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REPRODUCTION

Seasonality, estrous cycle characterization, estrus synchronization, semen cryopreservation, and artificial insemination in the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*)

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Abstract

The reproductive physiology of the Pacific white-sided dolphin, *Lagenorhynchus obliquidens*, was characterized to facilitate the development of artificial insemination (AI) using cryopreserved spermatozoa. Specific objectives were to: 1) describe reproductive seasonality of the Pacific white sided dolphins; 2) describe urinary LH and ovarian steroid metabolites during the estrous cycle; 3) correlate LH and ovarian steroidal metabolite patterns to ultrasound-monitored follicular growth and ovulation; and 4) assess the efficacy of synchronizing estrus, sperm collection/cryopreservation, and intrauterine insemination. Ovulations (64%, $n=37$) and conceptions (83%, $n=18$) occurred from August to October. Peak mean serum testosterone (24 ng/ml), cross-sectional testicular area (41.6 cm^2), and sperm concentration (144.3×10^7 sperm/ml) occurred in July, August, and September respectively. Spermatozoa were only found in ejaculates from July to October. Estrous cycles ($n=22$) were 31 d long and were comprised of a 10 d follicular and 21 d luteal phase. Ovulation occurred 31.2 h after the onset of the LH surge and 19.3 h after the LH peak. Follicular diameter and circumference within 12 h of ovulation were 1.52 and 4.66 cm respectively. Estrus synchronization attempts with altrenogest resulted in 17 (22%) ovulatory cycles with ovulation occurring 21 d post-altrenogest. Ten AI attempts using cryopreserved semen resulted in five pregnancies (50%). The mean gestation length was 356 days (range 348–367). These data provide new information on the Pacific white-sided dolphin's reproductive physiology and collectively enabled the first application of AI in this species.

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Introduction

Although a common species found throughout the northern Pacific, almost nothing is known about the reproductive physiology of the Pacific white-sided dolphin, *Lagenorhynchus obliquidens*. While these animals are prevalent in the wild (Hammond *et al.* 2008), few have been held in captivity. Out of the estimated 119 animals currently maintained in 23 zoos or aquaria around the world, only 10% of this population are residing in the USA. Despite the small numbers of animals held in USA aquaria, the majority of successful captive breeding (eight out of ten) has occurred in one USA facility with one male.

The successes that have been made through natural captive propagation are currently threatened by the lack of both available breeding males and cooperative genetic management practices among USA facilities. Inbreeding within small captive populations must be avoided to ensure long-term population health and enable the development of self-sustaining populations (Wildt 1992, Wildt *et al.*1997). Artificial insemination (AI) combined with semen cryopreservation tools may provide the vehicle necessary to avoid inbreeding depression within this captive population. However, information on the basic reproductive function of the species must be obtained before such assisted reproductive technologies can be implemented.

Previous efforts towards describing the reproductive biology of this species have relied exclusively on post-mortem analysis of reproductive tracts from animals caught as bycatch in fisheries or that died in captivity (Harrison *et al.* 1972, Harrison & McBrearty 1977, Walker *et al.* 1986, Ferrero *et al.* 1993, Ferrero & Walker 1996). Based on the presence of at least one corpus luteum, females reach sexual maturation at around 11 years of age and 170 cm in length. Conception occurs from June to August with an 11-month gestation (Harrison *et al.* 1972, Ferrero *et al.* 1993). Males reach sexual maturity at 170–180 cm from 10 to 11 years of age and exhibit seasonal changes in spermatogenesis (Harrison *et al.* 1972, Ferrero & Walker 1996).

Real-time evaluation of living animals is required to move beyond this basic biological information collected from post-mortem data. Analysis of urinary reproductive hormones combined with ovarian ultrasound exams has resulted in describing both follicular dynamics and hormonal profiles during estrous cycles and the perio-vulatory period in killer whale (Walker *et al.* 1988, Robeck *et al.* 1993, 2004), bottlenose dolphin (Robeck *et al.* 2005b), and beluga (Steinman *et al.* 2007). The description of the periovulatory period allowed for the development of methods for consistent prediction of ovulation. Ovulation prediction combined with insemination trials enabled the development of consistently repeatable methods for AI in these three cetacean species (Robeck *et al.* 2004, 2005b, O'Brien *et al.* 2008). Despite these successes in other cetaceans, virtually no data have been published on hormone profiles and follicular dynamics of the Pacific white-sided dolphin.

In addition to ovulation prediction, methods for ovulation control or estrus synchronization allow for practical application of AI to any species. Altrenogest has been used as both a contraceptive agent and to synchronize estrus for AI in the killer whale, bottlenose dolphin (Young & Huff 1996, Robeck *et al.* 2004, 2005b, O'Brien & Robeck 2006), and beluga (Robeck *et al.* 2007, O'Brien *et al.* 2008). Application of this technique for ovulation manipulation in the Pacific white-sided dolphins, if successful, would allow for improved management of natural breeding and for synchronizing estrus for AI.

Semen cryopreservation combined with AI enables long-term genetic management, since valuable male gametes, particularly from founder animals, can be reintroduced into the population long after the male's normal reproductive contribution (Wildt 1992, Wildt *et al.* 1997). This approach also maximizes the global genetic exchange of gametes among *ex situ* populations, but semen cryopreservation methods must be optimized for AI to reach its full potential as a management tool. Methods for semen cryopreservation have been developed for three cetacean species, the bottlenose dolphin, the killer whale, and the beluga (Seager *et al.* 1981, Schroeder & Keller 1990, Robeck & O'Brien 2004,

Robeck *et al.* 2004, 2005a, 2005b, O'Brien & Robeck 2006, 2007). Despite their accessibility in captivity, no information is available concerning semen production or cryopreservation in the Pacific white-sided dolphin.

The overall goal of this research was to gain a sufficient level of understanding of the Pacific white-sided dolphin's reproductive physiology to develop AI using cryopreserved semen. To accomplish this, specific objectives were to: 1) describe reproductive seasonality in captive male and female Pacific white-sided dolphins; 2) determine the excretory dynamics of urinary LH and ovarian steroid metabolites during the estrous cycle; 3) correlate LH and ovarian steroidal metabolite patterns to ultrasound-monitored follicular growth and ovulation; and 4) assess the efficacy of synchronizing estrus, sperm collection/cryopreservation, and intrauterine insemination.

Results

Seasonality

Female

Based on profiles of serum progesterone (P), a total of 37 ovulations ($n=8$ females) resulting in 17 conceptions were observed from 1980 to 2001 (Table 1). Twenty-four out of 37 ovulations (64.7%) and 14 out of 17 conceptions (82%) occurred from August to October (Fig. 1).

Male

Mean monthly testosterone (T) concentrations peaked in July (24.3 ± 1.3 ng/ml; $n=52$) and were greater ($P<0.05$) than the mean value from every other month of the year (range: February 0.09 ± 0.02 ng/ml, $n=6$, to June 5.6 ± 3.8 ng/ml, $n=7$; Fig. 1). Peak cross-sectional testicular area occurred in July and August, with the largest size occurring in August (41.6 ± 7.2 cm^2; Fig. 1). Testicular cross-sectional area in August was larger ($n=32$; $P<0.05$) than every month except July (36.2 ± 10.7 cm^2). The stromal parenchyma remained a homogeneous stippled appearance with a well-defined mediastinum and an oval cross-sectional shape throughout the year. However, relative to the echotexture of the hypaxialis lumborum muscle (HLM), the overall testicular echotexture gradually changed from slightly iso- to hypoechoic during nadir diameter months (January and February) to hyperechoic during months of maximal testosterone excretion (June–September; Fig. 2). Maximum hyperechogenicity subjectively appeared to correlate with maximum cross-sectional testicular diameter; while maximum hypoechogenicity was observed in conjunction with minimum testicular diameter (Fig. 2).

While differences ($P<0.05$) between the mean amounts of ejaculate collected each month existed, no correlation between amount of ejaculate collected and

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

Animal	Facility[a]	Sex	Birth date	Weight (kg)	Reproductive history[b]	Contribution[c]
1	SWT	F	1976[d]	104	Three abortions	P, US
2	SWT	F	1978[d]	124	Two calves, one abortion	P, US
3	SWT	F	1980[d]	131	One calf	P, US
4	SWT	F				
5	SWT	F	1978[d]	118	Four calves	P, US, UH, ES, AI
6	SWT	F	09/1993[e]	127	One calf	P, US, UH, ES, AI
7	SWT	F	1979[d]	129	Four calves	P, US, UH, ES, AI
8	SWT	F	10/1996[e]	107	One calf	P, US, UH, ES
9	Shedd	F	1985[d]	90	Nulliparous	P, US, UH, ES
10	Shedd	F	1988[d]	123	Nulliparous	P, US, UH, ES
11	Shedd	F	1988[d]	91	Nulliparous	P, US, UH, ES, AI
12	Shedd	F	1988[d]	101	Nulliparous	P, US, UH, ES, AI
13	Shedd	F	1988[d]	86	Nulliparous	P, US, UH, ES, AI
1	SWT	M	1978[d]	154	Sired 12 calves	Seasonality
2	KSW	M	1983[d]	140	No sired calves	Cryopreserved semen
3	KSW	M	1991[d]	120	One sired calf	Cryopreserved semen

P, serum progesterone; US, ultrasound exam; UH, urinary hormones; ES, estrous synchronization; AI, artificial insemination.
[a]SWT, SeaWorld Texas; Shedd, John G Shedd Aquarium; KSW, Kamogawa Sea World. [b]Reproductive history prior to start of urine collection data for animals. [c]Data that the animal contributed to the study. [d]Estimated age for wild caught. [e]Captive born.

the presence of spermatozoa ($P > 0.05$) was detected. Ejaculates containing spermatozoa were only found from July to October. Both mean concentration and total spermatozoa per ejaculate in September ($1443.0 \pm 776.4 \times 10^6$ spermatozoa/ml) were significantly greater ($P < 0.05$) than July ($86.0 \pm 57.3 \times 10^6$ spermatozoa/ml), August ($647.8 \pm 737.1 \times 10^6$ spermatozoa/ml), or October ($394.3 \pm 202.9 \times 10^6$ spermatozoa/ml; Fig. 1).

Endocrine monitoring

Hormone (estrogen conjugates (EC), LH, and urinary progestins (UP)) profiles during the periovulatory interval of 7 natural cycles and 15 post-altrenogest cycles were monitored during the study interval. Four out of the seven natural cycles occurred as repeat cycles after the animals (females 7, 5, and 8) had a non-conceptive post-altrenogest cycle. Female 13 cycled twice during a season without altrenogest pre-treatment. Based on continuously elevated progesterone and the presence of a CL (as determined by ultrasonography), female 11 had a retained CL post ovulation (August 18, 2002) for 86 days in 2002 and again post AI (August 16, 2003) without any ultrasound evidence of pregnancy for 103 days in 2003. The mean interval between successive EC and LH peaks ($n = 5$) was 31.0 ± 1.9 and 30.9 ± 1.7 d respectively. The luteal phase between successive ovulations was 20.8 ± 2.4 d.

For natural and synchronized estrous cycles, the mean length of the follicular phase and luteal phase was 10.0 ± 2.4 d ($n = 22$, range: 7–14 d) and 20.8 ± 2.4 d ($n = 9$, range: 17–24 d) respectively. The preovulatory EC rise was 3.2 ± 1.0 d ($n = 22$, range: 1–6 d). The interval between peak EC and peak LH was 16.7 ± 12.8 h ($n = 12$, range: 0–31 h). The interval from the onset of the LH surge to peak LH was 13.4 ± 4.8 h ($n = 7$, range: 8.0–18 h). The LH surge duration was 27.9 ± 3.1 h ($n = 6$, range, 24–29 h). Peak EC and LH concentrations were 14.5 ± 13.0 ng/mg Cr ($n = 22$, range: 3.8–62.7 ng/mg Cr)
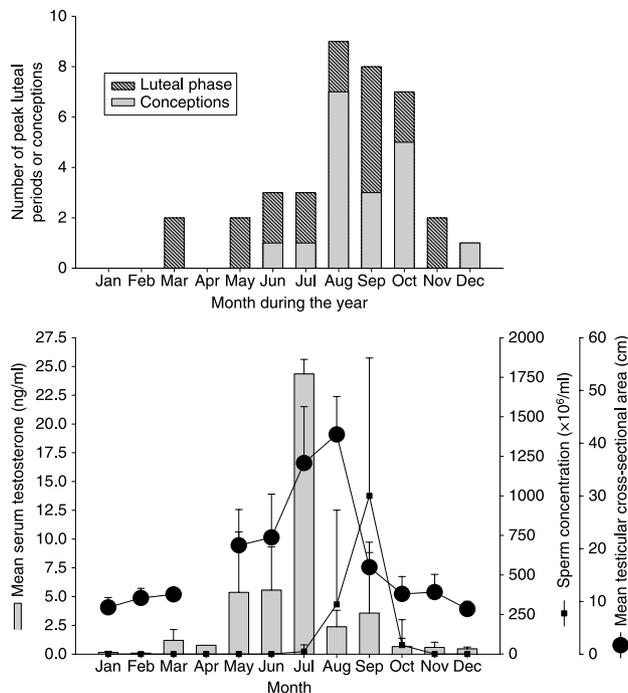

Figure 1 Demonstrates seasonal variations in female (top graph) and male (lower graph) reproductive parameters. For the female ($n = 7$), the bar charts illustrate frequency of luteal activity and conceptions. For the male ($n = 1$), profiles mean monthly serum testosterone ($n = 52$), sperm concentration ($n = 290$), and testicular cross-sectional area ($n = 32$) as determined by ultrasonography.
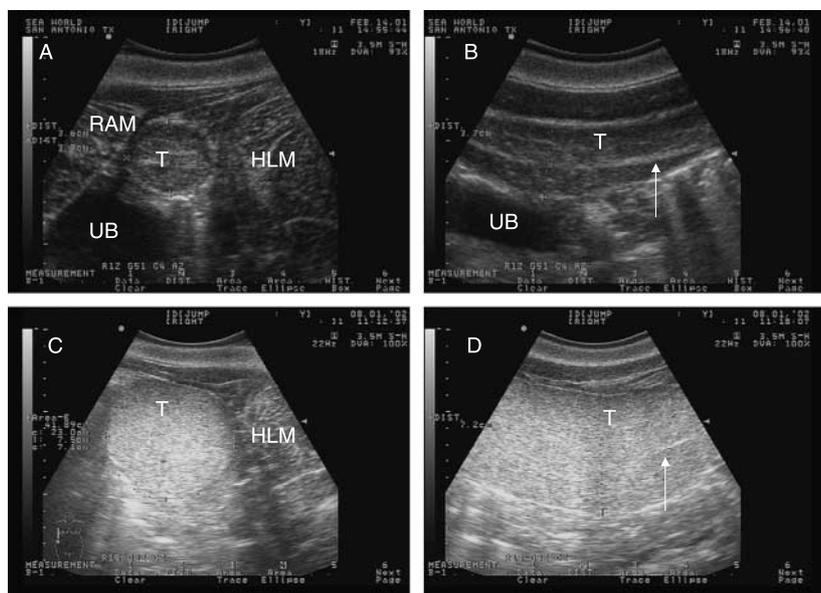
Figure 2 Transverse (A and C) and longitudinal (B and D) ultrasounds images of the testes in an adult Pacific white-sided dolphin, *Lagenorhynchus obliquidens*, during February (A and B) and August (C and D) of the same year. HLM, hypaxialis lumborum muscle; RAM, rectus abdominus muscle; T, testis; UB, urinary bladder. Scale for all images is in centimeters.

and 206.7±174.4 ng/mg Cr (*n*=22, range: 32.2–672 ng/mg Cr) respectively. The interval between peak LH and the first discernable post-ovulatory increase in UP was 1.8±1.0 d (*n*=17, range: 1–4 d). Hormone data from all animals were combined using day 0 as day of the peak LH to develop a composite dolphin estrous cycle (Fig. 3).

The canine LH (cLH) test empirical colorimetric descriptions when corresponding to the quantitative LH (qLH) test were as follows: slightly visible: 2.6±0.5 ng/ml; slightly less than: 15.5 ng/ml; equal to: 34.7 ng/ml; slightly greater than: 53.4±5.7 ng/ml; or maximal: 88.6±10.0 ng/ml. Results also demonstrated that subjective creatinine (Cr) concentrations based on urine color (low, medium, and high) were comparable with concentrations determined using the quantitative assay (low: 0.22±.03 mg Cr; medium: 0.25±0.03 mg Cr; high: 0.78±0.07 mg Cr).
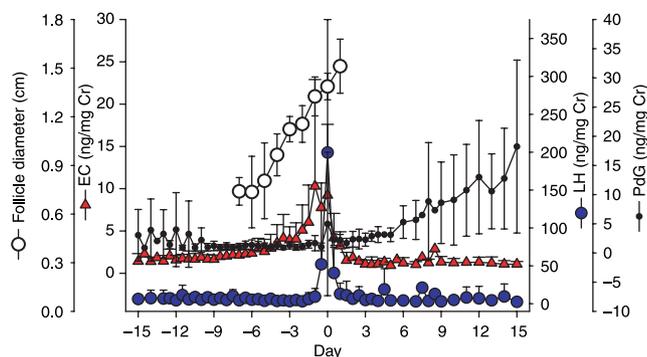

Figure 3 Mean values of the Pacific white-sided dolphin estrous cycle components, including urinary concentrations of EC (*n*=12), LH (*n*=12), UP (*n*=9), and normal primary follicle growth.

Estrous synchronization and follicular recruitment

Out of the 76 altrenogest treatments, 17 (22%) resulted in subsequent ovulation. Three of the females accounting for 19 treatments never ovulated in response to altrenogest or on their own. If such females are removed from analyses, the remaining six females responded 30% (17 ovulations/57 treatments) of the time. For the females that responded to synchronization, the mean time from the end of altrenogest treatment to the beginning of the follicular phase, the LH surge, and ovulation were 15.2±5.5 d (*n*=17, range: 7–22 d), 20.6±4.4 d (*n*=17, range: 14–29), and 21.6±4.4 d (*n*=17, range: 15–30) respectively. Out of the 76 altrenogest treatments, 18 were repeat treatments within the same year in females that did not respond to the first round or in one case where the animal (female 12) ovulated but was not inseminated. Ten ovulations (17%, 10 out of 58 total first treatments) occurred after the first round of altrenogest treatments, while six ovulations (27%, 6 out of 22) occurred after a second round of treatment, with female 12 ovulating after both the first and second treatments. Out of the 22 total ovulations, 5 (23%) were repeat ovulations that occurred spontaneously after the first altrenogest induced ovulation of that season.

Ultrasonographic evaluation of ovaries

Location and general appearance of ovaries were similar to what has previously been described for bottlenose dolphins and the Indo-Pacific dolphins (Brook 2001, Brook *et al.* 2004). As with these other species, the ovaries were ovoid in appearance with a relatively hyperechoic hilus and hypoechoic cortex (Fig. 4). In ten
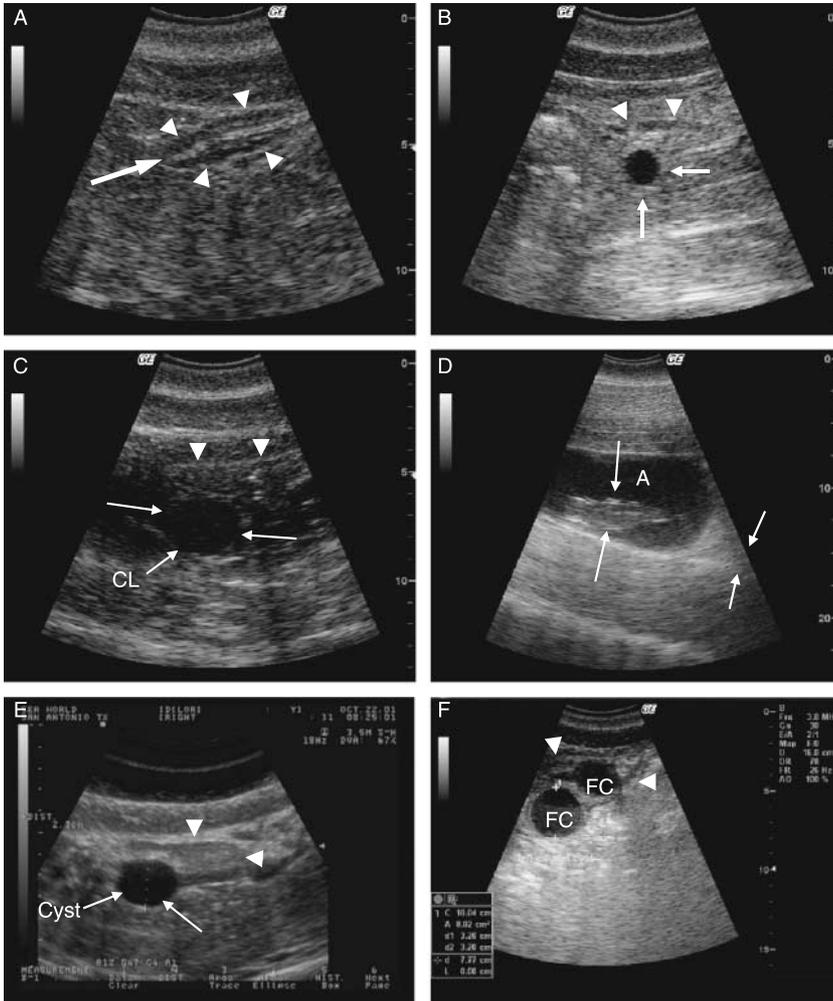
Figure 4 The Pacific white-sided dolphin ovarian (white arrow heads in all images) and uterine ultrasonography images. (A) Normal ovary with hypoechoic cortex and hyperechoic ovarian hilus (large arrow); (B) a round, turgid preovulatory follicle with thickened wall (long arrows); (C) a corpus luteum (CL: long arrows) of pregnancy; (D) day 87 pregnancy with amniotic fluid (A) and the fetus (white arrows); (E) ovarian cyst (white arrows); and (F) cystic follicles (FC).

females, multiple ultrasonographic measurements of the length and width of the right ($n=24$) and left ovaries ($n=36$) were 4.71 ± 0.8 by 1.6 ± 0.2 cm and 4.72 ± 0.9 by 1.8 ± 0.3 cm respectively. No significant differences between the sizes of the right and left ovaries were detected ($P>0.05$).

The mean number of days required for the eventual preovulatory follicle (POF) to develop from a diameter of 0.8 and 1.0 cm to ovulation was 6.3 ± 1.3 d ($n=10$) and 4.8 ± 1.1 d ($n=12$) respectively. The daily growth rate in circumference and diameter from 6 days prior to and until ovulation was 0.41 and 0.12 cm/d respectively (Figs 3 and 5). The POF maximum circumference and diameter were 4.66 ± 0.50 cm ($n=16$, range: 4.15–5.70) and 1.52 ± 0.16 cm ($n=16$, range: 1.25–1.83 cm) respectively. The POF consistently became round prior to ovulation and was located on the left ovary 100% of the time.

Secondary follicles at the time of ovulation on the ipsilateral ovary ($n=4$) to the POF were observed in 31% of the examinations (Fig. 5). In addition, two of these

females had two tertiary follicles >0.5 cm in diameter. The maximum diameter of secondary follicles was 1.18 ± 0.19 cm. While ovulations occurred exclusively on the left ovary, small follicles (0.5–0.8 cm) were often (not quantified) observed on the right or left ovary prior to the development of a dominant follicle. All the secondary and tertiary follicles on the right ovary, and most on the left ovary, regressed well before ovulation (Fig. 5).

Female 3 consistently had multiple ($n>5$) follicles, 0.5–1.0 cm in diameter, on both ovaries throughout the year. Despite the persistence of the 'cystic' ovarian structures, the female became pregnant through natural breeding. Female 7 had a large 2.0 cm cyst on her right ovary that was first observed in 1999 and remained unchanged throughout the study (Fig. 4).

The time of ovulation as determined by ultrasonography occurred 35.8 ± 9.2, 31.2 ± 8.6, and 19.4 ± 6.6 h after peak EC, LH surge onset, and peak LH respectively (Fig. 5). All pregnancies could be confirmed by ultrasonography between 6 and 8 weeks post AI.
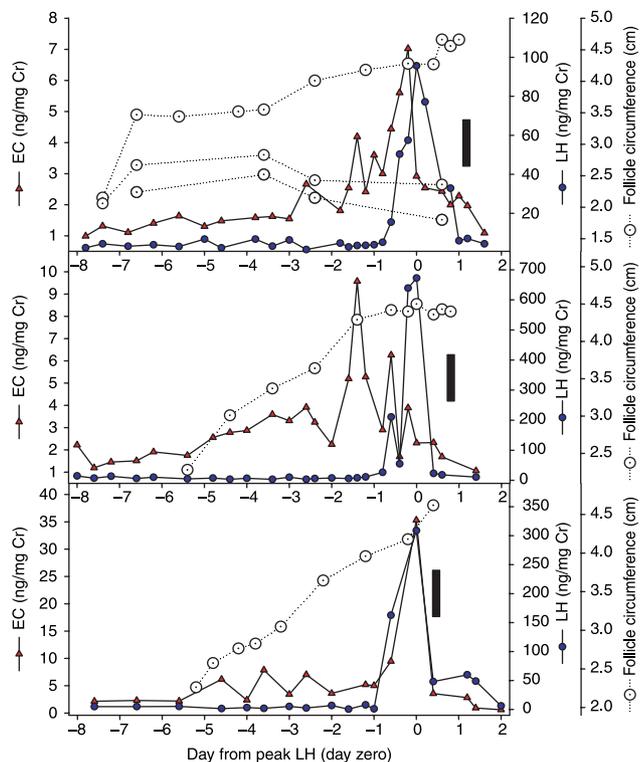
Figure 5 Follicular growth of primary and secondary follicles (top panel only) in three animals in relationship to urinary EC and LH. The black bar represents ovulation as determined by ultrasonography.

Ejaculate characteristics of raw and post-thawed sperm used during the AI trials

Owing to the over representation of male 1 in the US population, only ejaculates collected from male 2 and 3 were used for AI. The characteristics of these ten ejaculates are shown in Table 2. Overall, ejaculates from both males were of high quality with progressively motile (PM) spermatozoa and viability both >88%. For the straw-freezing method, total motility (TM), PM, and viability were all well maintained during the cryopreservation/thawing process (48, 47, and 64% of initial characteristics respectively; Table 2). For the directional freezing method, high levels of TM, PM, and viability were retained following cryopreservation/thawing (94.0, 91.6, and 99.5% of initial characteristics respectively).

Artificial inseminations

AI was performed in five females during ten estrous cycles from September 2001 to October 2008 (Table 3). During the development of AI, initial attempts (2001) relied on the presence of POFs after the animals had been administered altrenogest. As we developed both improved methods for semen deposition (uterine versus cervical) and increasingly rapid and accurate methods for determining urinary EC and LH

concentrations, we were able to decrease the number of inseminations prior to ovulation and increase the AI success rate (Table 3).

For all AI trials, the mean number of inseminations per cycle was 1.9 ± 1.5. The overall conception rate (five total conceptions/ten estrus periods $\times 100$) was 50%. However, if the first two attempts where semen was placed in the cervix are omitted, the conception rate (five total conceptions/eight estrus periods $\times 100$) was 63%. Four out of the five conceptions occurred after an altrenogest synchronization treatment. The lowest dose of frozen–thawed PM spermatozoa that resulted in conception was 26.6×10^7 spermatozoa, and the mean dose (only the last insemination closest to ovulation was used if multiple inseminations per estrus were preformed) for conceptive and non-conceptive inseminations were $184.7 \pm 117.0 \times 10^7$ and $111.98 \pm 71.2 \times 10^7$ spermatozoa respectively. The mean time from AI to ovulation in conceptive and non-conceptive cycles was -6.0 ± 3.8 h (range: -9.5–2.5 h) and -6.4 ± 8.2 h (range: -15.2–4 h) respectively.

Females 7, 12, 13, and 6 delivered their calves 367, 358, 354, and 348 (mean 356.7 ± 8.0 d) days post AI respectively. As of March 4, 2009, female 6 has an ultrasonographically normal 164-day-old fetus.

Discussion

The Pacific white-sided dolphin, one of the six species in the genus *Lagenorhynchus*, is found throughout the coastal regions of the Northern Pacific Rim from Baja California to the Sea of Japan and Taiwan (Walker *et al.* 1986). Although the species is relatively abundant by International Union for Conservation of Nature (IUCN; Hammond *et al.* 2008) estimates, the basic reproductive biologic data required to judge the potential for a species to withstand external pressures are lacking. However, their relative abundance places the species in an ideal situation where current *ex situ* reproductive research efforts may be employed if future environmental conditions threaten their survival. For example, the recent extinction of the Baiji (*Lipotes vexillifer*) could not be prevented because *ex situ* reproductive research efforts were delayed until the species was already critically endangered (Turvey *et al.* 2007). Thus, with no history of successful *ex situ* breeding or even a basic understanding of that species' biology, little could be done to prevent their demise.

In addition to developing an understanding of normal reproductive physiology of an *ex situ* population, more intensive management tools, like gamete cryopreservation and AI, can be developed to help maintain and possibly help preserve critically endangered species. In an effort towards reaching this goal, this research has used endocrine monitoring, with serum and urine, to characterize a range of reproductive events including reproductive seasonality, preovulatory follicular development,

Table 2 Characteristics of the Pacific white-sided dolphin semen used for artificial insemination.

Parameter	Neat ejaculate male 2[a] ($n=10$)	Neat ejaculate male 3[a] ($n=5$)	Post-thaw male 2 (0 h, $n=18$)	Post-thaw male 3 (0 h, $n=5$)	Post-thaw male 3 (0 h, $n=2$)
Freezing method			Straw/LN$_2$	Straw/LN$_2$	DS[b]
Semen characteristics					
Volume (ml)	5.51 ± 4.8	9.8 ± 3.6	11.5 ± 5.4[c]	14.0 ± 7.0[c]	8.8 ± 1.1[c]
Sperm concentration ($\times 10^7$/ml)	71.3 ± 45.9	53.7 ± 33.0	25.3 ± 11.9	18.7 ± 3.5	19.5 ± 7.0
Total spermatozoa per ejaculate ($\times 10^7$/ml)	319.1 ± 09.1	614.0 ± 605.6	129.7 ± 102.6	249.3 ± 82.7	171 ± 26.8
Sperm characteristics					
Total motility	95.3 ± 4.6	88.0 ± 4.5	50.8 ± 9.1	42.5 ± 10.6	82.5 ± 3.5
Percent progressive motility	98.1 ± 1.2	98.8 ± 0.4	96.2 ± 2.2	96.5 ± 2.1	96.5 ± 2.1
Progressive motility	93.5 ± 4.6	87.0 ± 4.8	48.9 ± 9.2	40.9 ± 9.3	79.7 ± 5.2
Kinetic rating (0–5)[d]	5 ± 0	5 ± 0	4.1 ± 0.7	4.6 ± 0.1	4.6 ± 0.1
Sperm motility index[e]	467.5 ± 4.9	434.8 ± 24.0	202.3 ± 50.7	185.8 ± 39.6	366.7 ± 35.0
Viability (%)	88.4 ± 1.91	92.2 ± 3.0	70.2 ± 11.8	59 ± 5.7	91.8 ± 0.4

Values are means ± S.D.
[a]A total of ten ejaculates were cryopreserved from males 2 and 3 and used during 18 inseminations. [b]DS, directional solidification freezing method. [c]Final volume of insemination dose. [d]Kinetic rating of spermatozoa graded subjectively: 0, no movement; 5, rapid forward progression. [e]Sperm motility index = progressive motility × kinetic rating.

and ovulation. The combination of endocrine evaluation with the development of semen collection, storage, and cryopreservation techniques, as well as ovarian ultrasound evaluation, has enabled us to develop the first successful and repeatable AI method in this species.

Previous to this research, no information had been published on the reproductive cycle of Pacific white-sided dolphins. However, methods used in this research were also developed or in some cases evolved simultaneously from research conducted with the killer whale and bottlenose dolphin, the first two cetaceans where the development of AI was successful (Robeck *et al*. 2004, 2005*b*). As with killer whales and bottlenose dolphins, training of the Pacific white-sided dolphins for daily urine collection was required before endocrine-monitoring techniques could be applied (Walker *et al*. 1988, Robeck *et al*. 1993, 2004).

Unlike killer whales and bottlenose dolphins, male and female Pacific white-sided dolphins exhibit distinct reproductive seasonality. The data of this study are in support of post-mortem research from wild populations, which identified a distinct 3-month pattern of reproductive activity (Harrison *et al*. 1972, Ferrero *et al*. 1993). However, females of the present study had the highest incident of both estrus activity and calving during August to October, while data from the wild Northern Pacific ocean population suggest peak reproductive activity occurs from June to August (Ferrero *et al*. 1993). This 2-month shift in seasonal reproductive activity may reflect the different geographical origin of founder animals in the captive population. Additional evidence in support of a geographical influence on seasonal reproductive patterns is the limited period of semen production by the two males located in Japan (incidentally caught in fisheries off Japan's Northern coast) to

June and early July (S Inoue, unpublished observations), while the USA male's peak seasonality corresponded with the females in this study. Also, the recent successful natural birth of a Pacific white-sided dolphin in Japan (E Katsumata, unpublished observations) has occurred in May. Recent mtDNA evidence suggests that distinct genetically isolated population groups exist even between the relatively close populations of the Sea of Japan and Northern Pacific coastal animals (Hayano *et al*. 2004). Varying evolutionary pressures based on geographic location have been hypothesized as being responsible for the existence of regional differences in peak reproductive activity across populations of bottlenose dolphins (Urian *et al*. 1996).

The seasonal changes in semen production were different from any cetacean described to date, with the Pacific white-sided dolphin male demonstrating a true seasonal decrease in serum testosterone concentrations, a corresponding seasonal azoospermia, and an ultra-sonographically documented significant decrease in testicular size. Both the bottlenose dolphin and the killer whale exhibit a diffuse seasonality, where females can breed throughout the year and males can exhibit seasonal peaks in T, which can vary within the animal and between years among animals. However, sperm production remains unchanged for both these species (Robeck & O'Brien 2004, Robeck & Monfort 2006). The beluga, which has a defined and repeatable reproductive seasonality, undergoes estrus activity from March to June (Robeck *et al*. 2005*a*). A male beluga exhibited periods of peak serum T (October–April) and sperm production (January–June). While the same male displayed a decrease in serum T and sperm production during T nadir months of July–September, ejaculates were never azoospermic (O'Brien *et al*. 2008). The Pacific

Table 3 Pacific white-sided dolphin artificial insemination data.

Parameter		Dates of artificial insemination									
	Sep 19–21 2001	Aug 7 2002	Sep 5–6 2002	Oct 7–8 2002	Oct 7–8 2002	Oct 14–15 2005	Oct 16 2005	Aug 26 2007	Nov 10 2007	Sep 20 2008
Female inseminated	7	7	7	12	13	5	6	12	11	6
Cryopreservation method	Str/N_2	Str/N_2	Str/N_2	Str/N_2	Str/N_2	Str/N_2	Str/N_2	Str/N_2	Str/N_2	Dir
Inseminations per estrus[a]	4	1	3	3	4	2	1	1	1	1
Frequency of inseminations (h)	1.2	NA	12	12	12	12	NA	NA	NA	NA
Maximum preovulatory follicle diameter (cm)	2.84	1.6	1.43	1.47	1.37	1.48	1.51	1.45	1.51	1.47
Site of semen deposition during insemination	Cervix	Cervix	Uterine body	Uterine body	Uterine body	L uterine horn	L uterine horn	L & R uterine horns	L & R uterine horns	L uterine horn
Insemination volume (ml)	3, 9, 8, 9	13	12, 4, 16	5, 12, 18	12, 17, 21, 21	12, 7	8	14	25	8
Total progressively motile spermatozoa per insemination ($\times 10^7$)	19.1, 71.8, 36.5, 41.0	231.2	128.5, 111.7, 218.1	47.3, 156.8, 187.0	181.3, 257.3, 307.0, 307.0	58.3, 94.1	26.6	85.9	107.7	115.5
Day of ovulation	Sep 22 2001	Aug 7 2002	Sep 6 2002	Oct 8 2002	Oct 9 2002	Oct 14 2005	Oct 16 2005	Aug 27 2008	Nov 10 2007	Sep 20 2008
The last insemination time to ovulation (h)	< −13	4	−3	−2.5	−9.5	−4.75	−9.0	−15.25	−9.75	−3.0
Length of observable estrus prior to ovulation (d)	–	1.5	1.5	7	1	2	4	8	3	4.5
Peak estrogen conjugates to ovulation (h)	–	−48	−34	−40	−42	−46.5	−26	−31	−34.5	−18
Start of LH surge to ovulation (h)	–	–	−17	−28.5	–	−39	−32.5	−43	−31.5	−43
Peak LH to ovulation (h)	–	–	−9	−15	−18	−21	−22	−31	−26.5	−18
Conception	No	No	Yes	Yes	Yes	No	Yes	No	No	Yes
Gestation length (d)	–	–	367	358	354	–	348	–	–	Pending

Str/N_2, straws (0.5 ml) held 4.5 cm over liquid nitrogen vapor for 10 min prior to plunging; Dir, directional freezing; NA, not applicable.
[a]Total number of inseminations per estrus period.

white-sided dolphin in the current study exhibited similar but more pronounced peak sperm production with a lag time after peak T production of 60 days. In contrast, peak testicular diameter had a lag time after peak T of only 30 days. This evidence supports a spermatogenic cycle of 60 days, similar to other domestic species (Amann & Schanbacher 1983).

During the study, animals ovulated twice during their season, demonstrating that this species can be seasonally polyestrus. The inter-ovulatory interval of 31 d ($n = 5$) is similar in length to that for the Indo-Pacific bottlenose dolphin (30 d, Brook *et al.* 2004) and the Atlantic bottlenose dolphin (33 days, Robeck *et al.* 2005*b*; TR Robeck & JK O'Brien 2008, unpublished observations).

Consistent detection of the start of the preovulatory LH surge required that urine samples were collected for monitoring at a minimum of three times a day. Samples collected every 4 h were used to define the temporal dynamics of the LH surge, which demonstrated an increase from baseline to peak of 14 h, longer than that which was observed for bottlenose dolphins (9 h; Robeck *et al.* 2005*b*). However, the length of time from the LH peak to ovulation in the Pacific white-sided dolphin of 17 h was 29 and 55% shorter than that observed for bottlenose dolphins (24 h: Robeck *et al.* 2005*b*) and killer whales (38 h: Robeck *et al.* 2004) respectively.

The strategy of inseminating every 12 h once estrus and/or a POF has been detected and until ovulation is not appropriate for species where semen is of limited supply or unique in value (e.g. sexed semen). To enable efficient use of semen, accurate prediction of ovulation timing is critical. During the initial development of AI for this species, a rapid LH test that could reliably predict ovulation was not available. Thus, we relied on our onsite EC assay to determine peak estrogen production by the POF, and use this point to predict approaching ovulation. As we improved the speed and portability of the LH assay (Steinman *et al.* 2003, O'Brien & Robeck 2006), we were able to rely on the more accurate and consistent LH surge to predict ovulation.

Once the LH surge had been characterized (2001–2005), we were able to use three samples per day at 6 hourly intervals (e.g. 0800, 1400, and 2000 h), with a 12 h gap during the night. However, if the surge commenced at night during the 12-h-sampling gap, this sampling strategy diminished our ability to accurately predict LH surge initiation. In order to address this problem, two methods were employed, 1) the use of the rapid (20 min) qualitative cLH test kit and 2) multiple urine tests (every 3–4 h) after the first positive test. The analysis of multiple urine samples allowed for a semi-quantitative indicator of the direction in which the LH concentration was heading (up or down). When this information was combined with the known interval from LH surge onset to peak LH concentration (14 h), we could estimate the timing of start of the LH surge, and thus predict the timing of approaching ovulation.

The use of altrenogest in marine mammals for contraception or as a synchronization agent has been well documented in killer whales, bottlenose dolphins, and beluga (Young & Huff 1996, Robeck *et al.* 2001, 2004, 2005*b*, Steinman *et al.* 2007). However, unlike killer whales and bottlenose dolphins, at the time of study, the Pacific white-sided dolphin was the first seasonally estrus cetacean where altrenogest had been used. As a result, when to administer the hormone (before or during the short breeding season) for optimum effectiveness was unknown.

In the horse, altrenogest is most effective at synchronizing estrus during the breeding season with little or no effect during seasonal anestrus (Webel & Squires 1982, Squires *et al.* 1983). Since the Pacific white-sided dolphins exhibited seasonal estrus activity from July to October, no attempts were made to synchronize them outside of this period. Even with this targeted administration plan, the overall success of this administration was only 16%. The reason for this poor response when compared with bottlenose dolphins, which typically have a 50% response to regumate (Robeck *et al.* 2005*b*), is unknown and may simply be an effect of the Pacific white-sided dolphin's tight reproductive seasonality.

The use of altrenogest as a synchronization agent in the Pacific white-sided dolphins results in a long delay from hormone withdrawal to ovulation of 20 d. This response is similar to the bottlenose dolphin (Robeck *et al.* 2005*b*). Both of these species have similar follicular dynamics, in that they do not have large numbers of recruitable follicles present on the ovary at any given time (i.e. follicle >4.0–4.5 mm for bovine; Guilbault *et al.* 1991, Ginther *et al.* 1998). Thus, they do not have continuous waves of follicles from which a portion is always receptive for recruitment post-progesterone administration. Since only small, non-growing follicles (<0.4 mm in diameter) are available for recruitment, the lack of continuous follicular waves in dolphins may account for the protracted response from progestogen withdrawal to ovulation when compared to the domestic pig (7–9 days; Kraeling *et al.* 1981, Pursel *et al.* 1981) and the horse (9 days for the normally cycling horse; Squires *et al.* 1983, Daels *et al.* 1996). Understanding the mechanism for the initiation of this recruitment may provide the solution for developing a more effective estrus induction and synchronization method.

Evaluation of ovaries using transabdominal ultrasonography has been previously reported in other cetacean species including the bottlenose dolphin (Robeck *et al.* 1998, 2005*b*, Brook 2001), Indo-Pacific humpback dolphin (Brook *et al.* 2004), killer whales (Robeck *et al.* 2004), and the beluga (Robeck *et al.* 2007). The circumferential daily growth rate of 0.41 cm and the maximum POF diameter of 1.5 cm in the Pacific white-sided dolphin were different from the bottlenose dolphin of 0.47 and 2.1 cm respectively. Since the follicular phase length is similar for both species,

the slightly faster growth rate for the bottlenose dolphin is reflective of a larger POF at ovulation. In addition, dominant follicle selection appears to occur around 0.8 cm in the Pacific white-sided dolphin as opposed to ~1 cm in the bottlenose dolphin. Similar to bottlenose dolphins (Robeck *et al.* 2005*b*), POF size in the Pacific white-sided dolphins did not correlate to urinary estrogen concentrations or animal body mass.

Ovulation occurred 100% of the time on the left ovary of the Pacific white-sided dolphin, this is an even greater degree of unilateralism than what was found in the bottlenose dolphin where ovulation occurred 82% on the left ovary (Ohsumi 1964, Robeck *et al.* 2005*b*). The reason for this unilateralism in ovulation is unknown. Also, as with bottlenose dolphins and Indo-Pacific dolphins (Brook *et al.* 2004), secondary follicles were observed; however, unlike bottlenose dolphins, secondary follicles did not regress but were present at ovulation in 31% of the observed ovulations. Cystic follicles developed in two different females during natural cycles. Two females had what appeared to be cystic ovaries throughout the study. One animal had a single large ovarian cyst that never changed in size and the other had multiple small cysts that remained on the ovary throughout the year. Abnormal ovarian cysts have been observed in bottlenose dolphins and in a killer whale. The origin and/or significance of these cysts are unknown, but all animals where this has been observed (including the animals in this study) were able to conceive.

The raw ejaculate characteristics presented herein are the first published data from this species, and during their season they are comparable in quality and concentration to those obtained from the bottlenose dolphin (Schroeder & Keller 1989, Robeck & O'Brien 2004). Owing to the limited number and overrepresentation of the male available for semen collection in the US, all the inseminations were completed using frozen–thawed semen imported from two males located in Japan. The minimum effective dose of 260 million PM spermatozoa required for conception in the Pacific white-sided dolphin is similar to that of cryopreserved bottlenose dolphin semen (270 million PM spermatozoa; Robeck *et al.* 2005*b*), but is considerably more than that achieved with sex-sorted, cryopreserved bottlenose dolphin spermatozoa (150 million PM spermatozoa; O'Brien & Robeck 2006). This suggests that future insemination efforts with the Pacific white-sided dolphin may require fewer PM spermatozoa for success.

The straw method for cryopreserving semen yielded similar results between the two males evaluated. Since ~50% of initial motility was maintained post-thawing, it appears that this method is adequate for gamete storage and use with AI. While not directly comparable, the directional freezing method enabled 94% of initial motility to be maintained post-thaw, thereby illustrating the potential vast improvement this technology can offer

over the conventional straw method. These findings are in agreement with recent cryopreservation studies using sexed bottlenose dolphin spermatozoa (O'Brien & Robeck 2006) and non-sexed beluga semen (O'Brien & Robeck 2007). Clearly, future-controlled experiments objectively evaluating the potential of directional freezing technology in the Pacific white-sided dolphin are warranted.

The mean gestation length of 356 days and range from 348 to 367 d is the first description of both the normal length and the potential range of gestation in this species. The length is slightly shorter than bottlenose dolphins that have a mean of 377 d and range from 357 to 399 d (Robeck *et al.* 2005*b*; JK O'Brien & TR Robeck unpublished observations).

The combination of years of research on basic endocrinology, semen collection, and cryopreservation has allowed for the first successful application of AI in the Pacific white-sided dolphin. Owing to the small North American population size, the ability to utilize and improve these techniques will largely influence whether or not this species remains in captivity. With that in mind, future efforts with the Pacific white-sided dolphin will be focused on the development of methodologies for sex-selecting spermatozoa for use with the AI techniques described herein. Sex selection will allow for a faster increase in the female population or, if necessary, the production of a genetically elite male by inseminating the least represented female with Y chromosome-bearing spermatozoa from an unrepresented male (O'Brien *et al.* 2002, 2009). While this species is not endangered in the wild, the degree of genetic and reproductive management required to maintain the diversity of this small *ex situ* population provides an ideal model for the restoration of any cetacean species that may become endangered in the future.

Materials and Methods

Animals

Twelve female and three male Pacific white-sided dolphins (*L. obliquidens*) located at three facilities were used for various combinations of endocrine monitoring, semen collection, and/or insemination trials (Table 1). During the collection of seasonality data (progesterone and conception data), females 1–8 had constant access to a male for breeding purposes. These data were collected from females 1–8 located at SeaWorld of Texas (SWT; San Antonio, TX 78251, USA) from 1980 to 2001 prior to the initiation of the urine collection for cycle monitoring. Once the AI trials began, the male was separated from candidate females (SWT) for a minimum of 1 month prior to the procedures. Male 1 and females 1–8 located at SWT were housed in an ~7445 m^3 manufactured saltwater enclosure (temperature: controlled from 17 to 20 °C). Females 8–12 were housed in a 15 141 m^3 indoor manufactured saltwater habitat (water temperature from 17 to 20 °C) at the John G Shedd Aquarium (Chicago Zoological Society, Chicago, IL 60605, USA).

Males 2 and 3 were housed with a mixed age group of males in a 1200 m³ outdoor facility containing natural saltwater (Ambient water temperature: 17–28 °C) located at Kamogawa Sea World (Kamogawa, Chiba, Japan).

Animals located in the US were fed a diet of frozen–thawed whole fish (herring, *Clupea harengus*; capelin, *Mallotus villosus*; and Columbia River smelt, *Thaleichthys pacificus*). The male in Japan was fed chub mackerel (*Scomber japonicus*), Arabesque greenling (*Pleurogrammus azonus*), and capelin (*M. villosus*). 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 on unrestrained animals. All procedures described within were reviewed and approved by the Sea-World Incorporated Institutional Animal Care and Use Committee, and were performed in accordance with the Animal Welfare Act for the care of Marine Mammals.

Female reproductive seasonality

Serum samples ($n=511$) for P concentrations were collected for 8–23 years from females 1–7 on a bimonthly to monthly schedule (Table 1). For each individual female, P concentrations that exceeded 3 ng/ml and were at least 2.0 times the mean non-pregnant P concentration for that particular individual were considered presumptive evidence of luteal activity (Robeck *et al*. 2005a). When a sample below this threshold was serially adjacent to a sample above the threshold, the beginning or end of a luteal phase was defined as median point between these two samples. The value with the highest concentration during a period of luteal activity was considered the peak. To determine the frequency during each month that reproductive activity occurred, the total number of a peak luteal phase that occurred during each month was combined across all sample years to develop a composite 12-month period.

Male reproductive seasonality

Testosterone concentrations were measured in 52 serum samples collected bimonthly for 1 year and during routine health exams over 7 years from male 1. Ultrasound evaluations ($n=17$; Aloka 900 machine with a 3.5 MHz wide footprint convex linear transducer (Corimetrics Medical, Charlotte, NC, USA)) of right and left testicles were conducted monthly (Oct–Mar) or biweekly (May–Sept) for 1.5 years to determine whether seasonal changes occur in maximum testicular diameter and stromal echogenicity. The echogenic tissue patterns were characterized by comparing the echogenicity of the testes during each examination to the surrounding musculature (HLM) as described for bottlenose dolphins (Brook *et al*. 2000). Ejaculates ($n=290$) were collected from male 1 (see Methods below) throughout the year for 3 years to determine seasonal production of spermatozoa. Ejaculate sperm concentration and total volume were determined using standardized techniques (Robeck & O'Brien 2004).

Urinary endocrine monitoring

Urine samples were collected from unrestrained female Pacific white-sided dolphins as previously described for bottlenose dolphins (Robeck *et al*. 2005b). Briefly, the animals were trained to lay on their back in the water with their flukes and peduncle resting in the lap of a trainer who was sitting on the edge of the pool. A second trainer would apply firm, steady pressure on the abdomen directly over the urinary bladder. The animals would urinate in response to the pressure and eventually became conditioned to urinate with only a slight touch in the same location. The urine was aspirated from the genital slit with a 10 ml syringe. All urine samples collected ($n=3388$) were used for monitoring of estrous activity, evaluating synchronization attempts, or for 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 UP, EC, and LH. Urine samples were collected daily until 10 days post-synchronization, then from 3 (minimum) to 5 times a day during the estimated periovulatory period.

Determination of total estrous cycle length 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 (Robeck *et al*. 2004, 2005b). Baseline concentrations (BC) were determined for all hormones in each individual animal by standard methods. Deviations above or a return to baseline for a minimum of two consecutive samples were used to define the beginning or the end respectively of a physiologically significant change in a particular hormone. Using this method, the following intra-estrous cycle endocrine components were determined: length of the luteal phase (UP concentrations > than BC for 2 consecutive days), follicular phase (EC concentrations > BC for 2 consecutive days), start of follicular phase to peak EC, and peak EC to peak LH were determined. The preovulatory rise in EC concentrations was subjectively defined as values >2 s.d.s. above baseline 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 a minimum thrice daily sample collection. The beginning of the surge was defined as any value >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 from all Pacific white-sided dolphins for all of the above-mentioned intervals.

Endocrine data were compared to 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.

Creatinine assay

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

EIA for estrogen conjugates

Urinary EC were measured by single antibody, direct EIA as previously described (Robeck *et al*. 2004, 2005*b*). 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 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 of 0.6 M H_2SO_4 was added. Intra-assay variation was <10% and inter-assay variation was 11.1% and 11.7% at 30 and 70% binding ($n=163$). Serial dilutions of the Pacific white-sided 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 the Pacific white-sided dolphin urine was $96.7\pm30.2\%$ ($y=0.94x+0.585$, $R^2=0.99$). Immunoassay of fractions separated by reverse-phase HPLC analysis revealed one major immunoreactive peak (fractions 17–23, 21% of total) that co-eluted with estrone-3-sulfate.

LH enzyme immunoassay

A rapid urinary LH assay was used which allowed LH concentration determination within 2.5 h using a single antibody, direct EIA (O'Brien & Robeck 2006) previously modified from the double antibody EIA developed by Graham *et al*. (2002). Intra-assay variation was <10% and inter-assay variation was 12.3 and 11.1% at 30 and 60% binding ($n=124$). Serial dilutions of the Pacific white-sided 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 the Pacific white-sided dolphin urine was $42.8\pm15.7\%$ ($y=0.62x-0.74$, $R^2=0.99$).

In addition to the qLH assay, the rapid semi-quantitative cLH kit (Witness Synbiotics Corp., Kansas City, MO, USA) was validated by comparing results to the qLH. The kits were validated by comparing colorimetric changes in the sample line (when compared to the sample control line) to urinary concentrations as described by the qLH assay and to the control. The sample lines were described as: slightly visible, slightly less than, equal to, slightly greater than, or maximal when compared with the control line. Subjective Cr values were assigned to each sample based on degree of yellow color (low, medium, or high).

Assay for urinary progestins

UP were measured by single antibody, direct EIA as previously described (Graham *et al*. 2001, Robeck *et al*. 2005*b*). Intra-assay variation was <10% and inter-assay variations were 12.3 and 8.7% at 30 and 70% binding ($n=173$). Serial dilutions of the Pacific white-sided 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 the Pacific white-sided dolphin urine was $91.6\pm22.8\%$ ($y=0.99x-1.57$, $R^2=0.99$). Immunoassay of fractions separated by reverse-phase HPLC analysis revealed an immunoreactive peak at fractions 67–71 that co-eluted with progesterone and two peaks at 73–76 and 77–80 that were unidentified.

Synchronization of ovulation

To evaluate the effects of an orally administered exogenous progestin, a synchronization tool for use with AI, animals were placed on 0.044 mg/kg p.o. of altrenogest (Regu-Mate, Intervet Inc., Millsboro, DE, USA) once a day for treatment periods lasting from 20 to 30 d. From 2001 to 2008, a total of 76 treatments were administered to ten female Pacific white-sided dolphins. 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, and at least twice daily after, cessation of altrenogest treatment.

Ovarian ultrasonography

Transabdominal ultrasonography was used to detail follicular activity during natural cycles, following altrenogest-induced cycles and to confirm pregnancy as previously described for bottlenose dolphins (Brook 2001, Robeck *et al*. 2005*b*). Ultrasonographic examinations were performed using either an Aloka 900 machine (SWT: Corimetrics Medical), GE Logibook Book (SWT: GE LogiqGE Medical Systems, Milwaukee, WI, USA), or a SonoSite 180 Plus (Shedd: SonoSite, Inc., Bothell, WA, USA) all with a 3.5 MHz transducer (wide footprint convex linear probe). Females were examined once on day 0 and 10 d post-altrenogest, then daily to thrice daily from day 11 to ovulation. In addition, any female exhibiting a follicle of 0.8 mm or greater in diameter was examined daily until the fate of follicular development could be determined. Once a potential POF was observed, the ovaries were visualized a minimum of three times daily with ultrasound. Follicular diameter and circumference and the time of ovulation were determined as previously described (Robeck *et al*. 2005*b*).

Semen collection and processing

Ejaculates were collected from male 2 ($n=10$) and male 3 ($n=5$) for cryopreservation and use during the AI trials (Table 2). Males were trained for unrestrained ejaculation as previously described (Keller 1986, Robeck & O'Brien 2004). Briefly, the animals received various tactile stimulations to elicit voluntary extrusion of the penis from the genital groove. After an erection was obtained, animals were conditioned to ejaculate by stimulation often directed towards the perineal area. Once the animals subjectively appeared in a pre-ejaculatory state, the penis was grasped with a gloved hand (Nitrisoft, Nitrile latex free examination glove; Sintex, Houston, TX, USA) and the ejaculate directed into a 24 oz WHIRL-PAK (NASCO, Fort Atkinson, WI, USA).

Ejaculate concentration, volume, sperm motility, and viability (plasma membrane integrity) were determined using standardized techniques (Robeck & O'Brien 2004,

Robeck *et al*. 2005*b*). The percentages of motile sperm were subjectively determined to the nearest 5% by analyzing four to five fields of diluted spermatozoa (35 °C, 1:25, spermatozoa:egg yolk citrate cryodiluent (EYC); 2.9% Na citrate; 20% egg yolk (v/v); and gentamycin 50 ug/ml) using bright-field optics (×400, Olympus, Tokyo, Japan). TM, PM, and kinetic rating (KR, 0–5 scale; 0, no forward movement; 5, rapid forward progressive movement) were subjectively determined. A sperm motility index (SMI:PM×KR) was used for comparisons of sperm quality between fresh and frozen–thawed spermatozoa (Robeck & O'Brien 2004).

For assessment of viability, 10 µl of semen was mixed with 40 µl of a 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 the 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 frozen storage

Semen was processed for cryopreservation over 5 years, and as advancements in cetacean sperm cryopreservation methods evolved they were applied to the species herein. Thus, two cryopreservation methods were applied to semen used in the AI trials.

Method 1

Ejaculates (male 2, $n=10$; male 3, $n=3$) were diluted 1:1 (v/v) with EYC cryodiluent and cooled from 21 to 5 °C over 1 h (-0.27 °C/min). At 5 °C, the sample was further diluted (1:1) with glycerolated EYC (6% v/v final glycerol concentration) slowly over 5 min. 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) for 10 min then plunged into liquid nitrogen.

The straws were thawed by plunging directly into a 35 °C water bath and shaken vigorously for 1 min (8.3 °C/s). Straws were combined and 100 µl aliquot of the combined sample were removed and diluted (1:1 over 5 min) with EYC (warmed to 35 °C), and used for post-thaw analysis as previously described for raw ejaculates. The remainder of the sample was stored at 21 °C until the insemination.

Method 2

Ejaculates (male 2, $n=2$) were diluted 1:1 (v/v) with Beltsville extender (BF5F; Pursel & Johnson 1975). BF5F was modified to contain 52.3 mM TES, 16.5 mM Tris, 105.4 mM fructose, 105.4 mM glucose, 20% v/v egg yolk, and gentamycin 50 µg/ml (330 ± 5 mOsm/kg and pH 7.0 ± 0.1). The sperm solution was cooled from 21 to 5 °C over 1.5 h (≈ -0.2 °C/min). At 5 °C, the sample was further diluted (1:1) with glycerolated BF5F (5% v/v final glycerol concentration) in a stepwise manner over 30 min. After equilibration for 1 h, sperm suspensions were transferred to 9 ml hollow tubes (IMT

International Ltd, Chester, UK) for cryopreservation using a directional solidification ('directional freezing') machine (MTG-516, IMT). The hollow tube was moved through the first block (5 °C) for 45 s at a constant velocity (3 mm/s) before reaching a distance of 2 mm into the opening of a second block (-50 °C) and held for 30 s for initiation of seeding (rapid induction of ice nucleation from the seeding point throughout the length of the glass tube). The tube was then moved at 1 mm/s across the second block for 5 min before entering the collection chamber (-100 to -110 °C), followed by immediate transfer to liquid nitrogen. Hollow tubes were thawed in air for 90 s, then transferred to a 35 °C water bath equipped with modifications to enable uniform sample thawing (Harmony CryoCare Activator IMT International). One 9 ml tube was required for each insemination. A 100 µl aliquot of the sample was removed, and diluted (1:1 over 5 min) with BF5F (warmed to 35 °C) and used for post-thaw analysis as previously described for raw ejaculates. The remaining sample was stored at 21 °C until the insemination.

Artificial insemination

The first five inseminations (females 7, 12, and 13) from 2001 to 2002 were based on the presence of a presumptive POF and the detection of peak urinary EC. For these inseminations, urinary estrogen levels were determined twice daily when a female possessed a consistently growing follicle that had reached a diameter of 1.2 cm. Once peak EC concentrations occurred (estimated to have occurred by a decrease in EC concentrations in the successive sample) the female was inseminated $\sim$12 h after the peak, continuing every 12 h until ovulation. Inseminations 6 and 7 (2005; females 5 and 6) and insemination 10 (2008; female 6) were timed to occur 24 h after the start of the urinary pre-ovulatory LH surge and every 12 h until ovulation. During 2007, the insemination method for females 11 and 13 was modified to include methodology under parallel development in beluga (Robeck *et al*. 2007, Steinman *et al*. 2007, O'Brien *et al*. 2008) and bottlenose dolphins (TR Robeck & JK O'Brien 2007, unpublished observations). Females with apparent POFs (>1.4 cm in diameter) and elevated urinary estrogens for greater than 10 days were administered three i.v. injections of a GnRH analog (Cystorelin, Merial, Duluth, GA, USA; 250 µg per injection, q. 1 h) to control the timing of ovulation. The insemination took place 22 h after the first GnRH injection.

All females were pre-medicated with diazepam (Abbott Lab; 0.1–0.2 mg/kg) 1–2 h prior to each procedure. The females were removed from the water and placed in lateral recumbency on 10.2-cm-thick closed cell foam pads. All females were kept wet during the procedure and vital signs were monitored throughout. Inseminations were performed using a flexible endoscope (either a 8.5 mm external diameter, 150 cm long; Karl Storz Veterinary Endoscopy America, Santa Barbara, CA, USA, or a 6.0 mm external diameter, 120 cm long Olympus America, Melville, NY, USA) equipped with a catheter in the working channel (2.2 mm external diameter (5 fr), 190 cm long; SurgiVet, Waukesha, WI, USA). For the procedures, the endoscope was advanced into the cranial vagina. The vagina was insufflated with air to visualize the cervical opening

(Robeck *et al*. 2005*b*). The semen was deposited in the cervix (inseminations 1 and 2), uterine body (inseminations 3–5), or the uterine horn ipsilateral to the POF (inseminations 6–10).

Statistical analyses

Hormone and sperm quality data were analyzed by ANOVA and means compared using Newman–Keuls' multiple comparisons, Mann–Whitney *U* test for non-parametric data, Kruskal–Wallis ANOVA on ranks and Dunn's multiple comparisons (SigmaStat, Version 2.0. SPSS Inc., San Rafael, CA, USA). Repeated-measures ANOVA on ranks and Holm–Sidak multiple comparisons were used to compare monthly changes in hormone or testes size for seasonality data. Data were considered significant if $P < 0.05$ and were presented as mean $\pm$ s.d.

Declaration of interest

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