic exchange, many aquaria are unwilling to

participate in this management practice. This reluctance to cooperate or share genetics between facilities has resulted in poor founder animal penetration and is often associated with over representation of dominant animals (Duffield *et al.* 2000). The development and application of artificial insemination (AI) in conjunction with genome resource banking would provide a mechanism to improve genetic management without animal transportation. However, before AI can be successfully developed in any

species, basic questions concerning the reproductive physiology of that species must be answered.

Similarly to killer whales (Duffield *et al.* 1995), bottlenose dolphins can be readily trained for unrestrained blood sample collections. Progesterone and estrogen analysis of these samples demonstrated that bottlenose dolphins could spontaneously ovulate, have an estimated 21 to 42 days estrous cycle (Benirschke *et al.* 1980, Kirby & Ridgway 1984, Schroeder 1990) and that estrogens are elevated from 5–7 days (Schroeder 1990). In addition, bottlenose dolphins have varying seasonal reproductive activity, ranging from polyestrous, seasonally polyestrous, to anestrus for one to two year intervals (Cornell *et al.* 1977, Cornell *et al.* 1987, Kirby & Ridgway 1984, Kirby 1990, Schroeder 1990). Recent data suggest that the time of year when females are reproductively active may depend on the geographical location where they or their founder was originally collected (Urian *et al.* 1996).

Efforts to fully define serum hormonal profiles (reproductive steroids and gonadotropins) of the bottlenose dolphin around ovulation have not been entirely successful (Sawyer-Steffan & Kirby 1980, Sawyer-Steffan *et al.* 1983, Schneyer *et al.* 1985, Yoshioka *et al.* 1986). Preovulatory estradiol (E2) levels observed in one animal ranged from 125 to 200 pg/ml (Yoshioka *et al.* 1986). With Indo-Pacific bottlenose dolphins (*Tursiops aduncus*), ovarian ultrasound was combined with serum hormone analysis to obtain similar E2 levels as reported previously (Yoshioka *et al.* 1986), but defined the serum estrogen profile as erratic and not useful for predicting ovulation (Brook 2000). Collection and analysis of urinary hormonal profiles as has been described for the killer whale (Walker *et al.* 1988, Robeck *et al.* 1993, 2004), would provide increased sampling frequency and may help resolve the endocrine dynamics during the peri-ovulatory period.

Ultrasonographic monitoring of ovarian follicular activity in bottlenose dolphin has been described previously (Brook 2000, Robeck *et al.* 1998). In the Indo-Pacific bottlenose dolphin, Brook (2000) defined the preovulatory follicle diameter (POF; range 17–23 mm) and documented a mean inter-ovulation interval of ~30 days. Importantly, the former study found that, for each animal POF sizes were similar between successive ovulations. Thus, predicting ovulation based on POF size was possible only after POF attributes had been characterized for that animal.

Altrenogest, a synthetic progestagen has been used to synchronize estrous in horses (Webel & Squires 1982) and pigs (Kraeling *et al.* 1981) without affecting fertility in the female (Squires *et al.* 1979, Stevenson & Davis 1982, Squires *et al.* 1983). In cetaceans, altrenogest has been used for long-term suppression of ovulation (Young & Huff 1996) and to synchronize estrous in the killer whale (*Orcinus orca*), Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) and bottlenose dolphin (Robeck *et al.* 2000, 2003, 2004). The ability to control the timing of ovulation

in bottlenose dolphins would allow for improved management of natural breeding and for timing of AI.

Methods of cryopreservation of bottlenose dolphin sperm using pellets or straws on dry ice or in liquid nitrogen vapor have been reported previously (Seager *et al.* 1981, Schroeder & Keller 1990, Durrant *et al.* 2000, Robeck *et al.* 2001, Robeck & O'Brien 2004). Despite these efforts, *in vivo* competence of frozen–thawed spermatozoa has never been demonstrated. The successful application of AI using frozen–thawed bottlenose dolphin spermatozoa would validate such preservation methods.

Artificial insemination has recently been successful using liquid-stored and cryopreserved spermatozoa in the killer whale and Pacific white-sided dolphin (Robeck *et al.* 2003, 2004) and using fresh extended sperm in the Indo-Pacific bottlenose dolphin (*Tursiops aduncus*; Robeck *et al.* 2001, FM Brook *et al.*, unpublished). Only ultrasound data were used in the latter study to determine timing for AI, as a result, multiple inseminations were required prior to ovulation. By relying on peak urinary estrogen conjugates (EC) and urinary luteinizing hormone (LH) to time inseminations in the killer whale and Pacific white-sided dolphin, respectively, fewer inseminations prior to ovulation were required (Robeck *et al.* 2003, 2004). The development of a urinary LH assay system for the bottlenose dolphin may provide a consistent predictor of ovulation and allow a more efficient use of valuable semen. In addition, the development of AI using cryopreserved semen would enable the global exchange of genetic material and provide a tool for future application of genome resource banking (Holt *et al.* 1996, Wildt *et al.* 1997). Furthermore, the application of other assisted reproductive technologies, such as sperm sexing, may then be integrated into dolphin captive breeding programs.

The overall goal of this research was to gain a sufficient level of understanding of bottlenose dolphin reproductive physiology to develop AI using cryopreserved semen. To accomplish this, specific objectives were: (i) to determine the excretory dynamics of urinary LH and ovarian steroid metabolites during the estrous cycle; (ii) to evaluate the effect of an exogenously administered synthetic progesterone analog (altrenogest) on reproductive hormone excretion; (iii) to correlate follicular growth and ovulation (as determined by transabdominal ultrasound) to urinary LH and ovarian steroid metabolites; (iv) to examine the *in vivo* fertilisation capacity of cryopreserved semen, and (v) to develop an intrauterine insemination technique.

Materials and Methods

Animals

Thirteen adult, proven breeding female bottlenose dolphins (*Tursiops truncatus*) located at five facilities were used in endocrine monitoring and in AI trials (Table 1). Female 1, located at the Dolphin Quest Facility in Oahu, Hawaii, was used only for endocrine monitoring. This female was housed in an approximately 1233 m³ natural

Table 1 Description of animals used and samples collected during the study.

Animal	Facility ^a	Sex	Age	Weight	Reproductive history	Sample date range ^b	No. of samples	Contribution ^c
1	DQ	F	14	167	Nulliparous	06-05-02 to 05-08-02	71	EC, LH, UP
2	SWC	F	22	286	1 stillborn, 1 calf	15-06-03 to 06-12-03 20-01-04 to 31-03-04 05-05-04 to 23-05-04	191 85 32	ES, ultrasound, EC, LH, UP
3	SWC	F	14	233	2 calves	15-06-03 to 06-12-03 20-01-04 to 31-03-04 05-05-04 to 23-05-04	159 86 31	ES, ultrasound, EC, LH, UP
4	SWC	F	15	145	1 calf	24-02-04 to 15-03-04 17-05-04 to 17-06-04	36 55	EC, LH, UP, ES, Ultrasound
5	SWC	F	24	218	5 calves	15-06-03 to 06-12-03 20-01-04 to 31-03-04 05-05-04 to 23-05-04	170 90 33	ES, ultrasound, EC, LH, UP
6	USN MMP	F	20	205	1 calf	14-06-04 to 30-06-04	27	ES, ultrasound, EC LH, UP
7	SWC	F	12	231	1 calf	07-05-02 to 27-06-02	27	ES, ultrasound, AI, EC, LH, UP
8	SWC	F	11	235	Nulliparous	07-05-02 to 27-06-02	26	ES, ultrasound, AI, EC, LH, UP
9	KSW	F	21	273	4 calves, 1 abortion	No urine samples		ES, ultrasound, AI
10	KSW	F	36	287	7 calves, 2 abortions	No urine samples		ES, ultrasound, AI
11	KSW	F	17	265	Nulliparous	No urine samples		ES, ultrasound, AI
12	GA	F	20	180	1 abortion 1 calf (1997)	16-07-03 to 07-10-03 15-04-04 to 03-07-04	67 81	ES, ultrasound, AI, EC, LH, UP
13	SWC	F	25	140	1 previous calf	10-08-03 to 08-12-03 25-01-04 to 13-03-04 16-05-04 to 08-07-04	111 56 75	ES, ultrasound, AI, EC, LH, UP
1	USN MMP	M	24	183	No previous sired calves	16-05-02	2 Ejac ^d	Cryopreserved semen
2	SWC	M	35	238	Sired 10 calves, and 1 stillborn	06-03-03	1 Ejac	Cryopreserved semen
3	KSW	M	17	330	2 calves, 1 abortion	02-07-02 to 04-07-02	3 Ejac	Liquid stored semen
4	KSW	M	20	365	8 calves, 1 abortion	06-11-92 21-06-93 to 07-07-93	1 Ejac 5 Ejac	Cryopreserved semen

^a DQ, Dolphin Quest; SWC, SeaWorld California; USNMMP, US Navy Marine Mammal Program; KSW, Kamogawa Sea World; GA, Genoa Aquarium.

^b Urine samples were collected from females and semen samples from males.

^c Data that the animal contributed to the study: EC, urinary oestrogen conjugate; ES, oestrous synchronisation; LH, luteinizing hormone; UP, urinary progestagen metabolites.

^d Ejac, number of ejaculates collected and used during the study.

salt-water enclosure (ambient temperature 24–28 °C) with three other dolphins of mixed age and sex.

All animals used for AI trials were held without access to males for a minimum of 1 month prior to the procedures. Three of the females (females 9, 10 & 11) used for AI trials were located at Kamogawa Sea World, Chiba, Japan. These three females were housed together in a 945 m³ enclosure with processed natural saltwater (ambient temperature 17–28 °C) with one other adult female and two male and two female juvenile animals. Two adult males (males 3 and 4) located at Kamogawa Sea World were semen donors for the AIs performed at that facility. They were housed separately in a 500 m³ pool with a mixed breeding group of dolphins. Seven females (females 2–5, 7, 8 and 13) were located at SeaWorld California (SWC), housed in an outdoor pool containing 850 m³ of natural processed salt water (ambient temperature 18 °C) during the study. The male (male 1) who donated the semen for the AIs at SWC and female 6 were located at the US Navy Marine Mammal Program in San Diego,

California, USA. They were housed in various natural saltwater interconnected enclosures ranging in size from 300 to 600 m³ (ambient temperature 13–21 °C). The remaining female (female 12) was located at the Acquario di Genova (Genova, Italy) in a 1700 m³ outdoor facility that relies on natural filtered salt water (ambient temperature 14 to 26 °C). Semen used for this animal was from male 2 housed at SWC as previously described. Animals located in the US and Italy were fed a diet of frozen–thawed whole fish (herring (*Clupea harengus*) capelin (*Mallotus villosus*) and Columbia river smelt (*Thaleichthys pacificus*)). In addition, the animal in Italy was also fed squid (*Loligo vulgaris*). The animals in Japan were fed chub mackerel (*Scomber japonicus*) and Arabesque greenling (*Pleurogrammus azonus*). All animals were fed at approximately 4–5% of their body weight per day.

Ethics of experimentation

All samples were collected using routine husbandry training and were obtained from unrestrained animals. All

procedures described within were reviewed and approved by the SeaWorld Incorporated Institutional Animal Care and Use Committee, and were performed in accordance with the Animal Welfare Act for the care of Marine Mammals.

Endocrine monitoring

Urine samples were collected from unrestrained animals as previously described (Lenzi 2000). Urine samples from female 1 were collected daily for 92 days for endocrine monitoring (EM) of estrous cycles. All other urine samples analyzed were associated with estrous synchronisation attempts or AI trials (Table 1). Samples were stored in duplicate at -70°C until analysis. Non-extracted urine samples were analyzed by enzyme immunoassay (EIA) for total immunoreactive levels of urinary progestins (UP), EC and LH. During the initial part of the study (2002) urine samples were collected twice daily for EM, estrous synchronisation (ES) or AI trials. However, during the latter part of the study (2003–2004), urine samples were collected three times a day as ovulation approached (Table 1). Urinary EC and LH were determined at the facility where the animal scheduled for AI was housed using our mobile endocrine laboratory (Steinman *et al.* 2003) and UP was determined retrospectively at the central laboratory (CRC, Front Royal, VA, USA).

Determination of total estrous cycle length (TCL) was based on either the interval between the beginning of successive LH peaks, or successive EC peaks. For the study, LH and EC peaks were defined as the maximum concentration for the respective hormones during the estrous period. In addition, intra-estrous cycle endocrine components were determined as follows: length of the luteal phase (UP concentrations >0.56 ng/mg Cr for 2 consecutive days); follicular phase (EC concentrations >0.93 ng/ml Cr for two consecutive days); start of follicular phase to peak EC, and peak EC to peak LH were also determined. The preovulatory rise in EC concentrations was subjectively defined as values >2 ng/mg Cr until the LH peak. The time from the beginning of the LH surge to peak LH and the total length of the LH surge were determined in animals with thrice daily sample collection. The beginning of the surge was defined as any value greater than 2 s.d. above baseline for that animal that was followed by the LH peak. If the LH surge began or ended between two sample periods, we subjectively assigned the beginning of the surge as occurring midway between the two samples. A 'normal' estrous cycle was determined by combining the mean values of all dolphins for all of the above-mentioned intervals.

Endocrine data were compared with the ultrasonographically estimated ovulation point (the midpoint between exams where the follicle is present in one and disappears in the next) to define the interval between the EC and LH peak and ovulation.

Cr assay

Urine samples were analyzed for Cr to account for varying concentrations of urine as described previously (Tausky 1954). Concentrations of urinary hormones and metabolites were expressed as mass of hormone per mg Cr excreted.

Assay for UP

UP were measured by single antibody, direct enzyme immunoassay as described previously (Graham *et al.* 2001). Briefly, neat urine samples (0.025–0.01 ml) and standards (range 200–0.79 pg/well; Sigma-Aldrich, St Louis, MO, USA) were added in duplicate to microtiter plates (Maxisorp, Nalge Nunc, Rochester, NY, USA) coated with a progestin antisera (polyclonal CL425, 1:10 000; C Munro, UC Davis, CA, USA). After 2-h room temperature incubation with enzyme conjugate (progesterone horseradish peroxidase, 1:40 000; C Munro), 0.1 ml substrate (azino-bis-3-ethyl benzthiazoline-6-sulfonic acid in citrate buffer; Sigma-Aldrich) was added and incubated for an additional hour. Plates were read at 405 nm (reference 540 nm) in a microplate reader (MRX, Dynex Technologies, Chantilly, VA, USA). Intra-assay variation was $<10\%$ and inter-assay variations were 9.1% and 15.7%, at 30% and 70% binding, respectively ($n=75$). Serial dilutions of bottlenose dolphin urine yielded displacement curves that were similar to the standard curve ($R^2=0.99$). The mean recovery of progesterone added to a pool of bottlenose dolphin urine was $84.3 \pm 3.8\%$ ($y=0.89x-0.25$, $R^2=0.99$). Immunoassay of fractions separated by reverse-phase HPLC analysis revealed three immunoreactive peaks at fractions 21–25 and fraction 46 which were unidentified and at fractions 67–71 that co-eluted with progesterone.

EIA assay for EC

Urinary estrogen conjugates were measured by single antibody, direct enzyme immunoassay as previously described (Robeck *et al.* 2004). Briefly, neat urine samples (0.025–0.0025 ml) and standards (range 200–0.79 pg/well; Sigma-Aldrich) were added to a microtiter plate coated with E1G antisera, and an enzyme conjugate was added to all wells. After incubation, 0.1 ml of substrate (tetramethylbenzidine in phosphate citrate buffer; Sigma-Aldrich.) was added to all wells and incubated at room temperature for 30 min. Finally, 0.05 ml 0.6 M H_2SO_4 were added. Intra-assay variation was $<10\%$ and inter-assay variation was 9.7% and 14.5% at 30% and 70% binding, respectively ($n=66$). Serial dilutions of bottlenose dolphin urine yielded displacement curves that were similar to the standard curve ($R^2=0.99$). The mean recovery of estrone glucuronide added to a pool of bottlenose dolphin urine was $126.9 \pm 22.6\%$ ($y=0.86x+1.07$, $R^2=0.99$). Immunoassay of fractions separated by reverse-phase HPLC analysis revealed one major immunoreactive peak (fractions 19–22, 16% of total) that co-eluted with estrone-3-sulfate.

LH EIA

Urinary LH was measured by single antibody, direct enzyme immunoassay modified from the double antibody EIA developed by Graham *et al.* (2002). Briefly, antisera (LH 518-B7, 1:400 000; J Roser, UC Davis, CA, USA) was added to 96-well flat-bottom microtiter plates and incubated at 4°C overnight. Neat urine samples (0.05–0.0005 ml) and standards (range 500–1.95 pg/well) were added to wells in duplicate and the plate incubated at 37°C for 3 h. Biotinylated LH (1:100 000, 0.1 ml) was then added to all wells and the plate was incubated for 30 min at 37°C. After incubation, plates were washed and then 0.2 ml streptavidin peroxidase added for an additional 30 min incubation. Substrate (0.2 ml tetramethyl benzidine in phosphate citrate buffer) was added, incubated for 40 min, and the reaction was stopped with 0.05 ml of 0.6 M H₂SO₄. Intra-assay variation was <10% and inter-assay variation was 12.2% and 15.8% at 30% and 60% binding, respectively ($n = 118$). Serial dilutions of bottlenose dolphin urine yielded displacement curves that were similar to the standard curve ($R^2 = 0.99$). The mean recovery of LH added to a pool of bottlenose dolphin urine was $53.0 \pm 15.7\%$ ($y = 1.52x - 0.74$, $R^2 = 0.99$).

Synchronisation of ovulation using progesterone analog for AI

To evaluate the effects of altrenogest as a synchronisation tool for use with artificial insemination, animals were placed on 0.044 mg kg^{-1} p.o. altrenogest (Regu-Mate, Intervet Inc., Millsboro, DE, USA) from 27–77 days. A total of 27 treatments were administered to 12 female dolphins (Fig. 1). The drug was administered by injecting directly into the coelomic cavity of a herring just prior to feeding. Immunoreactive UP, EC and LH were determined from urine samples collected daily during altrenogest treatment and at least twice daily after its cessation.

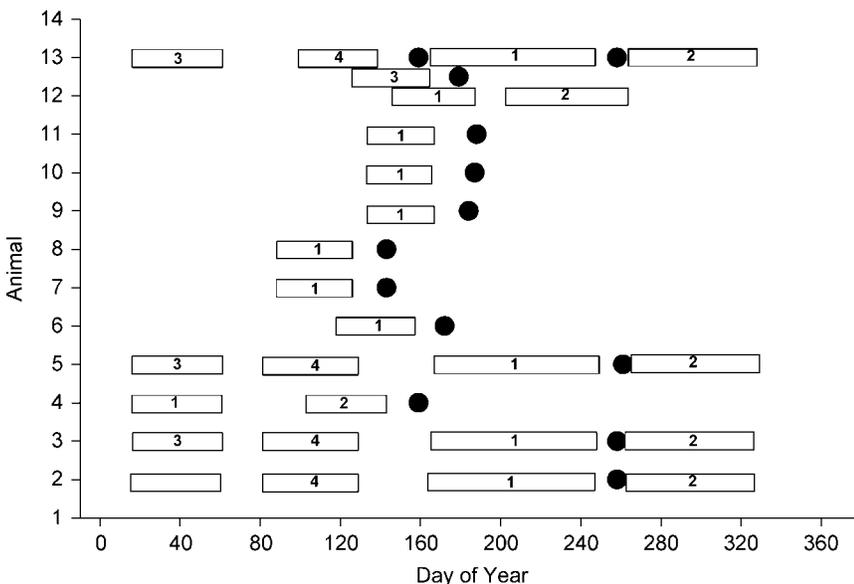


Figure 1 Yearly schedule of altrenogest administration. The animal numbers on the graph corresponds to the animal numbers in Table 1 and in the text. Animal 12 and 12.5 correspond to animal 12 as described in the Table 1. In this animal, altrenogest was administered during two consecutive years. Note the 4 altrenogest treatments given to 4 animals (females 2, 3, 5 and 13) during one year. Normal ovulations (black circle) only occurred from May to September.

Ultrasonography

Ultrasonography was used to detail follicular activity following altrenogest treatment and to confirm pregnancy. Ultrasonographic examinations were performed using an Aloka 900 machine (Corometrics Medical, Charlotte, NC, USA) and a 3.5 MHz transducer (wide footprint convex linear probe) or a EUB 405/Spazio Hitachi with a 3.5 MHz convex linear transducer (Acquario di Genova, Genova, Italy). Animals were examined once on day 0 and day 10 post-altrenogest, then daily to thrice daily from day 11 to ovulation. For examinations, the animals were trained to station in lateral recumbency adjacent to the edge of the pool. The ovaries were located trans-abdominally using a previously described technique (Brook 2000). Follicular size was determined by measuring its largest diameter, utilizing the anechoic margins as the border. Follicular circumference was calculated by software associated with the ultrasound machine or by determining the diameter in two planes and using the following formula (a & $b =$ radius):

$$C = 2\pi\sqrt{\frac{a^2 + b^2}{2}}$$

Ovulation was determined to have occurred when the follicle was not detectable in a subsequent exam. The time of ovulation was defined as the median time between the prior exam and the exam when the follicle could not be located. This interval between exams was a maximum of 12 h for twice daily and 8 h for three times daily examination.

Semen collection and processing

Ejaculates were collected from male 1 ($n = 2$), male 2 ($n = 1$) and male 4 ($n = 6$) and cryopreserved for later use. Ejaculates from male 3 ($n = 4$) were extended and

liquid stored for use during the AI trials (Table 1). All males were trained for unrestrained ejaculation as previously described (Keller 1986, Robeck & O'Brien 2004).

Ejaculate concentration, volume, sperm motility, and viability (plasma membrane integrity) were determined using standardized techniques (Robeck & O'Brien 2004). The percentages of motile sperm were subjectively determined to the nearest 5% by analyzing 4–5 fields of undiluted (male 4) or diluted spermatozoa (35 °C, 1 unit spermatozoa:25 units Biladyl Fraction A (Minitube of America, Verona, WI, USA); Tris (1210 gm), citric acid (690 gm), fructose (5 gm) and 20% egg yolk (v/v) per 500 ml) with antibiotics (Tylosin (0.5 mg ml⁻¹), Gentamycin (2.5 mg ml⁻¹), Lincomycin (1.5 mg ml⁻¹) and spectinomycin (3 mg ml⁻¹)) using bright field optics (x400, Olympus, Tokyo, Japan). Total progressive motility (PM) and kinetic rating (KR, 0–5 scale: 0, no movement; 5, rapid forward progressive movement) were subjectively determined. A sperm motility index (SMI; total progressive motility × kinetic rating) was developed for comparisons of sperm quality between fresh and post cryopreserved or liquid stored spermatozoa.

For assessment of viability, 10 µl semen was mixed with 40 µl live dead exclusion stain (eosin-nigrosin, IMV International Corp., Maple Grove, MN, USA) for 30 s. For each ejaculate an air-dried smear was used to evaluate 200 spermatozoa using bright field optics (× 1000). Spermatozoa were then placed into one of two groups based on stain uptake by the sperm head: live (no stain uptake) and dead (partial or complete stain uptake).

Processing of semen for liquid storage

Liquid stored semen was only used from male 3 during the insemination attempts in Japan. Ejaculates were diluted 1:2 (semen:diluent, v/v) over 5 min with Biladyl Fraction A. Biladyl was chosen due to its ease of preparation and preliminary studies that demonstrated its ability to maintain high levels of bottlenose dolphin sperm motility during storage for 24 h at 4 °C (T Robeck, unpublished). Sperm suspension (10 ml) was kept at 21 °C and used within 4 h for AI. The second half was cooled to 5 °C from 21 °C over 1 h (–0.27 °C min⁻¹) and used for AI within 24 h. After storage and prior to each insemination, a 15-µl portion was removed from each sample, warmed to 35 °C and re-evaluated (PM and KR).

Processing of semen for frozen storage

Semen cryopreservation of samples occurred over a 12 yr period, and as methods improved changes in freezing protocols were incorporated. Thus, three different methods were used to cryopreserve spermatozoa that were used during the AI attempts.

Method 1

Semen from male 4 was pelleted, based on a modified procedure first described by Seager *et al.* (1981). Sperm

samples were diluted 1:1 (v/v) with egg yolk citrate cryodiluent (EYC; 2.9% Na citrate, 20% egg yolk (v/v) and 8% glycerol (v/v)) and cooled from 21 °C to 5 °C over 1 h (–0.27 °C/min). At 5 °C the sample was further diluted (1:1) over 5 min with EYC for a final glycerol concentration of 6%. After incubation at 5 °C for 2 h, the sperm suspension was frozen as approximately 0.2 ml pellets on dry ice for 5 min prior to plunging in liquid nitrogen.

Method 2

Sperm samples from male 1 were diluted with Biladyl Fraction A (2:1, semen: diluent) slowly over 5 min. The sperm suspension was cooled from 21 °C to 5 °C over 1 h (–0.27 °C min⁻¹). Once at 5 °C, the sperm suspension was placed into an ice water bath (2 °C) for 1 h (cooling rate: –0.6 °C min⁻¹), then diluted 1:1 (v/v) slowly with Biladyl Fraction B (Fraction A with 14% glycerol (7% final glycerol concentration)). The sperm suspension was transferred to 0.5 ml straws (IMV International), sealed and frozen in liquid nitrogen vapor at a distance of 4.5 cm above the vapor (–12 °C min⁻¹) for 10 min then plunged into liquid nitrogen.

Method 3

Sperm samples from male 2 were frozen by the method previously described by Robeck & O'Brien (2004). Semen was diluted 1:1 (v/v) with a TES-TRIS yolk buffer (TYB) (Refrigeration Media, 320 mosm kg⁻¹, pH 7.2; Irvine Scientific, Santa Ana, CA, USA) without glycerol slowly over 5 min. The sperm suspension was cooled from 21 to 5 °C over 1 h (–0.27 °C min⁻¹). Once at 5 °C the sperm suspension was diluted 1:1 with TYB containing 6% glycerol (Freezing Media, Irvine Scientific, modified from 12% by dilution with Refrigeration Media; 3% final concentration) and loaded into 0.5 ml straws. Straws were frozen using a programmable freezer (Minidigicool, IMV International) as follows: 5 to –80 °C at –100 °C min⁻¹; –80 to –140 °C at –200 °C min⁻¹.

Semen was thawed using methods tailored for the pellet or straw freezing method, respectively. For pellets, 4 × 0.2 ml frozen pellets were added directly to 0.5 ml EYC pre-warmed to 35 °C. The sperm suspension was vigorously shaken in a 35 °C water bath for 1 min. The straws from methods 1 and 2 were thawed by plunging directly into a 35 °C water bath and shaken vigorously for 1 min (8.3 °C s⁻¹). Straws were combined and then either diluted (1:1) over 5 min with Androhep Enduraguard (Minitube of America, pH adjusted to 7.2, warmed to 35 °C) pre-warmed to 35 °C or used undiluted. Thawed sperm suspensions from all methods were evaluated for motility parameters (PM, KR) and then stored at 21 °C until the inseminations.

AI

The first two inseminations (females 7 and 8) were based on the presence of a POF and/or the detection of peak

urinary EC. Females 9–11 were inseminated based solely on follicle growth. Once the follicle subjectively appeared to be of preovulatory size (>2.0 cm), the animals were inseminated every 12 h until ovulation was confirmed by ultrasonography. Inseminations 6–8 (females 12 and 13) were timed to occur after the detection of the pre-ovulatory LH surge in the urine.

For each procedure, the use of liquid or frozen–thawed semen was based on availability. If cryopreserved semen was unavailable, fresh semen was collected 3 to 4 h prior to the insemination. One hour before the procedures, all females were pre-medicated with diazepam (0.1–0.2 mg/kg; Abbott Lab, Chicago IL, USA). The animals were removed from the water and placed in lateral recumbency on 10.2 cm thick closed cell foam pads. All animals were kept wet during the procedure and monitored for respiration rate. Inseminations were performed with a variety of flexible endoscopes (9–11 mm in diameter and 190–250 cm long) depending on endoscope availability at each facility. For the procedures, the endoscope was advanced into the cranial vagina. The vagina was insufflated with air to visualize the spermathecal fold and the cervical opening beyond (Robeck *et al.* 1994). A modified bullet tipped catheter (400 cm, 2.2 mm external diameter [6 French] Cook Vet Supplies, QLD, Australia) was placed in the working channel of the endoscope and used as a stylet to assist directing the endoscope into the cervix. Once in the uterus, the inseminations were initially performed at the uterine body or partially into each horn by the advancement of the catheter and deposition of half the semen dose in each horn (inseminations 1–5; females 7–11). The last three inseminations (6–8) were placed high in the ipsilateral horn to the preovulatory follicle (females 12 to 13).

Statistics

Hormone and sperm quality data were analyzed by analysis of variance and means compared using Newman-Keuls multiple comparisons and Mann-Whitney U tests (Sigma-Stat, Version 2.0. SPSS Inc., San Rafael, CA, USA). Data are presented as Mean \pm s.d.

Results

Endocrine monitoring

Hormone (EC, LH and UP) profiles during the peri-ovulatory interval of three natural cycles and nine post altrenogest cycles were monitored during the study interval. One of these cycles, as assessed by UP and ultrasound, did not exhibit a post-ovulation luteal phase. In addition, endocrine data were evaluated during and after 14 altrenogest treatments where ovulation post-treatment did not occur. For the natural cycles, female 12 had one normal cycle prior to being placed on altrenogest for AI trial, and female 1 had two successive natural cycles with the second cycle resulting in pregnancy (Fig. 1). Female 1 was

the only female that had two successive natural (non-synchronized) ovulations during the sampling period. Based on the three natural cycles, the interval between successive peak EC and LH was 35.5 days and 36.0 days, respectively, and the mean time between peak EC to peak LH was 4.0 ± 6.9 h (Fig. 2).

For natural and synchronized estrous cycles, the mean interval from peak EC to LH was 7.5 ± 3.8 h. The lengths of the follicular and luteal phases were 8.1 ± 3.0 days ($n = 10$, range 4–14 days) and 19.3 ± 2.8 days ($n = 6$, range 16–23 days), respectively. The preovulatory EC rise (i.e. the interval between when EC exceeded 2.0 ng/mg Cr until the pre-ovulatory EC peak) was 2.4 ± 1.8 days ($n = 10$, range 0–5 days). The interval between peak EC and peak LH was 7.5 ± 10.6 h ($n = 12$, range –0.3 to 24 h). The time from the onset of LH surge to peak LH was 9.4 ± 3.1 h ($n = 6$, range 5.5–12.5 h). The LH surge duration was 20.3 ± 5.1 h ($n = 6$, range 12–25 h). Peak EC and LH concentrations were 5.4 ± 3.8 ng/mg Cr ($n = 12$, range 2.1–13.7 ng/mg Cr) and 70.9 ± 115.7 ng/mg Cr ($n = 12$, range 10.2–429 ng/mg Cr), respectively. The interval between peak LH and the first discernable post-ovulatory increase in UP was 2.1 ± 0.6 days ($n = 9$, range 1–3 days). Mean estrous cycle phase durations, as described above, were used to develop a composite dolphin estrous cycle (Fig. 3).

Estrous synchronisation

Of the 27 altrenogest treatments, 13 (48%) resulted in subsequent ovulation. For the animals that responded to synchronisation, the mean time from the end of altrenogest treatment to the beginning of the follicular phase, the LH surge and ovulation were 11.6 ± 3.8 days ($n = 8$, range 7–17 days), 18.7 ± 3.1 days ($n = 9$, range 14–23) and 20.8 ± 3.2 days ($n = 13$, range 15–25), respectively. In females that did not ovulate, the post altrenogest increase in EC concentrations began 10.9 ± 3.1 days ($n = 14$, range 7–14.5 days) post altrenogest (Fig. 4). The interval from the end of altrenogest administration to first EC rise above baseline was similar ($P > 0.05$) between animals that ovulated and those that did not. Female 12 was placed on altrenogest twice during a three month period in 2003 (Fig. 1). She did not ovulate during the first attempt (July), but ovulated after the second attempt (September). Despite what appeared ultrasonographically and endocrinologically to be a normal ovulation, a corpus luteum (CL) could not be detected ultrasonographically 10 days post ovulation and circulating progesterone concentrations remained basal. Following a natural ovulation, female 12 was again placed on altrenogest and subsequently ovulated. Despite being treated with altrenogest shortly after her natural ovulation, she had a normal luteal phase of 20 days (Fig. 2).

Four females (females 2, 3, 5 and 13) were placed on altrenogest on four separate occasions. Their initial 77 day altrenogest treatment interval was used to achieve

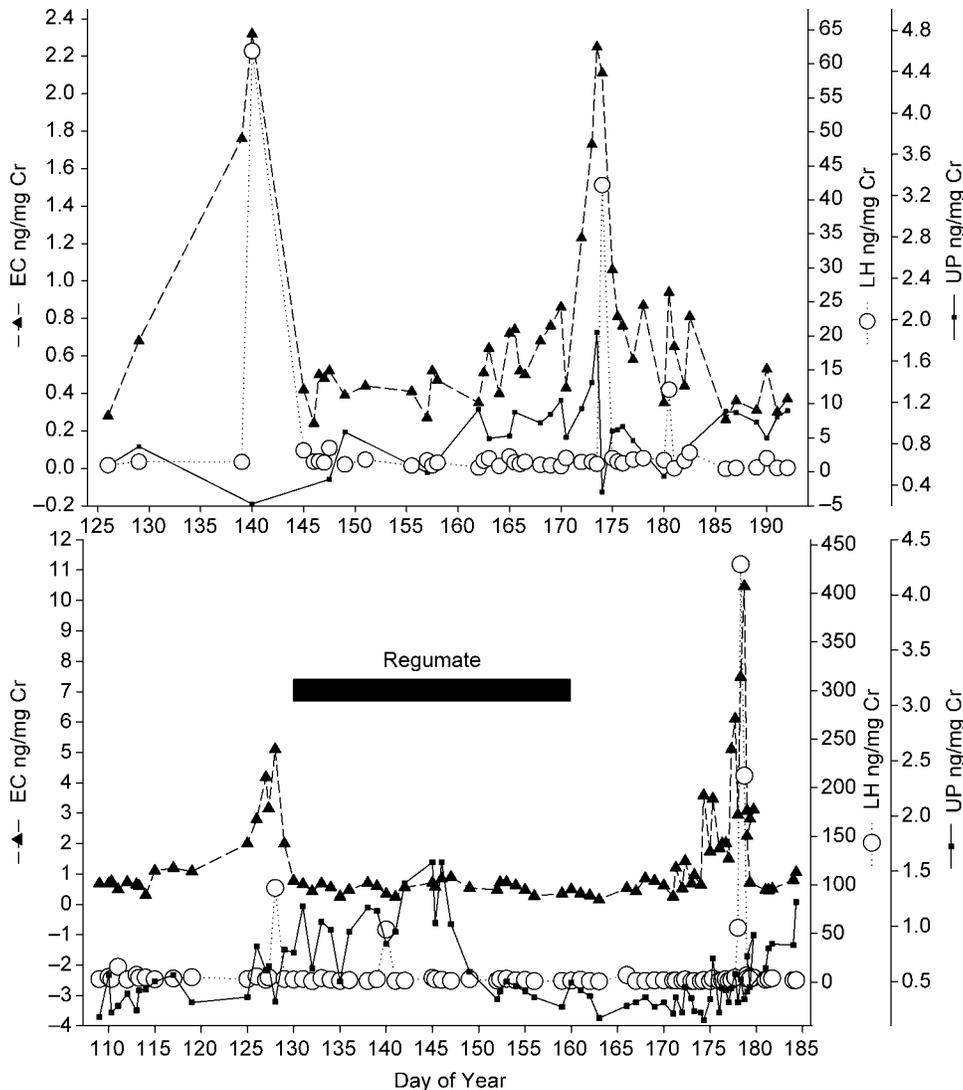


Figure 2 Three natural ovulations in females 1 (top panel) and 12 (bottom panel) characterized by urinary concentrations of LH, EC and UP. The black bar represents altrenogest administration in female 12. Note that UP concentrations continue to increase and are detected for 20 days post-ovulation, despite administration of altrenogest. Note the obvious LH surges detected during 12 h urine sampling periods for female 1 and during the second LH surge of female 12. The smaller LH surge of the first cycle for female 12 is reflective of once daily urine sampling.

both contraception and estrous synchronisation; all 4 females ovulated post-altrenogest treatment (Fig. 1). However, during the three subsequent treatments ($n=12$ treatments), only female 13 ovulated, and this occurred after the fourth treatment (Fig. 1). These failed attempts at repeated estrous synchronisation accounted for 11 of 14 regumate treatments that did not result in ovulation.

Ultrasonographic evaluation of ovaries

Dominant follicles (present less than 12 h prior to ovulation) were observed during 11 estrous cycles: eight during AI attempts and three during monitoring of natural ($n=1$) or synchronized ($n=2$) estrous cycles. Secondary follicles on the ipsilateral ($n=2$) and contralateral ovary ($n=4$) were observed in 54% of the examinations. Although these follicles were not measured in every exam, they were 1 cm or less in diameter and had regressed at the time of ovulation. The earliest time that

a dominant follicle was detected was 10.5 days prior to ovulation; the follicle diameter exceeded 1 cm in all instances. The mean time and circumference of a dominant follicle when it was first observed prior to ovulation was 5.5 ± 2.7 days and 4.2 ± 0.9 cm, respectively. The mean daily follicular growth rate in circumference was 0.47 ± 0.2 cm per day. The maximum circumference and diameter of a preovulatory follicle were 6.5 ± 1.5 cm (range, 4.23–9.6) and 2.1 ± 0.5 cm ($n=11$, range 1.7–3.1 cm), respectively. The preovulatory follicle consistently became turgid and round prior to ovulation and was located on the left ovary 82% of the time (Fig. 5). On several occasions the borders of the follicle appeared to thicken, however this was not consistently noted and did not correlate with impending ovulation. Maximum size of the preovulatory follicle had no significant correlation with peak urinary estrogen concentrations ($R^2 = 0.27$, $P > 0.05$), follicle growth rate ($R^2 = 0.65$, $P > 0.05$) or animal size ($R^2 = 0.11$, $P > 0.05$). The time of ovulation, as determined by ultrasonography, occurred

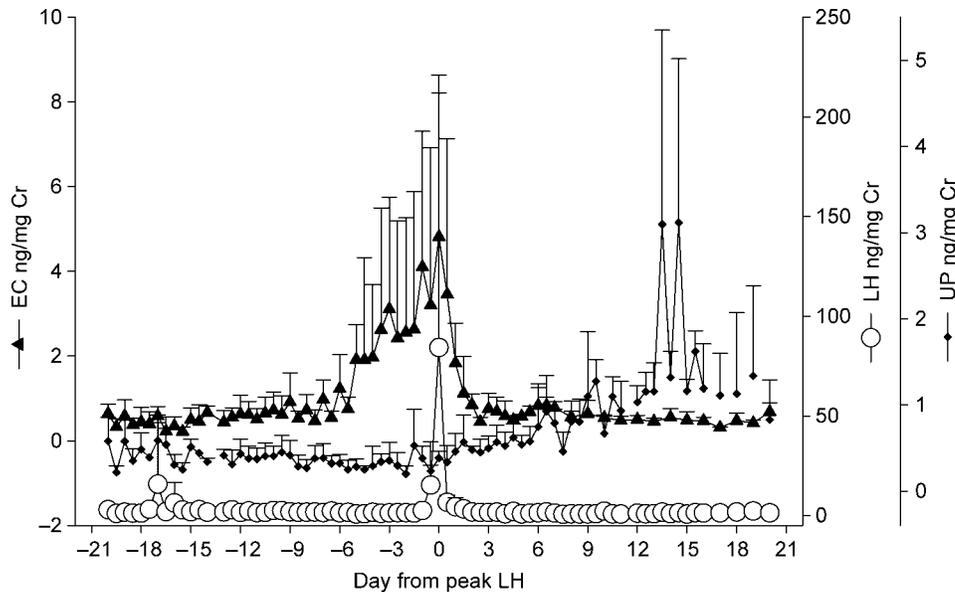


Figure 3 Mean values of bottlenose dolphin estrous cycle components, including urinary concentrations of LH ($n = 12$), EC ($n = 12$) and UP ($n = 9$). Note the LH peak occurs approximately the same time (8 h after) as the peak EC.

26.8 ± 7.1 h, 32.1 ± 8.9 h and 24.3 ± 7.0 h after peak EC, LH surge onset and peak LH, respectively (Fig. 6). All but one animal sonogrammed after ovulation (within 12 h) consistently had a 'donut shaped' structure consisting of a hyperechoic ring around a small anechoic center (Fig. 5). Female 7 was examined during ovulation with the follicle contracting into a stellate appearance (Fig. 5).

The stellate structure disappeared within 1 h, leaving a 'donut shaped' echosignature. The longevity of the 'donut shaped' structure was not determined because once ovulation had been confirmed the animal was not examined again for 1 wk. The earliest point that the CL could be detected was not determined, due to the infrequency of examinations. A cavitated hypoechoic CL was

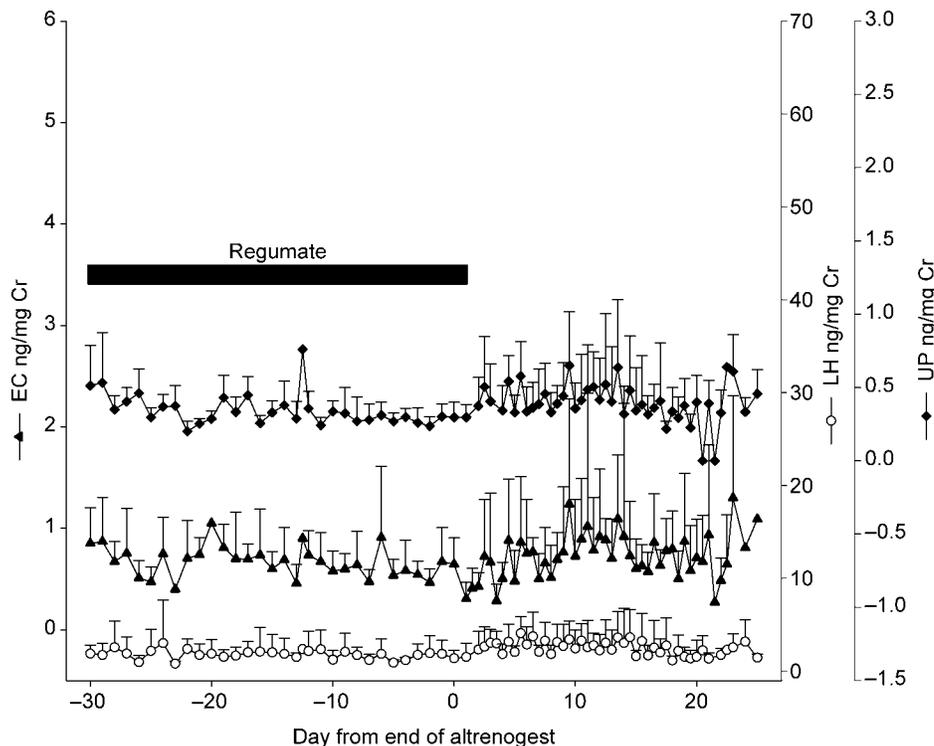


Figure 4 Mean LH, EC and UP concentrations from animals that did not ovulate after altrenogest treatment (black bar). Please note the increase in estrogen concentrations approximately 11 d after treatment ended.

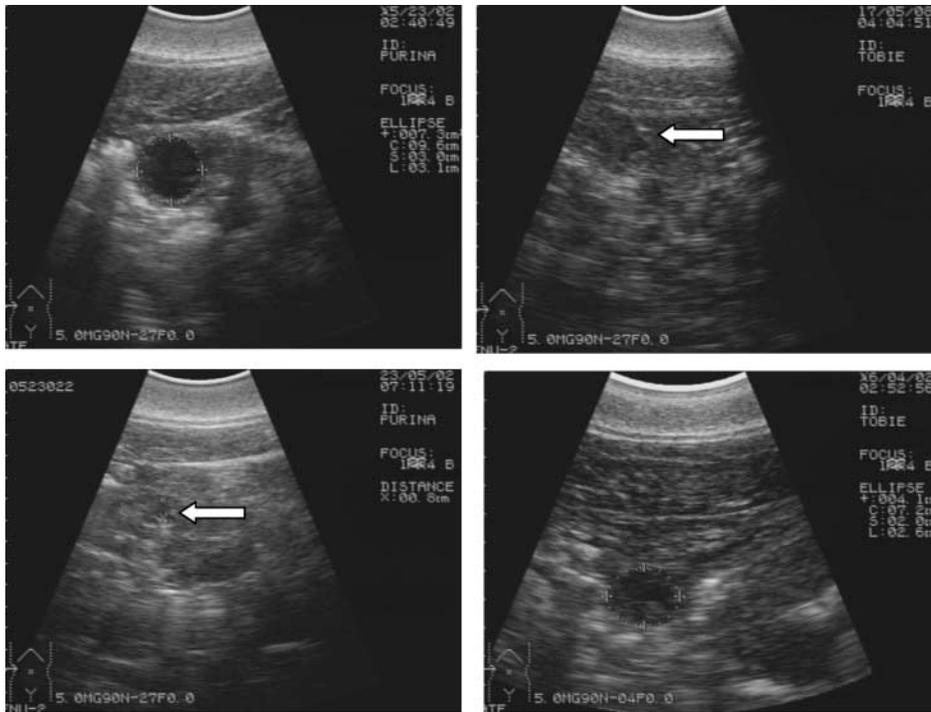


Figure 5 Bottlenose dolphin ovarian ultrasonographic images. A round, turgid preovulatory follicle (outlined by calipers, top left panel); the stellate (white arrow) appearance of an ovulating follicle (top right panel); the post-ovulatory 'donut shaped' signature (white arrow, bottom left panel); a corpus luteum (outlined by calipers) of pregnancy with a central fluid filled (anechoic) cavitation (bottom right panel).

observed during the early post-ovulatory interval in females 7, 8 and 12, which later appeared as a homogenous hypoechoic structure (Fig. 5). All non-pregnant animals had a homogenous CL. Fluid in the uterus was detected as early as 5 wk post AI. However, since each facility had differing access to an ultrasound machine and varying technical skills at interpreting the images, no conclusion could be made concerning the earliest time that pregnancy could be diagnosed. Overall, all pregnancies were confirmed by ultrasonography between 5 wk and 3 months post AI (Fig. 5).

Ejaculate characteristics in undiluted, liquid-stored, post-transport and post-thawed sperm

Characteristics of 13 ejaculates collected from four males are shown in Table 2. Overall, ejaculates were of high quality with total percent sperm motility and viability >84%. During liquid storage for up to 24 h post-collection, samples retained 94.6% of the SMI found in undiluted ejaculate (Table 2). The longest a sample was stored before being used for an insemination was 24 h, and this insemination in female 9 resulted in a pregnancy.

Motility parameters of dolphin spermatozoa frozen in straws either over liquid nitrogen (method 2: PM $56 \pm 12.1\%$) or using a programmable freezer (method 3; PM $61.8 \pm 3.2\%$) maintained a higher post-thaw percent SMI than straws frozen with pellets on dry ice (method 1: PM $25.7 \pm 12.4\%$). However, statistical comparisons could not be made between the freezing methods because samples were frozen years apart, with different diluents and different freezing rates (Table 2).

AI

AI was performed in seven animals during eight estrous cycles from May 2002 to June 2004 (Table 3). The first two animals were inseminated based on peak EC and follicle size and appearance (turgid round follicle, Fig. 5). The first animal (female 7) was inseminated as ovulation was occurring and the second (female 8) was inseminated within 4 h post-ovulation. Both animals were only inseminated once with frozen-thawed sperm and on both occasions they were inseminated directly into the uterus (Table 3). Both animals subjectively exhibited behavioral estrous 24 to 12 h prior to ovulation. Characteristics of behavioral estrous included displays of listing on the water surface, sinking and showing reduced responsiveness during standard training sessions.

The timing of inseminations for the next three animals (females 9–11) was based solely on the presence of a pre-ovulatory follicle. The first two of these females (females 9 and 10) were inseminated three to five times prior to ovulation (Table 3). The third animal (female 11) was inseminated twice beginning 24 h after behavioral estrous was first detected, but before ovulation. The remaining three animals (females 12 and 13) were inseminated based on the timing of the LH surge and the presence of a growing preovulatory follicle. All three inseminations were intra-cornual, occurring in the horn ipsilateral to the ovary with the preovulatory follicle (Table 3). As with the previous estrous cycles, all three exhibited subjective behavioral changes associated with estrous 48–12 hr prior to ovulation. For all trials, the mean number of inseminations per cycle were 1.9 ± 1.5 (Table 2).

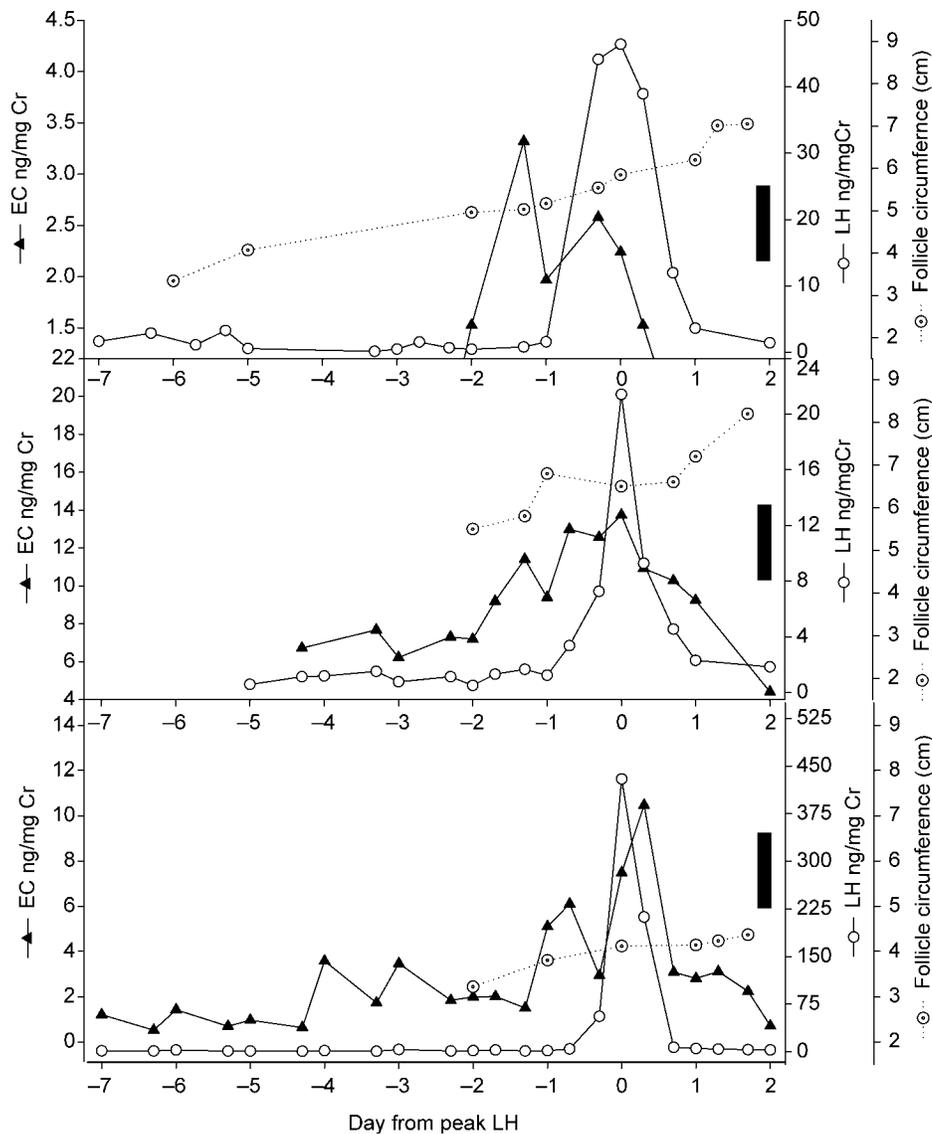


Figure 6 Follicular growth of primary follicles in three animals, female 4 (top graph), female 6 (middle graph) and female 12 (bottom graph), in relationship to urinary LH and EC. The black bar represents ovulation.

The overall conception rate (liquid stored (1) + cryopreserved semen (4) = 5 total conceptions/8 estrous periods \times 100) was 63%. For the two AIs using liquid stored semen, the mean number of progressively motile spermatozoa per insemination was 296 ± 143.7 and $100 \pm 27.3 \times 10^7$ spermatozoa, (females 9 and 10, respectively). For the six AIs using frozen-thawed semen, the mean number of progressively motile spermatozoa per insemination was $62.2 \pm 60.5 \times 10^7$ spermatozoa. The lowest dose of frozen-thawed progressively motile spermatozoa that resulted in conception was 27×10^7 spermatozoa, and the mean doses for conceptive and non-conceptive AIs were $75 \pm 81.0 \times 10^7$ and $35.5 \pm 14.8 \times 10^7$ spermatozoa, respectively. The conception rate using frozen-thawed spermatozoa was 67% (4/6; Table 3). The mean time from AI to ovulation in

conceptive and non-conceptive cycles was -2.4 ± 3.8 h (range -6.5 to 2 h) and -5.3 ± 2.1 h (range -3.0 to -6.0 h), respectively.

Females 7, 8 and 10 delivered their calves at 373, 361 and 376 days post AI, respectively. Female 11 aborted a fetus at an estimated 135 days post-conception and female 12 was diagnosed as pregnant by ultrasonography at 53 days post-conception.

Discussion

Successful development of AI procedures for *ex-situ* management of captive wildlife species first requires fundamental research of female and male reproductive physiology. As one step toward achieving this goal, the current study presents detailed information on

Table 2 Characteristics of bottlenose dolphin ejaculates used for artificial insemination^a.

Parameter	Neat ejaculates (<i>n</i> = 13) ^b	Liquid stored (0 to 24 h post collection; <i>n</i> = 8) ^c	Pellet post thaw (<i>n</i> = 3) ^d	Straws frozen over liquid nitrogen post thaw (<i>n</i> = 3) ^e	Straws frozen in programmable freezer (<i>n</i> = 2) ^f
Semen characteristic					
Volume (ml)	11.5 ± 8.5	10.0 ± 0.0 ^g	16.7 ± 2.9 ^g	11.8 ± 5.5 ^g	12.0 ± 2.8 ^g
Sperm concentration (× 10 ⁷ ml ⁻¹)	96.4 ± 93.4	41.3 ± 53.8	15.2 ± 12.1	15.0 ± 11.3	12.0 ± 0.0
Total spermatozoa per ejaculate (× 10 ⁷ ml ⁻¹)	572.2 ± 418.1	173.5 ± 128.9 ^h	61.1 ± 32.4 ^h	85.7 ± 95.7 ^h	43.5 ± 3.5 ^h
Sperm characteristic					
Total progressive motility (%)	84.4 ± 14.5	77.5 ± 16.6	29.3 ± 11.5	51.9 ± 9.8	55.0 ± 2.8
Kinetic rating (0–5) ⁱ	4.9 ± 0.2	4.6 ± 0.4	3.8 ± 0.3	4.8 ± 0.3	5.0 ± 0.0
Sperm motility index ^j	415.3 ± 73.4	92.8 ± 12.3 ^j	25.7 ± 12.4 ^k	56.0 ± 12.1 ^k	61.8 ± 3.2 ^k
Viability (%)	92.2 ± 4.9	93.5 ± 2.8	–	67.7 ± 6.7	68.6 ± 19.1

^a Values are the means ± s.d.

^b A total of 13 ejaculates were collected from 4 males.

^c A total of 8 inseminations were performed using 4 ejaculates from male 3; the ejaculates were held for 24 h or less at 4 °C.

^d A total of 6 ejaculates were cryopreserved and combined during thawing during 3 inseminations. Semen represent two pooled cryopreserved ejaculates.

^e A total of 2 ejaculates were cryopreserved and used during 3 inseminations.

^f A total of 1 ejaculate was cryopreserved and used during 2 inseminations.

^g Final volume of insemination dose.

^h Total progressively motile spermatozoa per insemination.

ⁱ Kinetic rating of spermatozoa graded subjectively: 0, no movement; 5, rapid forward progression.

^j Sperm motility index = total progressive motility × kinetic rating.

^k Mean percentage of neat sperm motility index for each sample, or % SMI.

the reproductive endocrinology of ten female bottlenose dolphins. By combining this knowledge with previously validated ultrasound technology (Brook 2001), this research has described the temporal relationship between reproductive hormones and ovulation, and applied the information, for the first time in any marine mammal species, to produce live offspring after AI with frozen-thawed spermatozoa.

Earlier work focusing on the reproductive cycle of bottlenose dolphins relied on measuring serum concentrations of total estrogens, progesterone and LH. Due to limitations of sampling frequency, it was not possible to adequately describe key endocrine events in the estrous cycle. Previous work characterizing the female reproductive cycle in killer whales using urinary monitoring provided a model for similar investigations in the bottlenose dolphin (Walker *et al.* 1988, Robeck *et al.* 1993, 2004).

Individual bottlenose dolphins tend to exhibit distinct reproductive seasonality, which varies depending on the animal's geographic origin (Urian *et al.* 1996). Bottlenose dolphin species that exhibit polyestrous activity (Indo-Pacific bottlenose dolphins) exhibit a mean inter-ovulatory interval (as defined by ultrasound) of 30.2 ± 1.7 days (Brook 2000). In the present study, the inter-ovulatory interval (defined as the interval between successive LH peaks) in a single Atlantic bottlenose dolphin was 36 days. More work is clearly needed to determine if this difference is due to individual animal variation or to differing characteristics between Indo-Pacific bottlenose

dolphin (a tropical species of bottlenose dolphin) and the Atlantic and Pacific bottlenose dolphins (temperate species).

As was described for the killer whale (Robeck *et al.* 2004), consistent detection of the LH surge appears to require twice daily sample analysis. In fact, the time from baseline to peak LH occurred within 9.4 ± 3.0 h, which may indicate that even twice-daily sampling (i.e. at 12 h intervals) may be insufficient for quantifying the LH surge. The period from peak LH to ovulation in the bottlenose dolphin (24 h) was 36% shorter than that for the killer whale (38 h; Robeck *et al.* 2004).

The ability to accurately detect the timing of the LH surge was critical for developing a reliable indicator of approaching ovulation and timing of AI in the bottlenose dolphin. Since the preovulatory estrogen peak is temporally broad-based and variable, this measure has limited use for pinpointing the time of ovulation. Conversely, urinary LH concentrations are basal until the preovulatory LH surge associated with impending ovulation. Our mobile endocrine laboratory (Steinman *et al.* 2003), which permits rapid on-site assessments of EC and LH, has eliminated the need to predict ovulation based on follicle size or appearance. As was demonstrated during the AI of females 9–11, wide individual variation in preovulatory follicle size prevented use of this parameter for predicting ovulation. As a result, more inseminations were required when AI timing was based on follicular size assessments (female 9, 3 inseminations; female 10, 5 inseminations;

Table 3 Bottlenose dolphin artificial insemination data.

Parameter	Dates of artificial insemination							
	23-05-02	23-05-02	01-07-02 to 03-07-02	03-07-02 to 06-07-02	06-07-02 to 07-07-02	04-10-03	08-06-04	27-06-04
Female inseminated	7	8	9	10	11	12	13	12
Cryopreservation method	Str/N ₂ ^a	Str/N ₂ ^a	Liquid stored	Liquid stored	Pellets ^b	Prog. freezer ^c	Str/N ₂ ^a	Prog. freezer ^c
Inseminations per oestrus ^d	1	1	3	5	2	1	1	1
Frequency of inseminations (h)	na	na	12	12	12	na	na	na
Maximum preovulatory follicle diameter (cm)	1.7	3.1	1.9	2.1	2.4	1.89	2.29	1.43
Maximum preovulatory follicle circumference (cm)	5.2	9.6	5.8	6.5	7.6	4.9	7.01	1.43
Side of ovulation	Left	Left	Right	Right	Left	Left	Left	Left
Site of semen deposition during insemination	Uterine body	Uterine body	Uterine body	Uterine body	Uterine body	Left uterine horn	Left uterine horn	Left uterine horn
Insemination volume (ml)	15	14.75	10, 10, 10	10, 10, 10, 10	15, 15	10	5.5	14
Total progressively motile spermatozoa per insemination (x 10 ⁷)	36	196	130, 379, 379	98, 98, 87, 72, 145	65, 27	46	25	41
Time of last insemination	08:30	17:00	06:00	17:00	10:00	20:30	00:30	11:30
Day of ovulation	23-05-02	23-05-02	03-07-02	06-07-02	07-07-02	05-10-03	08-06-04	27-06-04
Estimated time of ovulation	08:15	19:00	12:00	00:30	12:00	03:30	03:30	11:30
The last insemination time to ovulation (h)	+0.30	+2.0	-6.0	-6.0	-2.0	-7.0	-3.0	-6.5
Peak oestrogen conjugates to ovulation (h)	16.5	-	-	-	-	32	34.5	23.5
Start of LH surge to ovulation (h)	23.0	-	-	-	-	21.5	40	40
Peak LH to ovulation (h)	16.5	-	-	-	-	16.0	27	32
Conception	Y	Y	N	Y	Y	N	N	Y
Date of parturition	30-05-03	18-05-03	-	17-07-03	15-11-02 ^e	-	-	-
Gestation length (d)	373	361	-	376	135	-	-	-

^a Str/N₂ = method 2, straws (0.5 ml) held 4.5 cm over liquid nitrogen vapor for 10 min prior to plunging.

^b Pellets = method 1, 0.2 ml drops of sperm suspension on dry ice (modification of Seager *et al.* 1981).

^c Prog. freezer = method 3, straws frozen in programmable freezer (Minidigicool, IMV International) as follows: 5°C to -80°C at -100°C min⁻¹; -80°C to -140°C at -200°C min⁻¹ (Robeck & O'Brien 2004).

^d Total number and frequency of inseminations per oestrus period.

^e Animal aborted after 132 d of gestation.

female 11, 2 insemination per female) than with urinary LH determinations (females 12 and 13, 1 insemination per female).

Altrenogest has been used previously to synchronize estrous in killer whales and Pacific white-sided dolphins (Robeck *et al.* 2000, 2004). In domestic species, the period from end of altrenogest treatment to ovulation typically approximates the duration of the follicular phase. When altrenogest was administered to bottlenose dolphins for intervals ranging from 29 to 77 days, ovulation occurred 21 days post-withdrawal, which is nearly three times the duration of a normal follicular phase (8 days). A protracted period from hormonal withdrawal to ovulation was also observed in killer whales (25 days) and Pacific white-sided dolphins (21 days). The exact mechanism for this delay in delphinids is unknown, but may reflect differences in follicular recruitment compared with domestic species or duration of altrenogest treatment.

In the horse, altrenogest is most effective at synchronizing estrous during the transitional period and during the breeding season with little or no effect during seasonal anestrus (Webel & Squires 1982, Squires *et al.* 1983). Similarly, bottlenose dolphins tend to exhibit seasonal estrous activity from spring to autumn, and this is the time period in which normal altrenogest-induced ovulations occurred (i.e. May to September). While during the remaining months, animals appeared to exhibit a partial response (slight increase in EC without follicular development) to the hormone treatment. Similar partial responses (i.e. estrous behavior, but without ovulation post-altrenogest) have also been observed in horses during the anestrus period (Allen *et al.* 1980, Squires *et al.* 1983).

Evaluation of ovaries using trans-abdominal ultrasonography has been previously reported in bottlenose dolphins (Robeck *et al.* 1998, Brook 2000, 2001) and recently in killer whales (Robeck *et al.* 2004). Brook (2000, 2001) was the first to describe follicular growth during natural cycles in the Indo-Pacific bottlenose dolphin and reported a POF diameter of 19.9 ± 1.1 cm. A similar POF size (2.1 cm diameter) was found in this study, however, unlike Indo-Pacific bottlenose dolphins, POF size in this study did not increase with body size.

Histological and ultrasonographic evidence suggests that ovulation in bottlenose dolphins occurs predominantly on the left ovary (68–83%; Ohsumi 1964, Harrison & Ridgway 1971, Brook 2000). Results herein show that 82% of the ovulations occurred on the left ovary, thereby providing further support for the aforementioned studies. Similarly to earlier observations (Brook 2000), secondary follicles were observed during the exams, all of which regressed shortly before ovulation.

In addition to the turgid nature of the POF (Brook 2000), a follicle was observed with a stellate appearance (that had been previously turgid and round) whose form preceded the post-ovulation 'donut' ultrasonographic signature seen consistently within hours after ovulation. It is suspected that this was a follicle in the process of

ovulating and, if true, ovulation in bottlenose dolphins may occur over a period of less than 30 min (the time from initial follicle observation to post-ovulatory 'donut' shaped signature). Using once daily observations, Brook (2000) described ovulation as the disappearance of the POF, but the donut signature was not observed. Therefore, it is speculated that the donut signature is probably present for less than 24 h following ovulation. In agreement with Brook (2000), cavitated CL was observed only during pregnancy.

The raw ejaculate characteristics presented in this paper are similar to those of previous reports (Schroeder & Keller 1989, Robeck & O'Brien 2004). In this study, using liquid-stored semen, one of two animals conceived when inseminated within 12 h of ovulation. During the initial trials with liquid-stored semen, no effort was made to establish a minimum insemination dose. Based on the results of this study, it appears that if using liquid stored semen, fewer than 200 million progressively motile spermatozoa can be used for intrauterine AI in bottlenose dolphins.

Although not directly comparable, post-thaw sperm quality was highest using the programmable freezer in TYB extender. These results are in agreement with the recent report describing this method (Robeck & O'Brien 2004). Despite the variations in post-thaw motility among the cryopreservation methods employed in this study, all resulted in conceptions. These results suggest that less technological alternatives for banking bottlenose dolphin spermatozoa, such as pelleting sperm on dry ice or freezing sperm in straws over liquid nitrogen (in association with the appropriate cooling and freezing rates), can be used to successfully produce offspring following AI with frozen-thawed spermatozoa.

AI in bottlenose dolphins was first attempted by Schroeder & Keller (1990). That report described using an endoscope to deposit spermatozoa in the vaginal fold, just distal to the cervix (also called the spermathecal recess or pseudocervix). The lack of established pregnancies in this report led to speculation that intrauterine sperm placement would be required for AI in this species (Robeck *et al.* 1994). The unique structure of the dolphin cervix and pseudocervix (Green 1977, Robeck *et al.* 1994) requires an endoscope to gain access to the uterus. Accordingly, AI trials conducted previously (FM Brook *et al.*, unpublished, Robeck *et al.* 2001) and herein demonstrated that access to the cervix and uterus was possible providing that the pseudocervical opening could be adequately visualized.

During the development and improvement of the AI procedures described in the paper, the goals of the research were to improve the timing of AI, and as a consequence reduce the number of inseminations required per estrous and the number of spermatozoa required for each insemination trial. The development of the rapid onsite LH assay system improved the investigators' ability to time the inseminations around ovulation, resulting in offspring production following a single insemination. While the minimum dose of frozen-thawed spermatozoa required

for successful AI is unknown for the bottlenose dolphin, this study demonstrates that conception can occur from a dose as low as 270 million progressively motile spermatozoa. Defining the lowest dose of spermatozoa necessary for conception will be the focus of continued research.

Previous research had established effective methodologies for the long-term storage of bottlenose dolphin spermatozoa and now these data have demonstrated the capability to produce offspring following AI using cryopreserved semen. This is an important milestone for the captive genetic management of bottlenose dolphins and this study highlights the value of strategic and systematic investigation into the basic reproductive physiology of a wildlife species to allow the development of assisted reproductive technologies.

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