BIOLOGY OF REPRODUCTION **71,** 650–660 (2004)
Published online before print 28 April 2004.
DOI 10.1095/biolreprod.104.027961

Reproductive Physiology and Development of Artificial Insemination Technology in Killer Whales (*Orcinus orca*)[1]

T.R. Robeck,[2,3] K.J. Steinman,[4,7] S. Gearhart,[5] T.R. Reidarson,[6] J.F. McBain,[6] and S.L. Monfort[4]

SeaWorld Texas,[3] San Antonio, Texas 78251
Conservation and Research Center,[4] National Zoological Park, Smithsonian Institution, Front Royal, Virginia 22630
SeaWorld Orlando,[5] Orlando, Florida 32821
SeaWorld San Diego,[6] San Diego, California 92109
Center for Reproduction of Endangered Species,[7] Zoological Society of San Diego, San Diego, California 92112-0551

ABSTRACT

Research was conducted to define the basic reproductive physiology of killer whales (*Orcinus orca*) and to use this knowledge to facilitate the development of artificial insemination procedures. The specific objectives were 1) to determine the excretory dynamics of urinary LH and ovarian steroid metabolites during the estrous cycle; 2) to evaluate the effect of an exogenously administered, synthetic progesterone analog on reproductive hormone excretion; 3) to validate the use of transabdominal ultrasound for ovarian evaluation and timing of ovulation; 4) to examine the quality of semen after liquid storage and cryopreservation; and 5) to develop an intrauterine insemination technique. Based on urinary endocrine monitoring of 41 follicular phases and 26 complete cycles from five females, estrous cycles were 41 days long and comprised a 17-day follicular phase and a 21-day luteal phase. A consistent temporal relationship was observed between peak estrogen conjugates and the LH surge, the latter of which occurred approximately 0.5 days later. Two animals placed on oral altrenogest (three separate occasions for 30, 17, and 31 days, respectively) excreted peak urinary estrogen concentrations 25 days after withdrawal that were followed by sustained elevations in urinary pregnanediol-3α-glucuronide excretion. Mean preovulatory follicle diameter was 3.9 cm (n = 6), and ovulation occurred 38 h (n = 5) after the peak of the LH surge. Based on visual estimates of motility, liquid-stored semen maintained 92% of its raw ejaculate sperm motility index (total progressive motility × kinetic rating [0–5 scale, where 0 = no movement and 5 = rapid progressive movement]) when held at 4°C for 3 days postcollection. Semen cryopreserved using a medium freezing rate demonstrated good post-thaw total motility (50%), progressive motility (94%), and kinetic rating (3.5). Insemination during eight estrous cycles resulted in three pregnancies (38%), two from liquid-stored and one from cryopreserved semen. Two calves were delivered after gestation lengths of 552 and 554 days, respectively. These data demonstrate the potential of noninvasive endocrine monitoring combined with serial ultrasonography to improve our understanding of the reproductive biology of cetaceans. This fundamental knowledge was essential for ensuring the first successful conceptions, resulting in live offspring, using artificial insemination in any cetacean species.

assisted reproductive technology, follicular development, luteinizing hormone, ovulatory cycle, sperm

INTRODUCTION

Killer whales are one of the few marine mammals that are ubiquitous to ocean habitats around the globe. Despite their prevalence in the wild, the worldwide captive population of killer whales comprises less than 48 animals. The captive population is limited further by the size and space requirements of the species, resulting in the formation of numerous, small, genetically isolated groups. Despite this fractionated population, improved understanding of the environmental and social requirements of killer whales has led to successful natural breeding. Since 1985, when the first successful birth and rearing of killer whales occurred [1], approximately 26 births have followed at six facilities (B. Andrews, personal communication). As a result, more than half the present population (26/48) has been born in captivity, including second generations.

Despite these reproductive successes, long-term genetic management of this species necessitates moving animals between facilities, a maneuver that is both logistically difficult and expensive. Additionally, regulatory restrictions now preclude the international movement of animals between many countries. Development of artificial insemination (AI) capabilities for this species would enable genetic management without the need for animal transportation. However, a prerequisite for successful AI in any species is a fundamental understanding of their reproductive physiology [2].

The ability to train unrestrained killer whales for sample collection (blood and urine) and the subsequent application of endocrinological techniques have resulted in valuable data concerning their reproductive endocrinology. Duffield et al. [1] relied on twice-monthly serum progesterone from 18 females over a 10-year period to define a mean gestation period of 517 days (range, 468–539 d). Walker et al. [3] described urinary bioactive FSH, estrogen conjugates (EC), and progesterone metabolites in the killer whale. With only a limited number of estrous cycles studied (n = 6), those authors reported a 42- to 49-day estrous cycle, consisting of a 12-day follicular phase and a 21- to 28-day luteal phase. Although this was groundbreaking study for marine mammals, limited sample size and lack of ultrasound data precluded the description of endocrinological events as they related to ovulation. Analyzing once-daily urine samples from killer whales, Robeck et al. [4] validated that urinary

[1]This project was funded by SeaWorld Corporation and is a SeaWorld Technical contribution no. 2004-01-T.
[2]Correspondence: Todd R. Robeck, SeaWorld Texas, 10500 SeaWorld Drive, San Antonio, TX 78251. FAX: 210 523 3299;
e-mail: todd.robeck@seaworld.com

Received: 1 February 2004.
First decision: 23 February 2004.
Accepted: 14 April 2004.
© 2004 by the Society for the Study of Reproduction, Inc.
ISSN: 0006-3363. http://www.biolreprod.org

pregnanediol-3α-glucuronide concentrations paralleled circulating luteal phase progesterone secretion. In addition, those authors documented a single LH surge (using samples from five estrous cycles, with two being conceptive), and they suggested that twice-daily urinary hormone monitoring might permit more consistent detection of this important endocrine event.

Progestogens are commonly used to synchronize ovulation in numerous species [5]. In cetaceans, the progestin altrenogest has been used for long-term suppression of ovulation [6]. In addition, preliminary evidence suggests that it can be used to synchronize estrus in another cetacean, the bottlenose dolphin [7]. The ability to control the timing of ovulation in killer whales would allow improved management of natural breeding as well as improved timing of AI.

Ultrasound has been used to evaluate ovarian follicular activity [8–10] and to document the effects of an ovulatory induction protocol on follicular activity [8] in bottlenose dolphins. Brook [9] described follicular genesis in bottlenose dolphins and used this information to time natural breeding. The development of ultrasound for monitoring ovarian activity in killer whales would provide invaluable information concerning the temporal interrelationships between reproductive hormones and ovulation.

Collection with short-term liquid storage or long-term cryopreservation of semen has not been accomplished in killer whales. The only cetacean species in which this has been described is the bottlenose dolphin [11, 12]. Development of AI using liquid-stored semen would enable transfer of genes among populations within North America, whereas developing sperm cryopreservation would allow the global exchange of genetic materials and form the foundation for genome resource banking [13, 14].

The overall goal of the present study was to gain an understanding of killer whale reproductive physiology and then apply this knowledge to improve natural breeding success and to develop assisted reproductive techniques. To accomplish this, specific objectives were 1) to determine the excretory dynamics of urinary LH and ovarian steroid metabolites during the estrous cycle; 2) to evaluate the effect of an exogenously administered, synthetic progesterone analog on reproductive hormone excretion; 3) to validate the use of transabdominal ultrasound for ovarian evaluation and timing of ovulation; 4) to examine the quality of semen after liquid storage and cryopreservation; and 5) to develop an intrauterine insemination technique.

MATERIALS AND METHODS

Animals

Three female killer whales (females 1–3: age, 20, 18, and 13 yr, respectively; weight, 2343, 2454, and 2181 kg, respectively) were involved in the endocrine monitoring and estrous synchronization studies. For the AI trials, two additional females (females 4 and 5: age, 10 and 14 yr, respectively; weight, 2075 and 2513 kg, respectively) were included. One male (male 1: age, 20 yr; weight, 5254 kg) was used for semen collection. Of the females, only females 1 and 2 had previously calved. During the AI procedures, candidate females were kept isolated from any breeding-age males for 2 wk before and for 2 wk after the inseminations. In addition, the male used for semen collection was located in a different facility from the females used for AI. Animals were fed a diet of frozen-thawed, whole fish (herring [*Clupea harengus*], Columbia river smelt [*Thaleichthys pacificus*], and pink salmon [*Oncorhynchus gorbuscha*]) at approximately 2–3% of their body weight per day. All animals were housed with mixed (i.e., male and female) groups of various age classes. The animals were housed at three separate SeaWorld facilities that contained a minimum of 2.5 million gallons of saltwater cooled year-round (temperature range, 52–55°F).

Ethics of Experimentation

All samples were collected using routine husbandry training and were obtained from unrestrained animals. All animal procedures, including AI, were conducted without sedation or restraint. 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 U.S. Department of Agriculture Guiding Principles for the Care and Use of Marine Mammals.

Endocrine Monitoring

Blood and urine samples were collected from unrestrained animals as previously described [4]. Blood samples were collected from all animals on a monthly basis for routine health checks both before and during the present study. Routine analysis of these samples for serum progesterone concentrations (determined by a commercial laboratory; Quest Diagnostics, Irving, TX) in females 1–3 indicated that all were cycling for at least 3 yr before commencement of the urinary endocrine monitoring study [1]

Urine samples from females 1–3 were collected twice-daily for 731, 570, and 645 days, respectively. Samples were stored in duplicate at −70°C until analysis. Nonextracted urine samples were analyzed by RIA on an every-other-day basis for total immunoreactive levels of PdG (PdG) and EC (EC). The results for PdG and EC on every-other-day urine samples enabled us to delineate when the estrous cycles had occurred. We were then able to target our daily hormone analysis during estrus. To compared EC derived from RIA or enzyme immunoassay (EIA), we analyzed twice-daily urine samples from female 1 with both assay systems. Also, during these periods of hormonal estrus, LH concentrations were determined by RIA in twice-daily urine samples from females 1–3. Concurrent urinary EC values, as determined by both RIA and EIA from female 1, were positively correlated ($r = 0.75$, $P < 0.005$).

Determination of total cycle length (TCL) was based on either the interval between the beginning of successive luteal phases (interluteal phase interval), successive LH peaks, or successive EC peaks. For the present study, the LH and EC peaks were defined as the maximum concentration for the respective hormone during the estrous period. Length of the luteal phase (PdG concentrations > 0.1 ng/mg creatinine for three consecutive days), follicular phase (EC concentrations > 0.5 ng/mg creatinine for three consecutive samples), PdG nadir to EC peak, LH peak to start of the next luteal phase, start of follicular phase to LH peak, and peak LH to peak follicular phase EC were determined. The preovulatory rise in EC concentrations as determined by EIA was subjectively defined as values greater than 2000 pg/mg creatinine that occurred on two consecutive days during the follicular phase. Using this algorithm, the mean interval from preovulatory EC rise to LH peak was determined for all five whales. A ''normal'' estrous cycle was determined by combining the mean values from all five whales for all of the above-mentioned intervals.

After patterns of EC, PdG, and LH had been established in females 1–3, AI was attempted in all five females. During these attempts, EC and LH were analyzed in twice-daily urine samples. Based on the initial endocrine study, it was determined that EC data could be used to predict estrus for the purpose of preparing for AI. The data collected during the eight insemination attempts were combined with the initial endocrine monitoring study to determine cycle-length components as described above. Endocrine data collected during the AI attempts were compared to the ultrasonographically estimated ovulation point to define the interval between the EC and LH peak and ovulation.

Synchronization of Ovulation Using Progesterone-Analog Treatment

A total of three treatments were administered. Females 1 and 2 were placed on 0.025 mg/kg p.o. of altrenogest (Regu-Mate; Intervet, Inc., Millsboro, DE) for 30 and 17 days, respectively. Ten months after the first treatment, female 1 was administered a second round of treatment with 0.05 mg/kg for 31 days. Levels of PdG, EC, and LH were determined, from daily urine samples, both during and after the treatment with altrenogest. The mean time from altrenogest withdrawal to the first hormonally detected follicular phase was determined. Both females were cycling before altrenogest administration, and they were in mid to late luteal phase when administration began.

Creatinine Assay

Creatinine levels were determined for each urine sample to adjust for intersample differences in urine concentration [15]. Concentrations of uri-

nary hormones and metabolites were expressed as mass of hormone per milligram of creatinine excreted.

RIA for EC

Urinary EC was analyzed as previously described [16]. Initially, each urine sample was diluted (1:50) in PBS (0.1 M, 0.1% gelatin, pH 7.0) and a 50-μl aliquot was adjusted to a final assay volume of 300 μl in Tris buffer (0.1 M Tris, 0.9% NaCl, 0.1% NaN_3, and 0.1% gelatin, pH 8.4). Antiserum that cross-reacts 100% with estrone-glucuronide and estrone sulfate (anti-estrone-3-glucuronide serum, 100 μl, 1:1500; D. Collins, Emory University, Atlanta, GA) and [^{3}H]estrone sulfate (100 μl, 7000 cpm, specific activity = 55 Ci/mmol; Dupont-New England Nuclear, Wilmington, DE) were combined with unknowns and standards (4.9–2500 pg/tube; Sigma-Aldrich Chemical Co., St. Louis, MO) and incubated overnight at 4°C. Following the addition of 300 μl of charcoal-dextran (0.0625% Norit A charcoal and 0.00625% dextran in 0.1 M PBS, pH 7.0) and a 30-min incubation at 4°C, tubes were centrifuged (10 min, 1500 × *g*), decanted into scintillation vials, combined with 5.0 ml of Ready Solv HPb (Beckman Instruments, Inc., Fullerton, CA), and counted for 5 min.

Serial dilutions of pooled killer whale urine yielded displacement curves parallel to that obtained for estrone sulfate standards ($r = 0.99$). The mean ± SEM recovery of estrone sulfate (range, 4.9–2500 pg/tube) added to a pool of killer whale urine was 110.1% ± 10% ($y = 0.79x + 15.04$; $r^2 = 0.99$). Assay sensitivity was 4.9 pg/tube or 0.1 ng/ml. Interassay coefficients of variation for three separate internal controls (n = 18 assays) were 11% (20% binding), 13% (40% binding), and 18% (80% binding), and intraassay variation averaged less than 10%.

Assay for PdG

Urinary PdG was analyzed using methods previously described [17]. A 200-μl urine aliquot diluted (1:50) in PBS was combined with 100 μl each of PdG antiserum that cross-reacts 100% with PdG and 6.7% with pregnanediol (#02/Zoo, 1:20 000) and [^{3}H]-PdG (7000 cpm, specific activity = 42 Ci/mmol) supplied by Courtauld Institute of Biochemistry (London, U.K.). Urine samples and standards (19.5–5000 pg/tube; Sigma-Aldrich) were incubated overnight (4°C), and antibody-bound and free steroid were separated after a 45-min incubation with 300 μl of charcoal-dextran suspension and centrifugation for 10 min (1500 × *g*). Supernatants were combined with 5 ml of Ready Solv HPb and counted for 5 min.

Serial dilutions of killer whale urine yielded displacement curves parallel to that obtained with standard preparations ($r = 0.99$). Recovery of known amounts of PdG (range, 19.5–5000 pg/tube) added to a pool of diluted urine (100 μl, 1:16) gave a mean of 69% ± 9% ($y = 0.61x + 0.76$; $r^2 = 0.99$). Assay sensitivity was 19.5 pg/tube. Interassay coefficients of variation for two separate internal controls (n = 23 assays) were 10% (25% binding) and 14% (50% binding), and intraassay variation was less than 10%.

EIA for EC

To our knowledge, the EC EIA has not been previously used in this species. Urinary EC were measured by single-antibody, direct-enzyme immunoassay as previously described [18–20]. Briefly, E1G antisera (1:5000; C. Munro, UC Davis, CA) was added to 96-well, flat-bottom microtiter plates (Immulon 1; Dynex Technologies, Chantilly, VA) and incubated at 4°C overnight. Neat urine samples (0.01 ml) and standards (range, 3.1–400 pg; Sigma-Aldrich) were added to wells in duplicate. Enzyme conjugate (E1G horseradish peroxidase, 1:100 000; C. Munro) was then added to all wells and the plate incubated at room temperature for 2 h. After incubation, plates were washed and 0.1 ml of substrate (tetramethyl benzidine in phosphate citrate buffer; Sigma-Aldrich) added to all wells before incubation at room temperature for 30 min. Finally, 0.05 ml of 0.6 M H_2SO_4 was added to all wells, and plates were read at 450 nm (reference, 650 nm) in a microplate reader (Benchmark; Bio-Rad, Hercules, CA). Intraassay variation was 5%, and interassay variation was 15.9% (n = 28; 55% binding). Serial dilutions of killer whale urine yielded displacement curves that were similar to the standard curve ($r = 0.99$). Immunoassay of fractions separated by reverse-phase high-performance liquid chromatographic analysis revealed one minor immunoreactive peak (fractions 21–24, 16% of total) that coeluted with estrone-1-sulfate and a second, much larger immunoreactive peak (fractions 62–67, 67% of total) that coeluted with estrone.

Significantly elevated EC concentrations as determined by RIA and EIA were subjectively defined as those greater than 500 and those greater than 800 pg/mg creatinine, respectively. The follicular phase was defined as the length of time during which at least three consecutive days had significant EC concentrations during the PdG nadir.

RIA for LH

The assay for LH was a ^{125}I double-antibody RIA that has been previously described [21]. An anti-bovine LH antiserum (518-B_7, 1:500 000) and ovine LH (oLH-26, 25 and AFP 861-4B), provided by NIH (Bethesda, MD), was used as the radioligand and standard, respectively. Then, 100 μl of sample or standard (0.039–5 ng/ml) were added, in duplicate, to the antiserum (100 μl). After incubation at room temperature for 24 h, approximately 25 000 cpm (in 100 μl) was added to each tube and incubated at room temperature for another 24 h. Finally, 100 μl of goat-anti-mouse antibody (1:800; Sigma-Aldrich) were added. Following a 1-h incubation at room temperature, the tubes were centrifuged at 3000 × *g* for 25 min and decanted, and the precipitate was counted for radioactivity in a gamma counter (Cobra II Auto Gamma; Packard).

Serial dilutions of pooled killer whale urine yielded displacement curves parallel to that obtained with standard preparations ($r = 0.96$). Sensitivity of the assay was 0.3 ng/ml, and interassay coefficients of variation for two separate controls were 6.2% (50% binding) and 11.8% (80% binding).

The preovulatory LH peak was defined as the highest LH value during the follicular phase.

Ultrasonographic Exams

Ultrasonographic examinations were performed using an Aloka 500 (Corimetrics Medical, Charlotte, NC) and a 3.5-MHz transducer (either a 17-cm, linear-array probe or a wide-footprint, convex-linear probe). Animals were examined once daily during the predicted period of estrus. This period was predicted by adding 40 days to the previous ovulation or hormonally detected estrus. On the estimated (based on historical hormonal data and on data collected during the current cycle) day of peak EC, ultrasound exams were increased to three times daily. For exams, the animals were trained to station in lateral recumbency adjacent to the edge of the pool. The ovaries were located using a technique first described for use in small delphinids [10]. The transducer was placed in a transverse plan on the lateral aspect of the whale dorsal to the leading edge of the genital slit. The commissure created where the rectus abdominus muscle overlaps the hypaxial muscles was first located and followed cranially until the ovaries were visualized. The probe was then rotated to a longitudinal plane for evaluation. Follicular size was determined by measuring its largest diameter, utilizing the anechoic margins as the border. For ovoid follicles, diameter in two planes was determined, and circumference was calculated using the following formula (a, B=radius):

$$C = 2\Pi \mathrm{sqrt}((a^2 + B^2)/2)$$

Thus, follicular size reflected follicular antral diameter. Follicular size was quantified either immediately, using the ultrasound machine's electronic calipers, or later, when the videotaped sonograms were reviewed. Ovulation was determined to have occurred when the follicle was not detectable in a subsequent exam [10]. The time of ovulation was defined as the median time between the previous 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 thrice-daily examination.

Semen Collection and Processing

Nine ejaculates were collected for use during a total of eight AI attempts or estrous periods in four whales. Five were collected from September 1998 to August 2001 and cryopreserved for later use, and four were collected and shipped fresh for the AI procedures. The semen was collected from the adult male trained to voluntarily ejaculate [22]. This male, positioned in dorsal recumbency adjacent to the edge of the pool, was trained to present his penis. Before training sessions, the male was sexually stimulated by the presence of a receptive female or another male killer whale. Once arousal ensued (as assessed by partial extrusion of penis from the genital groove), operant conditioning and positive-reinforcement techniques were used to achieve ejaculation, which occurred at variable intervals before, during, or after penile presentation. Semen was collected into a 125-ml, cylindrical, plastic collection container (Nalgene; Nalge Nunc International, Rochester, NY).

Ejaculate concentration, volume, color, pH (pH indicator strips; Whatman, Inc., Ann Arbor, MI), and sperm motility, viability (plasma membrane integrity), and morphology were determined using standardized techniques [12]. The percentages of motile sperm were subjectively de-

termined to the nearest 5% by analyzing four to five fields of diluted sperm (35°C) (1:25, sperm:Hepes-buffered human tubal fluid medium; Irvine Scientific, Santa Ana, CA) using bright-field optics (400× Olympus, Tokyo, Japan). Total progressive motility (PM), and kinetic rating (KR: 0–5 scale, where 0 = no movement and 5 = rapid forward progressive movement) were subjectively determined. For data analysis and sample comparisons, these values were then transformed into a sperm motility index (SMI; modified from that described by Howard et al. [23]) as follows:

$$\text{SMI} = \text{TM (\%)} \times \text{PM (\%)} \times \text{KR}$$

For assessment of viability, 10 μl of semen were mixed with 40 μl of a live-dead exclusion stain (eosin-nigrosin; IMV International Corp., Maple Grove, MN) for 30 sec. 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).

For acrosomal and morphologic analysis, 2 μl of the sperm sample were diluted with 10 μl of Hepes-TALP medium (Androhep, modified Tyrode medium with Hepes and BSA; Minitube of America, Verona, WI) at room temperature on a glass slide, smeared, and allowed to air-dry for 5 min. The slide was then fixed with formal saline and stained (Spermac Stain; Minitube of America; a differential stain used with light microscopy) within 2 wk of fixation. Gross structural morphology was evaluated in 200 sperm/sample under bright-field optics (1000×). Acrosomes were evaluated with bright-field optics (1000×) and were classified as normal or abnormal (100 per sample). A normal acrosome had a distinct outline and was stained blue-green. Acrosomes were classified as abnormal if they were partially or completely lost or if obvious membrane irregularities (e.g., pitting, vacuolation) were present [24].

Processing of Semen for Liquid Storage

Ejaculates were diluted (2:1, semen:diluent) over 5 min with a commercially available bovine extender, Biladyl (Fraction A: 1210 g of Tris, 690 g of citric acid, 5 g of fructose, and 20% egg yolk [v/v] per 500 ml; Minitube of America), with antibiotics (0.5 mg/ml of tylosin, 2.5 mg/ml of gentamicin, 1.5 mg/ml of lincomycin, and 3 mg/ml of spectinomycin). Biladyl was chosen because of its ease of preparation and because of the results of preliminary studies that demonstrated its ability to maintain high levels of killer whale sperm motility during storage for 36 h at 4°C (unpublished results). The diluted sample was transferred to a sterile, 4-ounce Whirl-Pak (Nasco, Fort Atkinson, WI) and placed in a storage container (Equitainer; Hamilton Research, Inc., South Hamilton, MA). If necessary, extra extender was placed in an additional Whirl-Pak and added to the storage container to obtain a final fluid volume of 120–150 ml. The storage container was sealed and sent counter-to-counter via a commercial airline to the site where AI was conducted. Once on location, the sample was divided into three aliquots and stored at 4°C until AI. After transport and before each insemination, a 15-μl portion was removed from each sample, warmed to 35°C, and re-evaluated for TM, PM, and KR.

Processing of Semen for Frozen Storage

Ejaculates were extended with Biladyl (Fraction A; 2:1, semen:diluent) over 5 min. The sealed tube containing the sperm suspension was placed in a beaker with 250 ml of water (21°C) and cooled to 5°C over 1 h (cooling rate, $-0.27°C\ min^{-1}$). 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) over 10 min with Biladyl (Fraction B: Fraction A + 14% glycerol; 2°C) and incubated for an additional 30 min at 2°C. The sperm suspension was transferred to 0.5-ml straws (IMV International Corp.) and frozen in liquid nitrogen vapor at a distance of 13.5 cm above the vapor (cooling rate, $-6°C\ min^{-1}$) for 5 min, then placed at 7.5 cm above the vapor (cooling rate, $-7°C\ min^{-1}$) for an additional 5 min before plunging. This freezing rate was selected based on a preliminary freezing trial that indicated killer whale sperm had better in vitro postthaw motility after freezing using a slow (cooling rate, $-6°C\ min^{-1}$) compared with a fast (cooling rate, $-35°C\ min^{-1}$) freeze (unpublished results). Straws were then plunged into liquid nitrogen.

Artificial Insemination

The first insemination was based on the presence of a preovulatory follicle (POF) and/or the detection of peak urinary EC. Inseminations were repeated once daily with fresh semen and every 12 h with cryopreserved semen until ovulation was confirmed by ultrasonography.

For each procedure, the use of liquid or frozen-thawed semen was based on availability. If fresh semen was unavailable, cryopreserved semen was thawed 1 h before insemination. The straws were placed in a water bath at 35°C for 30 sec (thawing rate, $8.3°C\ sec^{-1}$ [12]), then diluted 1:1 (v/v) over 5 min with Biladyl (Fraction A). Total motility, PM, KR, and sperm concentration were determined for each sample before insemination.

For all procedures, females were trained to station themselves poolside in dorsal recumbency. To overcome the substantial technical challenges associated with intrauterine sperm deposition, insemination methods were modified, as appropriate, during successive AI trials. For the initial insemination trial, an endotracheal tube (inner diameter, 22 mm; outer diameter, 30 mm; length, 90 cm; Cook Veterinary Products, Spencer, IN) was introduced into the vagina until resistance was encountered (~70 cm). A gastroscope (length, 183 cm; outer diameter, 11 mm) with a polyurethane tube (outer diameter, 5 mm) affixed along its length using tape was introduced into the endotracheal tube. The cervix was visualized with the aid of the gastroscope. A modified 8-French canine urethral catheter (two catheters were connected via a blunted, 19-gauge needle to create a 400-cm tube) was then inserted into the polyurethane tube and introduced through the external cervical os, and semen was deposited beyond the first of two cervices. Killer whales have two cervices in series of similar morphology and appearance to an equine cervix [25]. The two cervices, of approximately 5 cm in length each, are composed of longitudinal folds; the folds of the proximal cervix are continuous with the endometrial folds of the uterus. The internal os of the distal cervix is separated by approximately 1 cm from the external os of the proximal cervix. Both the distal and cervical os are surrounded by a fornix approximately 5 cm in depth (unpublished results).

For the remaining insemination attempts, substantial modifications were made. First, a catheter was developed based on killer whale reproductive tract dimensions (length, 400 cm; external diameter, 2.8 mm [8 French]; bullet-tipped catheter; catalog no. V-WIC-8.0-400; Cook Veterinary Products). Additional procedural modifications were made: 1) The endoscope was inserted directly into the vagina without the aid of a speculum, 2) insufflation of the vagina was performed to visualize the cervical opening before advancing the endoscope into the caudal uterine body, and 3) once the endoscope was in the uterine body, the catheter was advanced 10 to 15 cm beyond the endoscope for semen deposition.

Statistics

Hormone- and sperm-quality data were analyzed by analysis of variance and means compared using Newman-Keuls multiple comparisons and the Mann-Whitney *U* test (SigmaStat, Version 2.0.; SPSS, Inc., San Rafael, CA). Results are presented as the mean ± SD.

RESULTS

Endocrine Monitoring

Two (females 1 and 2) of the three whales involved in the endocrine monitoring and estrous synchronization studies continued to cycle during the entire sampling period and account for 22 complete estrous cycles. A complete cycle was defined as one in which PdG, EC, and LH were measured. Female 3 experienced three prolonged interestrous intervals, with baseline PdG and EC, of 105, 88, and 91 days. In addition, this female, which was not housed with a breeding-age male, had continuously elevated progesterone, or pseudopregnancy, for 193 days.

During the entire study period (endocrine monitoring and AI trials), 41 follicular phases (LH and EC) and 26 complete estrous cycles (PdG, LH, and EC) were evaluated for the five whales. Three typical cycles for female 4 are illustrated in Figure 1. The TCL between consecutive peak estrogens was 40.7 ± 6.0 days (n = 41; range, 35.5–68 days). No significant differences ($P > 0.05$) were observed between median TCL determined by the interval between successive LH peaks (42 ± 7.2 days; median, 39 days), EC peaks (median, 39 days), or the interluteal phase interval (41.7 ± 6.4 days; median, 40 days; as determined by PdG levels). Lengths of the follicular phase and luteal phase were 17.1 ± 3.5 days (n = 41; range, 9–27 days) and 21.6

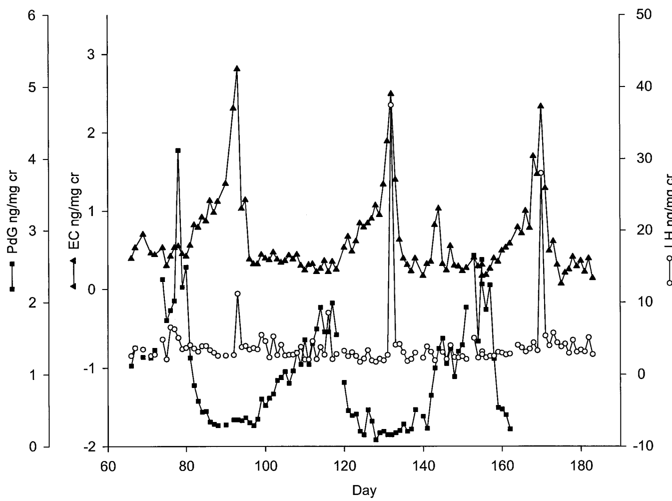

FIG. 1. Three typical cycles from female 4 characterized by urinary concentrations of LH, EC, and PdG. Note the obvious LH surge in two of the three cycles. The level of PdG was not measured beyond Day 165.

± 3.9 days (n = 31; range, 14–33 days), respectively. The time from PdG nadir (luteal regression) to peak EC was 13.9 ± 1.8 days (n = 26; range, 12–18 days). The interval between peak estrogens and peak LH was 0.46 ± 0.49 days (n = 29; range, 0–2 days). The interval between peak LH and increased PdG was 5.5 ± 2.5 days (n = 28; range, 1–14 days). The time of ovulation, as determined by ultrasonography, occurred at 58.0 ± 10.4 and 38.0 ± 29.0 h of the EC and LH peaks, respectively. The mean cycle phase durations were used to develop a composite killer whale estrous cycle (Fig. 2). All reproductive cycle intervals described above were similar between animals ($P > 0.05$).

Estrous Synchronization

The whale treated with altrenogest for 17 days (female 2) excreted maximal EC concentrations 22 days after altrenogest withdrawal, but no EC-associated LH peak was detected. However, this female did have a rise in PdG concentrations, suggesting the presence of luteal tissue, at the expected time after maximal EC concentrations were obtained. Increased LH activity in this female was only observed during the presumed luteal phase that followed (Fig. 3). This female did not cycle again for 102 days (data not shown) after this initial cycle. The female treated initially with altrenogest for 30 days (female 1) had maximal EC excretion 29 days after altrenogest withdrawal (Fig. 3). An LH peak was associated with peak EC, and a subsequent luteal phase was observed. Thereafter, the ovarian cycle length of female 1 averaged 40.8 ± 5.8 days (n = 5). During her next treatment (using twice the altrenogest dose for 31 days), peak EC excretion occurred at 24 days after altrenogest withdrawal. Although an LH peak was not observed, a presumptive luteal phase, characterized by prolonged excretion of PdG, followed. Ovarian cycle length for the 10 subsequent cycles (sampling was discontinued after the 10th cycle) was 39.4 ± 2.4 days. Overall, peak EC excretion was detected 25.0 ± 3.6 days after altrenogest withdrawal (n = 3 treatments). An LH peak was not detected after two of three altrenogest treatments; however, luteal phases were detected in all three. Luteal phases that were ongoing at the onset of altrenogest treatment lasted 20.0 ± 2.0 days.

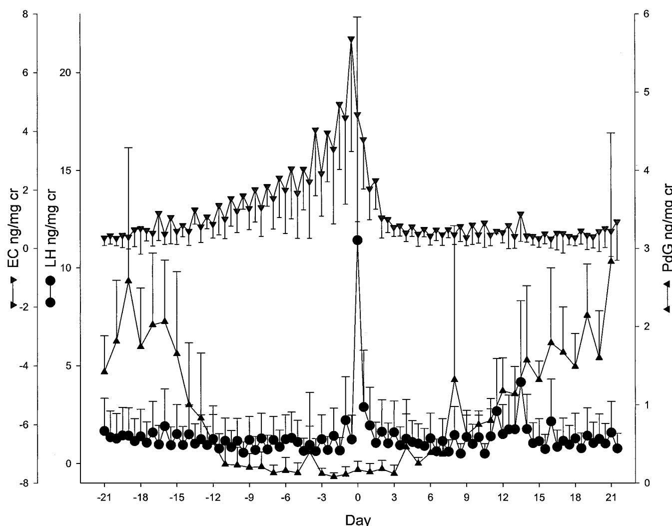

FIG. 2. Mean values of killer whale estrous cycle components, including urinary concentrations of LH, EC, and PdG. Note the peak LH at approximately 0.5 d following the EC peak.

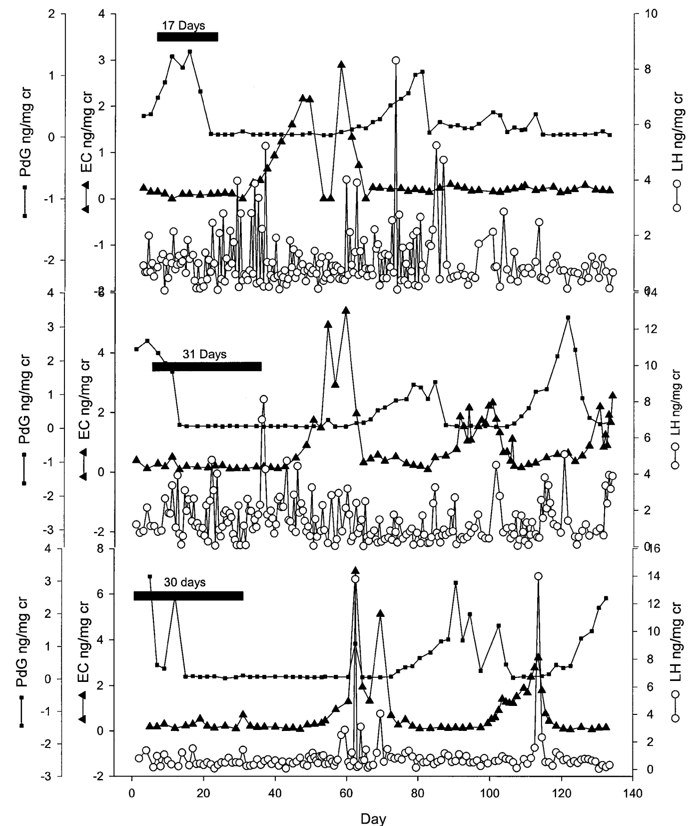

FIG. 3. Altrenogest treatment (black bar) in two killer whales and effects on urinary concentrations of PdG, EC, and LH. Results are shown for female 2 (**top panel**) and for female 1 (**bottom two panels**). Note the lack of an EC-associated LH surge in female 2. Also note that at the start of all three treatments, the animals excreted elevated PdG, representative of circulating progesterone.

Ultrasonographic Evaluation of Ovaries

Growing follicles were observed during all eight AI attempts, and the POF was observed in six of eight instances (Fig. 4). The two instances in which the POF was not observed resulted from observations being discontinued before ovulation. These two AI attempts were aborted because

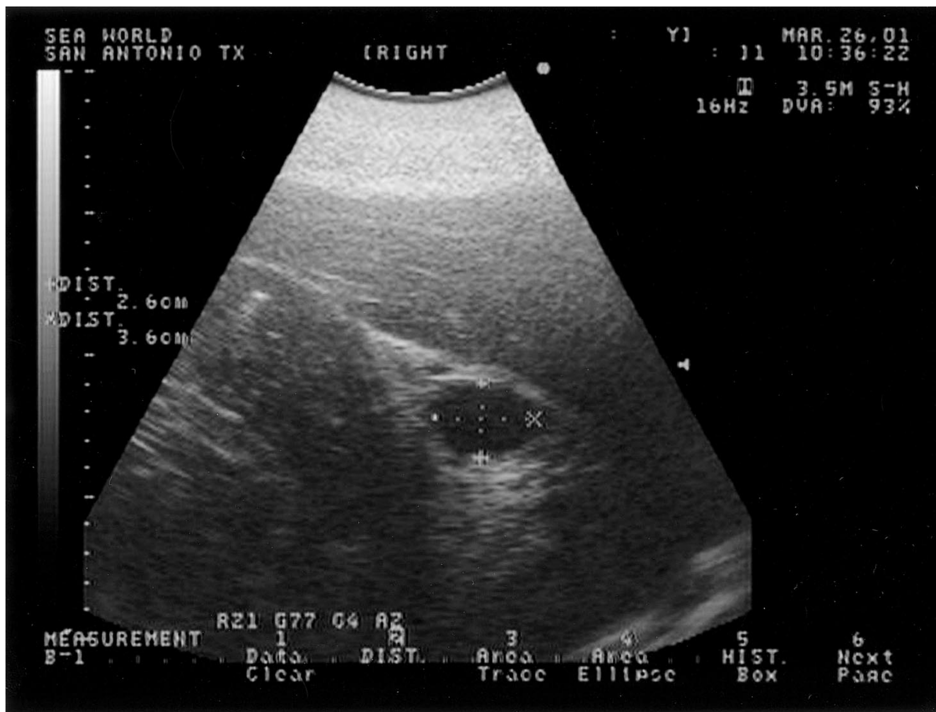

FIG. 4. Killer whale preovulatory follicle (diameter noted by hash marks). Note the oblong shape of the follicle and the depth (13 cm) within the body cavity.

of lack of semen. For the other six AI attempts, growth of the dominant and secondary follicle was recorded for up to 20 days before ovulation (Fig. 5). A secondary follicle was observed three times on the ipsilateral ovary of the dominant follicle and twice on the contralateral ovary; none was observed in female 4. These secondary follicles were observed up to 10 days postovulation. Female 5 had three follicles, two on the right (the side of ovulation) and one on the left. The circumference and maximum diameter of the secondary follicle at the time of ovulation was 9.7 ± 2.4 cm and 3.3 ± 0.9 cm, respectively. The POF circumference and maximum diameter was 11.2 ± 2.6 and 3.9 ± 0.6 cm, respectively. The rate of growth of the POFs was 0.98 ± 0.5 cm/d. During growth to ovulation, the follicles were anechoic and varied in shape from oblong to spherical. Ovulation was detected only by the disappearance of the POF. The ovulations occurred on the left ovary in female 1 (n = 1 ovulation) and in female 4 (n = 3 ovulations) and on the right ovary in female 2 (n = 3 ovulations) and in female 5 (n = 1 ovulation).

Corpora hemorrhagica were not visualized during ultrasounds performed after disappearance of the POF. A corpus luteum (circumference, 26.1 ± 1.5 cm) was only detected in two animals 2 wk postconception. Each detected corpus luteum was a spherical structure with heterogeneous echogenicity (anechoic, fluid-filled structure with many hair-like, hyperechoic lines within the fluid). They could be repeatedly detected during random pregnancy exams. Their echogenicity did not change; however, they continued to grow, with the maximum observed size (circumference 46.5 ± 0.86 cm) occurring 90 days postconception. After maximum circumference was reached, the size did not appear to decrease during pregnancy. Exams were not conducted postpartum.

Ultrasonography indicated that female 2 had approximately four nongrowing cysts (all ~2.5 cm in diameter) associated with the left ovary. Following complications related to a cerebral fungal infection, postmortem examina-

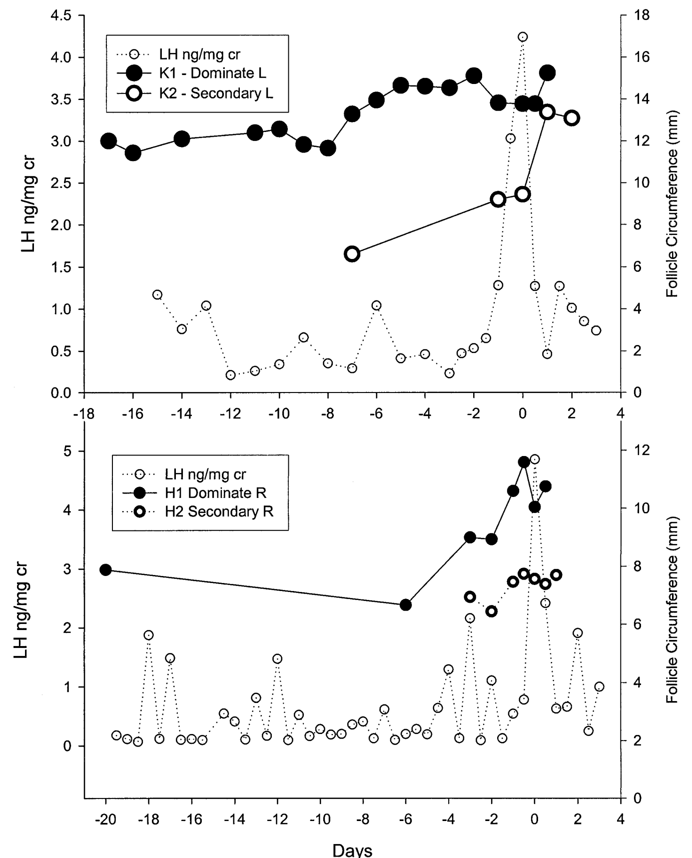

FIG. 5. Follicular growth of primary and secondary follicles in two killer whales in relationship to urinary LH. Note the slow growth of the dominant follicle (solid circle).

TABLE 1. Characteristics of killer whale ejaculates used for artificial insemination.[a]

Parameter	Neat ejaculate (n = 13)	Liquid-stored semen post-transport (12- to 24-h postcollection, n = 5)[b]	Frozen-thawed (0-h postthaw, n = 8)[c]
Semen characteristics			
Volume (ml)	13.9 ± 11.5	41.1 ± 5.1[d]	39.9 ± 9.9[d]
Sperm concentration ($\times 10^7$ ml^{-1})	98.8 ± 69.7	8.8 ± 75.9	5.4 ± 5.2
Total spermatozoa per ejaculate ($\times 10^7$ ml^{-1})	1315 ± 1379.1[e]	262.5 ± 181.2[efg]	88.0 ± 88.3[fg]
Sperm characteristics			
Total motility (%)	90.5 ± 4.7[e]	87.3 ± 6.6[e]	50.3 ± 5.0[f]
Percent progressive motility (%)	94.2 ± 3.8	95.0 ± 0.0	94.0 ± 4.3
Kinetic rating (0–5)[h]	4.6 ± 0.4[e]	4.4 ± 0.4[e]	3.5 ± 0.4[f]
Sperm motility index[i]	391.3 ± 49.7	92.2 ± 5.9[j]	46.2 ± 12.9[j]
Viability (%)	89.5 ± 6.7	94.3 ± 4.0[k]	90.6 ± 8.7[k]
Intact acrosomes (%)	87.4 ± 10.6	—	—
Morphologically normal (%)	88.4 ± 4.8	—	—

[a] Values are the means ± SD.
[b] A total of 10 inseminations were performed using five ejaculates; the ejaculates were held for 3 days or less at 4°C.
[c] A total of eight ejaculates were cryopreserved and used during 20 inseminations.
[d] Final volume of insemination dose.
[e,f] Data in the same row with different superscript are significantly different ($P < 0.001$).
[g] Total progressively motile sperm.
[h] Kinetic rating of spermatozoa graded subjectively: 0 = no movement; 5 = rapid forward progression.
[i] Sperm motility index = percent progressive motility × kinetic rating.
[j] Mean percentage of neat sperm motility index for each sample, or %SMI.
[k] Mean percentage of neat percentage live or percentage of original sample.

tion (24 mo after study onset) confirmed the existence of four cysts occupying the majority of the left ovary.

Acrosome Evaluation

The Spermac Stain was effective at differentially staining the killer whale acrosome, midpiece, and tail a deep green, whereas the nucleus stained red. Exposure time of the sperm initial red stain was increased from 2 min (as recommended by the manufacturer) to 3 min to improve both resolution of the sperm nucleus and ease of acrosome evaluation.

Characteristics of Neat, Liquid-Stored Postthawed Semen

Characteristics of 13 ejaculates collected from one male are shown in Table 1. Overall, ejaculates were of high quality, with sperm motility, viability, intact acrosomes, and normal morphology all greater than 87%. Five ejaculates were processed for liquid storage, and the remaining eight were cryopreserved for use in AI procedures.

During liquid storage for up to 3 days postcollection, samples retained 92% of the SMI found in undiluted ejaculate (Table 1). The longest a sample was stored before being used for any insemination was 36 h, and this insemination resulted in a pregnancy.

Motility parameters of killer whale spermatozoa were well maintained after cryopreservation, thawing, and dilution (SMI, 46.2%; TM, 50.3%; PM, 94%; KR, 3.5%) (Table 1). The postthaw TM and KR were significantly ($P < 0.001$) lower than those obtained for the neat ejaculate and liquid-stored semen.

Artificial Insemination

Artificial insemination was performed during eight cycles over a 2-yr period. Eleven inseminations were performed during the first cycle attempted; after this, an average of 2.6 ± 1.1 inseminations were performed per cycle (Table 2). Semen was deposited in the cervix during the first two inseminations trials, whereas intrauterine inseminations were achieved in the remaining six trials. The distance from the opening of the vagina to the first cervix ranged from 50 to 70 cm, and the distance remained relatively constant among females.

The overall conception rate was 38% (three of eight). Using liquid-stored semen, conceptions were achieved (two of three) when inseminations were conducted within 24 h of ovulation. Using frozen-thawed semen, one of the six attempts was successful when insemination was performed within 12 h of ovulation. Of the five unsuccessful trials, two were associated with only one or two inseminations using frozen-thawed semen. These inseminations had to be discontinued before ovulation, because additional fresh or frozen-thawed semen was unavailable (Table 2). In another AI trial, endocrine analysis revealed that insemination had been conducted 72 h after the preovulatory LH surge. Of the three conceptions (two male and one female offspring), two females delivered live calves at 552 and 554 days, respectively. The third animal (female 2) died from an unrelated cause with a normal 129-day, 136-g, 19-cm (total length) male fetus. Paternity testing confirmed the sire was from the AI procedures (D. Duffield, unpublished data).

DISCUSSION

For numerous reasons, many exotic species are housed in small, isolated groups. In some cases, AI is potentially the most effective and practical assisted reproductive technology available for managing the genetics of these populations [14, 26–29]. To our knowledge, our results represent the first successful conceptions, resulting in live offspring, using AI in any cetacean species.

Earlier work conducted in killer whales [3, 4] and long-

TABLE 2. Killer whale artificial insemination data.

Animal	IPE[a]	SOD[b]	SS[c]	TPMS ($\times 10^7$)[d]	LHO[e] (h)	LHI[f] (h)	PFC[g] (cm)	IO[h] (h)	Conception
Female 1	11 q 24 h	Cervix	L	128.9	−36	48	15.4	−24	Yes
Female 2	2 q 24 h	Cervix	F	19.8	−84	72	8.1	−24	No
Female 4	2 q 24 h	Uterine	L	100.0	?	48	?	?	No
Female 4	1[i]	Uterine	F	67.5	−60	−36	11.3	−96	No
Female 2	2[i] q 24 h	Uterine	F	50.0	?	−24	?	?	No
Female 4	3 q 12 h	Uterine	L	100.0	0	0	10.5	0	Yes
Female 2	4 q 12 h	Uterine	F	69.8	−24	12	10.6	−12	Yes
Female 5	4 q 12 h	Uterine	F	17.0	−24	24	11.2	−24	No
Mean ± SD	3.3 ± 3.1	—	—	64.1 ± 40.0	−38 ± 29	24 ± 39	11.2 ± 2.6	−30 ± 33	—
Mean conception ± SD	3.5 ± 4.4	—	—	85.0 ± 29.6	−12 ± 18	6 ± 25	10.6 ± 2.8	−6 ± 12	—
Mean NC[j] ± SD	2.0 ± 1.1	—	—	46.3 ± 32.9	−56 ± 30	26 ± 47	9.7 ± 2.2	−48 ± 41	—

[a] IPE = inseminations per estrus (the total number and frequency of inseminations per estrus period).
[b] SOD = site of deposition (site where semen was deposited during insemination).
[c] SS = semen storage (method of storage for semen used closest to ovulation; L = Liquid state (slowly diluted in Biladyl extender v/v, cooled and transported via equitainer to location of insemination); F = frozen (cryopreserved).
[d] TPMS = total progressively motile sperm per insemination dose.
[e] LHO = LH peak to ovulation (the estimated time between the LH surge and ovulation). Ovulation was determined by the disappearance of the follicle from one exam to the next. The time between exams was divided to approximate when ovulation occurred.
[f] LHI = LH peak in relation to the farthest temporally located insemination that was closest to ovulation.
[g] PFC = preovulatory follicle circumference.
[h] IO = insemination to ovulation (closest temporally [h] related insemination to ovulation).
[i] Total numbers of inseminations were limited by the availability of semen. Thus, inseminations were stopped before ovulation.
[j] NC = no conception.

term systematic evaluations of female reproductive physiology performed in the present study are both essential to the successful development of AI in this species. The latter entailed simultaneous monitoring of endocrine events and ovarian activity. Although urinary LH levels have been reported previously in killer whales [4], more accurate timing of the LH surge in relation to urinary estrogens was determined in the present study by using twice-daily samples. Elevated EC data can be used to confirm that an animal is in estrus, but this information alone cannot accurately predict the timing of AI. By basing the timing for our inseminations on the time between peak levels of urinary hormones (EC and LH) and ovulation (58 and 38 h, respectively), we used less frozen or liquid-stored semen, because inseminations were commenced only after the peak EC had been detected. Efforts are ongoing to develop a more rapid, semiquantitative LH assay that can be used poolside to further improve our ability to predict ovulation, as opposed to the current test with its 3-day turnaround time.

Altrenogest has been used effectively in several species to regulate the estrous cycle without reducing fertility, including horses [30–32] and pigs [33, 34]. Long-term (>1 yr) use of altrenogest has been used to suppress estrus without affecting subsequent fertility in the killer whale [6] and Pacific white-sided dolphin (unpublished results). Similarly, both animals (females 1 and 2) in the synchronization trial of the present study were used for AI trials in subsequent cycles and became pregnant. In domestic animals, treatment with synchronization protocols that rely on altrenogest is usually followed by follicular recruitment and ovulation processes (postwithdrawal) that mimic a normal follicular phase (postluteal regression). However, in killer whales, altrenogest caused a delay or suppression of follicular growth both during and for a protracted period after hormone withdrawal that was longer (25 days) than the interval from normal luteal regression to peak EC excretion (13 days). A protracted interval between hormonal withdrawal and subsequent ovulation was also observed in bottlenose and Pacific white-sided dolphins [7]. Although this interval is prolonged and more variable compared to traditional estrous synchronization methods used with domestic species, altrenogest has been useful for coordinating ovulation in a group of females during natural or AI breeding trials in bottlenose and Pacific white-sided dolphins [35].

An estrogen-associated LH surge was only observed in one of the three postaltrenogest cycles. However, they all exhibited a subsequent luteal phase, which provided presumptive evidence of ovulation. This probably resulted from a urine sampling frequency (daily) during the altrenogest trials that was insufficient to enable consistent detection of the LH surge. As referenced previously [4], twice-daily sampling is required to consistently capture the short-lived LH surge in killer whales.

The characteristics of semen in the present study represent the only information currently available for killer whales. These data demonstrate that killer whale semen is generally of excellent quality, with ejaculates containing high numbers of morphologically normal and progressively motile spermatozoa. The mean ejaculate volume (13.9 ml) recorded in the present study is not an accurate reflection of normal semen production in the killer whale, however, because some ejaculate was invariably lost during the technically difficult collection procedure. Anecdotal reports from animal staff cite killer whales being observed to ejaculate ‘‘large volumes’’ in the water during sexual activity. Indeed, mean killer whale ejaculate volumes in the present study were considerably less than that reported for bottlenose dolphins (20.4 ml [12]). Further studies with additional males are necessary to establish reliable normative ejaculate characteristics in manually stimulated males.

The differential stain for use with light microscopy, Spermac, has been used with canine spermatozoa [36, 37] to evaluate acrosomal status, and the results have correlated highly with the fluorescent stain FITC-PSA [38]. It has also recently been validated for use in bottlenose dolphins [12]. In the present study, Spermac was effective for differentially staining the acrosome, nucleus, midpiece, and tail of killer whale spermatozoa. Although this stain has thus far

only been applied to fresh sperm, it is likely to prove useful for evaluating postthaw acrosomal status in the killer whale.

Similar to dolphin sperm stored at 4°C [12], killer whale sperm maintained greater than 90% of its original SMI for up to 3 days postcollection during liquid storage. The ability of liquid-stored sperm to fertilize in vivo is a factor of two aging periods: in vitro storage time and in vivo storage after insemination, which increases if the insemination to ovulation interval goes from 12 to 24 h [39]. Porcine sperm can be stored for up to 5 days before a significant reduction in fertility is observed [40]. Susceptibility to aging appears to be dependent either on the male or on ejaculate quality before storage [40, 41], and it can be affected by the storage temperature ($<$5°C vs. 18–24°C [42]). Based on our success using semen from one male, it appears that the fertility of killer whale spermatozoa stored in Biladyl at 4°C will allow once-daily intrauterine inseminations with semen for up to at least 3 days postejaculation.

The simple method described for cryopreservation of killer whale semen resulted in adequate PM after thawing (47%), which was biologically competent. In view of the small number of ejaculates evaluated thus far in killer whales, further studies are required to optimize liquid storage methods (i.e., semen extender, cooling rates, and holding temperatures) and cryopreservation methods (i.e., cryodiluent, freezing rates, and thawing rates) with the aim of enhancing in vitro sperm-quality parameters.

Because of the difficulties encountered when attempting to cannulate the cervix of small ruminants and companion animals, intrauterine insemination in those species has usually been accomplished using laparoscopy (for ovine and caprine, see [43]; for canine and feline, see [44]). However, nonsurgical endoscopic methods have been reported in the dog [45]. In addition, endoscopic methods have recently been used in the horse to reduce the total number of sperm necessary for fertilization as compared to traditional, nonsurgical vaginal cervical cannulation methods [46–48]. Endoscopic semen deposition in cetaceans was first described in the bottlenose dolphin by Schroeder and Keller [11]. These early inseminations involved cervical placement of spermatozoa and did not result in a confirmed pregnancy. Because these attempts were unsuccessful, it was hypothesized that intrauterine insemination would be required for successful AI [49]. Thus, we did not attempt intracervical inseminations in the killer whale. Instead, an endoscopic intrauterine insemination method was developed based on the cervical conformation of the killer whale reproductive tract, which differs significantly from that of the bottlenose dolphin. The most reliable and repeatable insemination method involved placement of the endoscope in the uterus and deposition of spermatozoa within the lumen of the uterine horn.

In the present study, no effort was made to determine a minimum amount of semen required to ensure fertility. In the cow, the minimum number of sperm traditionally required for a successful AI is approximately 10-fold greater when using cryopreserved sperm than when using fresh sperm [50]. However, recent reports have shown that intrauterine deposition of spermatozoa, either at the midbody or the uterine horn, can dramatically reduce the number of spermatozoa required for conception (for bovine, see [51]; for equine, see [52]). In killer whales, the ability to perform deep cornual inseminations should lend itself to a reduced sperm number being required per insemination and, thus, to more efficient use of valuable genetic material. This may be especially important for future attempts to inseminate killer whales with sexed spermatozoa, which is prohibitively expensive to produce in large quantities [51, 53].

Transabdominal ultrasonographic ovarian observations have been reported in Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) and Atlantic bottlenose dolphins [8, 10]. Using the Indo-Pacific bottlenose dolphin, Brook [9] was the first to describe normal follicular waves associated with natural estrous cycles in any cetacean species. In that study, the time from initial follicle development (diameter, $>$0.4 mm) to ovulation was 12.0 $\pm$ 1.3 days. In the present study, follicles less than 1 cm in diameter were not identified, nor was any attempt made to consistently track individual follicles throughout their entire cycle. However, two animals each had follicles less than 2.0 cm in diameter that were followed for 20 days before ovulation. These preliminary data suggest that follicular growth and recruitment occurs over a greater period of time in killer whales than in dolphins. Another difference between the two species is in the size of the POF, which is larger in the killer whale (39 $\pm$ 58 mm) compared to the bottlenose dolphin (19.9 $\pm$ 1.1 mm [9]).

Based on histological identification of corpora albicantia in the bottlenose dolphin, ovulation and pregnancy occurred in the left ovary and the left uterine horn, respectively, more than 68% of the time [54, 55]. In support of this, 83% of ovulations occurred in the left ovary in the Indo-Pacific bottlenose dolphin [9]. In the present study, developing follicles and ovulations were observed on the right and left ovaries of four killer whales. However, definition of the symmetry of ovulation in killer whales is not yet possible, because the ovulations observed in this small number of females were not serially monitored.

Similar to the bottlenose dolphin [9], follicles in the killer whale assumed a turgid, spherical shape as ovulation approached. Equine antral follicles and POFs are similar in size to those of the killer whale, and their shape has been reported to change between examinations and as ovulation approaches [55]. In the mare, this change in shape is believed to be the result of pressure from adjacent ovarian structures or of impending ovulation. In killer whales, the follicles are located on the stromal surface, and the change in shape between exams may result from movement of the ovary and pressure from the surrounding viscera.

In 26% of bottlenose dolphin estrous cycles, secondary follicles on either ovary regressed shortly after ovulation [9]. We also observed secondary follicles during all examinations in both the ipsilateral and contralateral ovary. However, in contrast to those in the bottlenose dolphin, secondary follicles in the killer whale did not regress and were still present up to 10 days postovulation. We hypothesize that the secondary follicles observed at the time of ovulation were destined to become primary follicles during the subsequent estrous cycle. As reported in this and other studies [1, 3, 4], killer whales often experience prolonged anestrus. We theorize that prolonged anestrous intervals may be predicted by the absence of a secondary follicle during the previous periovulatory interval. Despite using a 3.5-MHz probe, insufficient resolution caused by a thick blubber layer prevented the detection of antral follicles less than 1 cm in diameter. Therefore, the size and number of antral follicles initially recruited could not be determined. Further serial ultrasonographic evaluations paired with endocrine monitoring will help to define these questions concerning follicular recruitment.

The development of any AI methodology requires an ability to determine the appropriate time and site for semen

deposition to ensure optimum fertility. Whereas the overall conception rate following AI in the present study was only 38%, the success rate was hampered by failures encountered during the method development stage. Because killer whales do not exhibit consistent signs of behavioral estrus, we relied on physiologic indicators to predict ovulation. In our first attempt, a large follicle (diameter, >4.0 cm) was assumed to be preovulatory based, in part, on extrapolations from bottlenose dolphins, in which 3.0 cm (mean, 1.9 cm [9]) is considered to be the maximum diameter for a POF. Thus, our protocol initially included daily inseminations (11 total inseminations) (Table 2) using ovulation or follicle disappearance [9] as our end point for discontinuing inseminations. However, it soon became apparent that follicular growth was slower in killer whales compared to bottlenose dolphins and that endocrine data were essential for determining whether we were, in fact, visualizing a functional follicle. Despite achieving an initial pregnancy, we subsequently relied more heavily on peak urinary EC measures to fine-tune the timing of insemination. Despite these improvements, two subsequent inseminations (females 2 and 4) were, unfortunately, stopped before ovulation, because fresh or frozen semen was unavailable.

Genome resource banking and assisted reproductive technology represent important tools for maintaining maximal genetic diversity of captive marine mammal populations [12, 13]. We have now developed a methodology that provides a vehicle for the creation of a captive killer whale genome resource bank. The widespread use of this technology in captive killer whale populations will depend, in part, on the ability to collect and cryopreserve semen from additional males at other facilities and on the training of females for sample collection, ultrasound, and AI procedures. Overall, we have demonstrated the potential of utilizing systematic approaches to improve our understanding of the reproductive biology of the killer whale, information that we believe was essential for ensuring the first successful conception resulting in offspring using AI in any cetacean species.

ACKNOWLEDGMENTS

We thank the veterinary, animal laboratory, animal care, and animal training staff at SeaWorld Orlando, San Antonio, and San Diego for their consistent support in making this research possible. We especially thank Laura Surovik and the animal training staff at SeaWorld Orlando for consistent semen collection and Dr. J.K. O'Brien for her editorial comments. We thank Dr. Fiona Brook for demonstrating that her ultrasonographic techniques developed in bottlenose dolphins could be applied to killer whales to locate the ovaries. We also recognize the technical support provided by Kendall Mashburn in conducting endocrine analyses and Nancy Czekala for enzyme immunoassay technical advice.

REFERENCES

1. Duffield DA, Odell DK, McBain JF, Andrews B. Killer whale (*Orcinus orca*) reproduction at Sea World. Zoo Biol 1995; 14:417–430.
2. Wildt DE, Schiewe MC, Schmidt PM, Goodrowe K, Philips JG, O'Brien S, Bach M. Developing animal model systems for embryo technologies in rare and endangered wildlife. Theriogenology 1986; 25:33–51.
3. Walker LA, Cornell L, Dahl KD, Czekala NM, Dargen CM, Joseph BE, Hsueh AJW, Lasley BL. Urinary concentrations of ovarian steroid hormone metabolites and bioactive follicle-stimulating hormone in killer whales (*Orcinus orchus*) during ovarian cycles and pregnancy. Biol Reprod 1988; 39:1013–1020.
4. Robeck TR, Schneyer AL, McBain JF, Dalton LM, Walsh MT, Czekala N, Kraemer DC. Analysis of urinary immunoreactive steroid metabolites and gonadotropins for characterization of the estrous cycle, breeding period, and seasonal estrous activity of captive killer whales (*Orcinus orca*). Zoo Biol 1993; 12:173–188.
5. Wright PJ, Malmo J. Pharmacologic manipulation of fertility. Vet Clin North Am Food Anim Pract 1992; 8:57–89.
6. Young SJF, Huff DG. Fertility management in a female killer whale (*Orcinus orca*) with altrenogest (Regu-mate). Proc Intl Ass Aquat Anim Med (Abstr) 1996; 27:66.
7. Robeck TR, Jensen E, Brook F, Rouke N, Rayner C, Kinoshita. Preliminary investigations into ovulation manipulation techniques in delphinids. Proc Am Assoc Zoo Anim Vet Int Assoc Aquat Anim Med 2000; 31:222–225.
8. Robeck TR, McBain JF, Mathey S, Kraemer DC. Sonographic evaluation of the effects of exogenous gonadotropins on follicular recruitment and ovulation induction in the Atlantic bottlenose dolphin, *Tursiops truncatus*. J Zoo Wildl Med 1998; 29:6–13.
9. Brook FM. Sonographic testicular and ovarian assessment in the bottlenose dolphin, *Tursiops truncatus aduncus*. In: Duffield D, Robeck TR (eds.), The Bottlenose Dolphin Breeding Workshop. Silver Springs, MD: AZA Marine Mammal Taxon Advisory Group; 2000: 7–15.
10. Brook FM. Sonographic imaging of the reproductive tract of the female bottlenose dolphin, *Tursiops truncatus aduncas*. Reproduction 2001; 121:419–428.
11. Schroeder JP, Keller KV. Artificial insemination of bottlenose dolphins. In: Leatherwood S, Reeves RR (eds.), The Bottlenose Dolphin. San Diego, CA: Academic Press; 1990:447–460.
12. Robeck TR, O'Brien JK. Effect of cryopreservation methods and precryopreservation storage on bottlenose dolphin (*Tursiops truncates*) spermatozoa. Biol Reprod 2004; 70:1340–1348.
13. Holt WV, Bennet PM, Volobouev V, Watson PF. Genetic resource banks in wildlife conservation. J Zool Soc London 1996; 238:531–544.
14. Wildt DE, Rall WF, Critser JK, Monfort SL, Seal US. Genome resource banks: "living collections" for biodiversity conservation. Bioscience 1997; 47:689–698.
15. Taussky HH. A micrometric determination of creatine concentration by Jaffe reaction. J Biol Chem 1954; 208:853–861.
16. Monfort SL, Wemmer C, Kepler TH, Bush M, Brown JL, Wildt DE. Monitoring ovarian function and pregnancy in Eld's deer (*Cervus eldi thamin*) by evaluating urinary steroid metabolite excretion. J Reprod Fertil 1990; 88:271–281.
17. Monfort SL, Bush M, Wildt DE. Natural and induced ovarian synchrony in golden lion tamarins (*Leontopithecus rosalia rosalia*). Biol Reprod 1996; 55:875–882.
18. Munro C, Stabenfeldt G. Development of microtiter plate enzyme immunoassay for the determination of progesterone. J Endocrinol 1984; 101:41–49.
19. Munro CJ, Stabenfeldt GH, Cragun JR, Addiego LA, Overstreet JW, Lasley BL. Relationship of serum estradiol and progesterone concentrations to the excretion profiles of their major urinary metabolites as measured by enzyme immunoassay and radioimmunoassay. Clin Chem 1991; 37:838–844.
20. McGeehan L, Li X, Jackintell L, Huang S, Wang A, Czekala NM. Hormonal and behavioral correlates of estrus in captive giant pandas. Zoo Biol 2002; 21:449–466.
21. Brown JL, Schmitt DL, Bellem A, Graham LH, Lehnhardt J. Hormone secretion in the Asian elephant (*Elephas maximus*): characterization of ovulatory and anovulatory luteinizing hormone surges. Biol Reprod 1999; 61:1294–1299.
22. Surovik LP, Lacinak CT, Tompkins CD, Scarpuzzi MR, Mairot JS, Andrews J, Robeck TR. Training techniques utilized in the first successful artificial insemination of a cetacean. Proc Int Mar Anim Trainer Assoc (Abstr) 2001; 29:19.
23. Howard JG, Bush M, de Vos V, Schiewe MC, Pursel VG, Wildt DE. Influence of cryoprotective diluent on postthaw viability and acrosomal integrity of spermatozoa of the African elephant (*Loxodonta africana*). J Reprod Fertil 1986; 78:295–306.
24. Pursel VG, Johnson LA, Rampacek GB. Acrosome morphology of boar spermatozoa incubated before cold shock. J Anim Sci 1972; 34: 278–283.
25. Ginther OJ. Chapter 1: Reproductive Anatomy. In: Reproductive Biology of the Mare Basic and Applied Aspects. Cross Plains, WI: Equiservice; 1992:1–40.
26. Howard JG, Bush M, Morton C, Morton F, Wentzel K, Wildt DE. Comparative semen cryopreservation in ferrets (*Mustela putorius furo*) and pregnancies after laparoscopic intrauterine insemination with frozen-thawed spermatozoa. J Reprod Fertil 1991; 92:109–118.

27. Loskutoff NM, Bartels P, Meintjes M, Godke RA, Schiewe MC. Assisted reproduction technologies in non-domestic ungulants: a model approach to preserving and managing genetic diversity. Theriogenology 1995; 43:3–12.
28. Loskutoff NM. Role of embryo technologies in genetic management and conservation of wildlife. In: Holt WV, Pickard AR, Rodger JC, Wildt DE (eds.), Reproductive Science and Integrated Conservation. Cambridge, U.K.: Cambridge University Press; 2003:183–194.
29. Vishwanath R. Artificial insemination: the state of the art. Theriogenology 2003; 59:571–584.
30. Squires EL, Stevens WB, McGlothlin DE, Pickett NW. Effects of an oral progestin on the estrous cycle and fertility of mares. J Anim Sci 1979; 49:729–735.
31. Webel SK, Squires EL. Control of the estrous cycle in mares with altrenogest. J Reprod Fertil Suppl 1982; 32:193–198.
32. Squires EL, Heesemann SK, Webel SK, Shideler RK, Voss VL. Relationship of altrenogest to ovarian activity, hormone concentrations and fertility of mares. J Anim Sci 1983; 56:901–909.
33. Kraeling RR, Dziuk PJ, Pursel VG, Rampacek GB, Webel SK. Synchronization of estrus in swine with ally trenbolone (RU-2267). J Anim Sci 1981; 52:831–835.
34. Stevenson JS, Davis DL. Estrous synchronization and fertility in gilts after 14- or 18-day feeding of altrenogest beginning at estrus or diestrus. J Anim Sci 1982; 55:119–123.
35. Robeck TR, Atkinson S, Brook F. Chapter 11: Reproduction. In: Dierauf L, Gulland F (eds.), CRC Handbook in Marine Mammal Medicine, 2nd ed. Boca Raton, FL: CRC Press; 2002:193–236.
36. Oettle EE. Using a new acrosome stain to evaluate morphology. Vet Med 1986; 81:263–266.
37. Rota A, Strom B, Linde-Forsberg C. Effects of seminal plasma and three extenders on canine semen stored at 4°C. Theriogenology 1995; 44:885–900.
38. Pena AI, Quintela LA, Herradon PG. Flow cytometric assessment of acrosomal status and viability of dog spermatozoa. Reprod Domest Anim 1999; 34:495–502.
39. Soede NM, Wetzels CCH, Zondag W, de Koning MAI, Kemp B. Effect of time of insemination relative to ovulation, as determined by ultrasonography, on fertilization rate and accessory sperm count in sows. J Reprod Fertil 1995; 104:99–106.
40. Johnson LA, Weitze KF, Fiser P, Maxwell WMC. Storage of boar semen. Anim Reprod Sci 2000; 62:143–172.
41. Vishwanath R, Shannon P. Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperatures. Reprod Fertil Dev 1997; 9:321–331.
42. Shannon P, Curson B. Effect of storage temperature on the viability and fertility of bovine sperm diluted and stored in Caprogen. N Z J Agric Res 1984; 27:173–177.
43. Evans G, Maxwell WMC. Salamon's Artificial Insemination of Sheep and Goats. Sydney, Australia: Butterworths; 1987.
44. Farstead W. Current state in biotechnology in canine and feline reproduction. Anim Reprod Sci 2000; 60–61:375–387.
45. Johnston SD, McGowan MR, Carrick FN, Tribe A. Nonsurgical intrauterine artificial insemination in bitches using frozen semen. J Reprod Fertil Suppl 1993; 307–311.
46. Morris LHA, Hunter RHF, Allen WR. Hysteroscopic insemination of small numbers of spermatozoa at the uterotubal junction of preovulatory junction of preovulatory mares. J Reprod Fertil 2000; 118:95–100.
47. Lindsey AC, Morris LH, Allen WR, Schenk JL, Squires EL, Bruemmer JE. Hysteroscopic insemination of mares with low numbers of nonsorted or flow sorted spermatozoa. Equine Vet J 2002; 34:128–132.
48. Lindsey AC, Schenk JL, Graham JK, Bruemmer JE, Squires EL. Hysteroscopic insemination of low numbers of flow sorted fresh and frozen/thawed stallion spermatozoa. Equine Vet J 2002; 34:121–127.
49. Robeck TR, Curry BE, McBain JF, Kraemer DC. Reproductive biology of the bottlenose dolphin (*Tursiops truncatus*) and the potential application of advanced reproductive technologies. J Zoo Wildl Med 1994; 25:321–336.
50. Shannon P. Factors affecting semen preservation and conception rates in cattle. J Reprod Fertil 1978; 54:519–527.
51. Hunter RHF. New breeding opportunities with deep corneal insemination: exploiting modern sperm technologies in cattle. Reprod Domest Anim 2001; 36:217–222.
52. Brinsko SP, Rigby SL, Lindsey AC, Blanchard TL, Love CC, Varner DD. Pregnancy rates in mares following hysteroscopic or transrectally guided insemination with low sperm numbers at the uterotubal papilla. Theriogenology 2003; 59:1001–1009.
53. O'Brien JK, Hollinshead FK, Evans KM, Evans G, Maxwell WMC. Flow cytometric sorting of frozen-thawed spermatozoa in sheep and nonhuman primates. Reprod Fertil Dev 2004; 15:367–375.
54. Harrison RJ, Ridgway SH. Gonadal activity in some bottlenose dolphins (*Tursiops truncatus*). J Zool 1971; 165:355–366.
55. Ginther OJ. Chapter 10: Follicles. In: Ultrasonic Imaging and Reproductive Events in the Mare. Cross Plains, WI: Equiservice: 1986:142–149.