

**Title.** Reproductive physiology and development of artificial insemination technology in killer whales (*Orcinus orca*)

**Short title.** Artificial insemination in killer whales

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## 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 to: i) determine the excretory dynamics of urinary LH and ovarian steroid metabolites during the estrous cycle; (ii) evaluate the effect of an exogenously administered synthetic progesterone analog on reproductive hormone excretion; (iii) validate the use of transabdominal ultrasound for ovarian evaluation and timing of ovulation; (iv) examine the quality of semen after liquid storage and cryopreservation, and (v) 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 d long, comprised of a 17 d and 21 d follicular and luteal phase, respectively. A consistent temporal relationship was observed between peak estrogen conjugates (iEC) and the LH surge, which occurred ~0.5 d later. Two animals placed on oral altrenogest (3 separate occasions for 30, 17 and 31 d, respectively) excreted peak urinary estrogen concentrations 25 d post-withdrawal that were followed by sustained elevations in urinary pregnanediol-3 $\alpha$ -glucuronide (iPdG) 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 (SMI = total progressive motility x kinetic rating (0-5 scale, 0 = no movement, 5 = rapid progressive movement) when held at 4°C for 3 d post-collection. Semen cryopreserved using a medium freezing rate demonstrated good post-thaw total motility (50.3%), progressive motility (94%) and kinetic rating (3.5 out of 5). Insemination during 8 estrous cycles resulted in three

pregnancies (38%), 2 from liquid stored and one from cryopreserved semen. Two calves were delivered after gestation lengths of 552 and 554 d, respectively. These data demonstrate the potential of noninvasive endocrine monitoring combined with serial ultrasonography for improving our understanding of the reproductive biology of cetaceans. This fundamental knowledge was essential for ensuring the first successful conceptions, resulting in live offspring, using AI in any cetacean species.

## **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 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 6 facilities (B. Andrews, personal communication). As a result, more than half of the present population has been captive born (26/48), 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 to successful AI in any species is the 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 has resulted in valuable data concerning the species' reproductive endocrinology. Duffield *et al.* [1] relied on twice monthly serum progesterone from 18 females over a 10-y period to define a mean gestation of 517 d (468 to 539 d). Walker *et al.* [3] described urinary bioactive FSH, estrogen conjugates and progesterone metabolites in the killer whale. With limited numbers of estrous cycles (n = 6), these authors reported a 42 - 49 d estrous cycle, consisting of 12 and 21 – 28 d follicular and luteal phase durations, respectively.

Although a 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 pregnanediol-3 $\alpha$ -glucuronide concentrations paralleled circulating luteal phase progesterone secretion. In addition, Robeck *et al.* [4] documented a single LH surge (out of 5 estrous cycles, 2 conceptive), 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 for improved management of natural breeding and for timing of AI.

Ultrasound has been used to evaluate ovarian follicular activity [8, 9, 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 interrelationship between reproductive hormones and ovulation.

The collection, short-term liquid storage or long-term cryopreservation of semen has not been accomplished in killer whales. The only cetacean species where 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, while 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 this research was to gain an understanding of killer whale reproductive physiology and apply this knowledge to improve natural breeding success and to develop assisted reproductive techniques. To accomplish this, specific objectives were to: i) determine the excretory dynamics of urinary LH and ovarian steroid metabolites during the estrous cycle; (ii) evaluate the effect of an exogenously administered synthetic progesterone analog on reproductive hormone excretion; (iii) validate the use of transabdominal ultrasound for ovarian evaluation and timing of ovulation; (iv) examine the quality of semen after liquid storage and cryopreservation, and (v) develop an intrauterine insemination technique.

## **MATERIALS AND METHODS**

### **Animals**

Three female killer whales, 1, 2 and 3 (20, 18 and 13 yrs of age, 2343, 2454 and 2181 kg, respectively) were involved in the endocrine monitoring and estrous synchronization studies. For the AI trials, two additional females, Female 4 and 5 (10 and 14 yrs of age and weighing 2075 and 2513 kg, respectively) were included. One 20 yr old male, Male 1, weighing 5254 kg, was used for semen collection. Of the females, only Female 1 and 2 had previously calved. During the AI procedures, candidate females were kept isolated from any breeding age males two weeks before and 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 to 3% of their body weight per day. All animals were housed with mixed 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 salt water, cooled year round with temperatures ranging from 52 to 55°F.

### **Ethics of Experimentation**

All samples were collected using routine husbandry training and were obtained on 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 USDA 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 prior to and during the study. Routine analysis of these samples for serum progesterone concentrations (determined by a commercial laboratory; Quest Diagnostics, Irving, TX) in females 1, 2, and 3 indicated that all were cycling for at least 3 yrs prior to commencement of the urinary endocrine monitoring study [1]

Urine samples from females 1, 2 and 3 were collected twice daily for 731, 570 and 645 d, respectively. Samples were stored in duplicate at  $-70^{\circ}\text{C}$  until analysis. Non-extracted urine samples were analyzed by radioimmunoassay (RIA) on an every other day (e.o.d.) basis for total immunoreactive levels of pregnanediol-3 $\alpha$ -glucuronide (iPdG) and estrogen conjugates (iEC). The results for iPdG and iEC on e.o.d. urine samples enable us to delineated when the estrous cycles had occurred. We were than able to target our daily hormone analysis during estrus. To compared iEC derived from RIA or enzyme immunoassay (EIA) we analysed twice-daily urinary urine samples from Female 1, with both assay systems. Also during these periods of hormonal estrus, luteinizing hormone (LH) concentrations were determined by RIA in twice-daily urine samples from all three whales. Concurrent urinary iEC determined by both RIA and EIA from female 1 were compared for significance of positive correlation by Pearson's correlation ( $r = 0.75$ ,  $p < 0.005$ ).

Determination of total cycle length (TCL) was based on either the interval between the beginning of successive luteal phases (inter luteal phase interval), successive LH peaks, or successive iEC peaks. For the study, LH and iEC peaks were defined as the maximum concentration for the respective hormones during the estrous period. In addition, intra-endocrine estrous cycle components were determined in an attempt to improve our ability to predict ovulation within each female's cycle. Length of the luteal phase (iPdG concentrations  $> 0.1$  ng/mg cr for 3 consecutive days), follicular phase (iEC concentrations  $> 0.5$  ng/ml cr (creatinine) for 3 consecutive samples), iPdG 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 iEC were determined. The preovulatory rise in iEC concentrations as determined by EIA was subjectively defined as values  $> 2000$  pg/mg cr that occurred on 2 consecutive days during the follicular phase. Using this algorithm, the mean interval from preovulatory iEC rise to LH peak was determined for all whales. A "normal" estrous cycle was determined by combining the mean values from all whales for all of the above-mentioned intervals.

After patterns of iEC, iPdG and LH had been established in Females 1, 2, and 3, artificial insemination was attempted in all 5 females. During the artificial insemination attempts, iEC and LH were analyzed in twice daily urinary samples. Based on the initial endocrine study, it was determined that iEC data could be used to predict estrus for the purpose of preparing for AI. The data collected during the 8 insemination attempts was combined with the initial endocrine monitoring study to determine cycle length components as describe above. Endocrine data collected during the artificial insemination



attempts was compared to the ultrasonographically estimated ovulation point to define the interval between the iEC and LH peak and ovulation.

### Synchronization of ovulation using Progesterone Analog Treatment

A total of 3 treatments were administered. Females 1 and 2 were placed on 0.025 mg/kg p.o. of altrenogest (Regu-Mate, Intervet Inc., Millsboro, Delaware 19966, USA) for 30 and 17 d, respectively. Female 1 was administered a second round of treatment with 0.05 mg/kg for 31 d, 10 mo after the first treatment. Immunoreactive iPdG, iEC and LH were determined, from daily urine samples, during and after the treatment with altrenogest. The mean time from altrenogest withdrawal to the first hormonally detected follicular phase was determined. Both killer whales were cycling prior to altrenogest administration, and they were in mid to late luteal phase when administration began.

### Creatinine assay

Creatinine (cr) levels were determined for each urine sample to adjust for inter-sample urine concentration differences [15]. Concentrations of urinary hormones and metabolites were expressed as mass of hormone per mg cr excreted.

### RIA Assay for Estrogen Conjugates (iEC)

Urinary iEC 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) 1:10,000, and a 50  $\mu$ l aliquot was adjusted to a final assay volume of 300  $\mu$ l in TRIS buffer (0.1 M TRIS, 0.9% NaCl, 0.1% NaN<sub>3</sub>, 0.1% gelatin, pH 8.4). Antiserum which crossreacts 100% with estrone-glucuronide and estrone-sulfate (anti-estrone-3-glucuronide serum, 100  $\mu$ l, 1:1500, D.

Collins, Emory University, Atlanta, GA) and  $^3\text{[H]}$ -estrone sulfate (100  $\mu\text{l}$ , 7,000 c.p.m., 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  $\mu\text{l}$  charcoal-dextran (0.0625% Norit A charcoal, 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 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  $\pm$  S.E.M. recovery of estrone sulfate (range, 4.9-2500 pg/tube) added to a pool of killer whale urine was 110.1  $\pm$  10% ( $y = 0.79x + 15.04$ ;  $r^2 = 0.99$ ). Assay sensitivity was 4.9 pg/tube or 0.1 ng/ml, inter-assay coefficients of variation for three separate internal controls ( $n = 18$  assays) were 11% (20% binding), 13% (40% binding), and 18% (80% binding), and intra-assay variation averaged <10%.

### Assay for Pregnanediol-3 $\alpha$ -Glucuronide (iPdG)

Urinary PdG immunoreactivity was analyzed using the methods previously described [17]. A 200  $\mu\text{l}$  urine aliquot diluted (1:50) in PBS was combined with 100  $\mu\text{l}$  each of PdG antiserum which crossreacts 100% with PdG and 6.7% with pregnanediol (#02/Zoo, 1:20,000) and  $^3\text{[H]}$ -PdG (7000 c.p.m., specific activity 42 Ci/mmol) supplied by Courtauld Institute of Biochemistry, London, England. Urine samples and standards (19.5-5000 pg/tube, Sigma-Aldrich Chemical Co.) were incubated overnight (4°C), and antibody bound and free steroid were separated after a 45 min incubation with 300  $\mu\text{l}$  charcoal-dextran suspension and centrifugation for 10 min (1500 g). Supernatants were combined with 5 ml 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  $\mu$ l, 1:16) gave a mean of  $69 \pm 9\%$  ( $y = 0.61x + 0.76$ ;  $r^2 = 0.99$ ). Assay sensitivity was 19.5 pg/tube, and inter-assay coefficients of variation for two separate internal controls ( $n = 23$  assays) were 10% (25% binding and 14% (50%% binding), and intra-assay variation was <10%.

### EIA Assay for Estrogen Conjugates (EC)

The EC EIA has not been previously used in this species. Urinary estrogen conjugates were measured by single antibody, direct enzyme immunoassay as previously described [18, 19, 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 (0.01ml) and standards (range 400pg – 3.1pg, Sigma-Aldrich) were added to wells in duplicate. Enzyme conjugate (E1G horseradish peroxidase, 1:100,000, C. Munro, UC Davis, CA) was then added to all wells and the plate incubated at room temperature for two hours. After incubation, plates were washed and 0.1ml of substrate, tetramethyl benzidine in phosphate citrate buffer (Sigma-Aldrich) added to all wells and incubated at room temperature for 30 min. Finally, 0.05ml of 0.6M H<sub>2</sub>SO<sub>4</sub> was added to all wells and plates read at 450nm (reference 650nm) in a microplate reader (Benchmark, Biorad, Hercules, CA). Intra assay variation was 5% and interassay variation was 15.9% ( $n = 28$ ) at 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 HPLC analysis revealed one minor immunoreactive peak (fractions 21-24, 16% of

total) that co-eluted with estrone-1-sulfate, and a second, much larger immunoreactive peak (fractions 62-67, 67% of total) that co-eluted with estrone.

Significantly elevated iEC concentrations determined by RIA and EIA were subjectively defined as those > 500 pg/mg cr and 800 pg/mg cr, respectively. The follicular phase was defined as the length of time during which at least 3 consecutive days had significant iEC concentrations during iPdG nadir.

### Luteinizing Hormone RIA

The assay for LH was an I<sup>125</sup> double antibody RIA that has been previously described [21]. An anti-bovine LH antiserum (518-B<sub>7</sub>, 1:500,000) and ovine LH (26), provided by NHPP, NIADDK, was used as the radioligand and standard. Then, 100ul of sample or standard (0.039 to 5 ng/ml) were added, in duplicate, to the antiserum (100ul). After incubation at room temperature for 24 h, approximately 25,000 c.p.m. (in 100ul) was added to each tube and incubated at room temperature for another 24 h. Finally, 100ul of goat-anti-mouse antibody (1:800, Sigma-Aldrich Chemical Co.) was added. Following a one-hour room temperature incubation, the tubes were centrifuged at 3000g for 25 minutes, decanted, and the precipitate 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 inter-assay coefficients of variation for two separated 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 machine (Corometrics 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 data collected during the current cycle) day of peak iEC, ultrasound exams were increased to three times daily. For examinations, 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 evaluations. Follicular size was determined by measuring its largest diameter, utilizing the anechoic margins as the border. For ovoid follicles, diameter in two planes were determined, and circumferences was calculated from the following formula:

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

Thus, follicular size reflected follicular antral diameter. Follicular size was quantified immediately using the ultrasound machine's electronic calipers, or later when

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 prior exam and the exam when the follicle could not be located. This interval between exams was a maximum of 12 hours for twice daily and 8 hours for three times daily examination.

### Semen Collection and processing

A total of nine ejaculates were collected for use during a total of 8 AI attempts or estrus 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]. The 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 Int., 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 determined to the nearest 5% by analyzing 4-5 fields of diluted sperm

(35°C) (1:25, sperm: HEPES-buffered HTF medium, Irvine Scientific, Santa Ana, CA USA) using bright field optics (x400, Olympus, Tokyo, Japan). Total motility (TM), percent progressive motility (PPM) and kinetic rating (KR, 0-5 scale, 0 = no movement, 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 Howard *et al.*, [23]):  $SMI = (\text{percentage of total motility}) \times \text{percent progressive motility} \times \text{kinetic rating}$ .

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) for 30s. For each ejaculate, an air-dried smear was used to evaluate 200 spermatozoa using bright field optics (x1000). 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 was diluted with 10 µl of HEPES-TALP Media (Androhep; modified Tyrodes media 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 (x1000). Acrosomes were evaluated with bright field optics (x1000) 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 (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 (Minitube of America), Fraction A (1210 gm Tris, 690 gm citric acid and 5 gm fructose, and 20% egg yolk (v/v) per 500 ml) with antibiotics (0.5 mg ml<sup>-1</sup> Tylosin, 2.5 mg ml<sup>-1</sup> Gentamycin, 1.5 mg ml<sup>-1</sup> Lincomycin, and 3 mg ml<sup>-1</sup> spectinomycin). Biladyl was chosen due to its ease of preparation and preliminary studies that demonstrated its ability to maintain high levels of killer whale sperm motility during storage for 36 h at 4°C (T. Robeck, unpublished). The diluted sample was transferred to a sterile 4 oz 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 to 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 prior to each insemination, a 15- $\mu$ l portion was removed from each sample, warmed to 35°C and re-evaluated (TM, PPM, and KR).

### Processing of semen for frozen storage

Ejaculates were extended with Biladyl Fraction A (2:1, semen: diluent) over five min. The sealed tube containing the sperm suspension was placed in a beaker with 250 ml water (21°C) and cooled to 5°C over 1 h (-0.27°C min<sup>-1</sup>). Once at 5°C, the sperm



suspension was placed into an ice water bath (2°C) for 1 h (cooling rate:  $-0.6^{\circ}\text{C min}^{-1}$ ), then diluted 1:1 (v/v) over 10 min with Biladyl Fraction B (at 2°C) containing 14% glycerol (7 % final glycerol concentration) 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 ( $-6^{\circ}\text{C min}^{-1}$ ) for 5 min, then placed at 7.5 cm above the vapor ( $-7^{\circ}\text{C min}^{-1}$ ) for an additional 5 min prior to plunging. This freezing rate was selected based a preliminary freezing trial that indicated killer whale sperm had better in-vitro post thaw motility after freezing using a slow ( $-6^{\circ}\text{C min}^{-1}$ ) compared to with a fast ( $-35^{\circ}\text{C min}^{-1}$ ) freezing rate (T. Robeck, unpublished). Straws were then plunged into liquid nitrogen.

## Artificial Insemination

The first insemination was based on the presence of a preovulatory follicle and/or the detection of peak urinary iEC. 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 prior to insemination. The straws were placed in a water bath at 35°C for 30 seconds ( $8.3^{\circ}\text{C s}^{-1}$  [12]) then diluted 1:1 v/v over 5 min with Biladyl Fraction A. Total motility, PPM, KR and sperm concentration were determined for each sample prior to insemination.

For all procedures, females were trained to station themselves poolside in dorsal recumbency. To overcome the substantial technical challenges associated with intra-

uterine sperm deposition, insemination methods were modified, as appropriate, during successive AI trials. For the initial insemination trial, an endotracheal tube (22 mm I.D., 30 mm O.D., 90 cm length; Cook Veterinary Products, Spencer, Indiana 47460 USA) was introduced into the vagina until resistance was encountered (approximately 70 cm). A gastroscope (183 cm x 11 mm O.D.) with a polyurethane tube (5 mm O.D.) affixed along its length with 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 inserted into the polyurethane tube, introduced through the external cervical os, and semen was deposited beyond the first of two cervixes (T. Robeck, unpublished data). Killer whales have two cervixes in series of similar morphology and appearance to an equine cervix [25]. The two cervixes, of approximately 5 cm in length each, are composed of longitudinal folds; the folds of the proximal cervix are continuous with 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 cervical Os are surrounded by a fornix approximately 5 cm in depth (T. Robeck, unpublished).

For the remaining insemination attempts, substantial modifications were made. First, a catheter was developed based on killer whale reproductive tract dimensions (400 cm, 2.8 mm external diameter [8 French], bullet tipped catheter, Cook Vet Supplies, Cat Number, V-WIC-8.0 – 400). Additional procedural modifications were made, including: (i) the endoscope was inserted directly into the vagina without the aid of a speculum; (ii) insufflation of the vagina was performed to visualize the cervical opening prior to advancing the endoscope into the caudal uterine body; and (iii) 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 Newmann-Keuls Multiple Comparisons and Mann-Whitney U test (SigmaStat, Version 2.0. SPSS Inc., San Rafael, CA USA). Results are presented in Mean  $\pm$  standard deviation (SD).

## RESULTS

### Endocrine Monitoring

Two (Female 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 iPdG, iEC, and LH were measured. Female 3 experienced three prolonged inter-estrus intervals, with baseline iPdG and iEC, of 105, 88 and 91 d. In addition, this female, not housed with a breeding age male, had continuously elevated progesterone, or pseudopregnancy for 193 d.

During the entire study period, endocrine monitoring and AI trials, 41 follicular phases (LH and iEC), and 26 complete estrous cycles (iPdG, LH and iEC) were evaluated from the five whales. Three typical cycles for female 4 are illustrated in Fig.1. The mean TCL between consecutive peak estrogens was  $40.7 \pm 6.0$  d (n=41, range: 35.5 to 68 d). No significant differences ( $P > 0.05$ ) were observed between median TCL determined by

the interval between successive LH peaks ( $42 \pm 7.2$  d, median 39 d), iEC peaks (39 d) or the inter-luteal phase interval ( $41.7 \pm 6.4$  d, median 40 d; as determined by iPdG levels). Length of the follicular phase and luteal phase were  $17.1 \pm 3.5$  d ( $n = 41$ , range = 9 to 27 d) and  $21.6 \pm 3.9$  d ( $n = 31$ , range = 14 to 33 d), respectively. The time from iPdG nadir (luteal regression) to Peak iEC was  $13.9 \pm 1.8$  d ( $n = 26$ , range = 12 to 18 d). The interval between peak estrogens and peak LH was  $0.46 \pm 0.49$  d ( $n = 29$ , range = 0 to 2 d). The interval between peak LH and increased iPdG was  $5.5 \pm 2.5$  d ( $n = 28$ , range = 1 to 14 d). The time of ovulation as determined by ultrasonography occurred a mean  $58.0 \pm 10.4$  and  $38.0 \pm 29.0$  h of the iEC 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 female treated with altrenogest for 17 d (Female 2) excreted maximal iEC concentrations 22 d after altrenogest withdrawal, but no iEC-associated LH peak was detected. However, this female did have a rise in iPdG concentrations, suggesting presence of luteal tissue, at the expected time after maximal iEC 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 d (data not shown) after this initial cycle. The female treated initially with altrenogest for 30 d (Female 1) had maximal iEC excretion 29 d after altrenogest withdrawal (Fig. 3). An LH peak was associated with peak iEC, and a subsequent luteal phase was observed. Thereafter, Female 1's ovarian cycle length ( $n = 5$ ) averaged  $40.8 \pm 5.8$  d. During her next treatment (using twice the altrenogest dose for 31 d), peak iEC excretion occurred at

24 d after altrenogest withdrawal. Although an LH peak was not observed, a presumptive luteal phase, characterized by prolonged excretion of iPdG, followed. Mean ovarian cycle length for the 10 subsequent cycles (sampling was discontinued after the 10th cycle) was  $39.4 \pm 2.4$  d. Overall, peak iEC excretion was detected  $25.0 \pm 3.6$  d after altrenogest withdrawal ( $n = 3$  treatments). An LH peak was not detected in 2 of 3 altrenogest cycles; however, luteal phases were detected in all three. Luteal phases ongoing at the onset of altrenogest treatment lasted  $20.0 \pm 2.0$  d.

### Ultrasonographic evaluation of ovaries

Growing follicles were observed during all eight AI attempts and the pre-ovulatory follicle (POF) was observed in 6/8 instances (Fig. 4). The two instances where the POF were not observed was because observations were discontinued prior to ovulation. These two AI attempts were aborted due to lack of semen. For the other six AI attempts, growth of the dominant and secondary follicle was recorded for up to 20 d before ovulation (Fig. 5). A secondary follicle was observed on the ipsilateral ovary of the dominant follicle three times, twice on the contra-lateral ovary, and none were observed in Female 4. These secondary follicles were observed up to 10 d post-ovulation. Female 5 had three follicles, two on right, the side of ovulation, and one on the left. The mean circumference and maximum diameter of the secondary follicle at the time of ovulation was  $9.7 \pm 2.4$  cm and  $3.3 \pm 0.9$  cm, respectively. The mean preovulatory follicle circumference and maximum diameter were  $11.2 \pm 2.6$  cm, and  $3.9 \pm 0.6$  cm, respectively. The mean rate of growth of the POFs was  $0.98 \pm 0.5$  cm d<sup>-1</sup>. 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 preovulatory follicle. The ovulations occurred on the left ovary

in Female 1 (n = 1 ovulation) and 4 (n = 3 ovulations) and on the right ovary in Female 2 (n = 3 ovulations) and 5 (n = 1 ovulation).

Corpora hemorrhagica were not visualized during ultrasounds performed after the disappearance of the POF. A CL (mean  $26.1 \pm 1.5$  cm diameter) was only detected in two animals two weeks post conception. Both CLs were spherical structures with heterogeneous echogenicity (anechoic fluid filled structure with many hair like hyperechoic lines within the fluid). The CLs could be repeatedly detected during random pregnancy exams. The echogenicity did not change; however, they continued to grow with the maximum observed size ( $46.5 \pm 0.86$  cm diameter) occurring 90 days post-conception. After maximum diameter was reached, the size did not appear to decrease during pregnancy. Exams were not conducted post-partum.

Ultrasonography indicated that Female 2 had approximately four non-growing cysts (all approximately 2.5 cm in diameter) associated with the left ovary. Following complications related to a cerebral fungal infection, post-mortem examination (at 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, while the nucleus was stained red. Exposure time of sperm initial red stain was increased from 2 min (as recommended by the manufacturer) to 3 min to improve resolution of the sperm nucleus and ease of acrosome evaluation.

## Ejaculate Characteristics in undiluted, liquid-stored post-transported and post-thawed sperm

Characteristics of 13 ejaculates collected from one male are shown in Table 1. Overall, ejaculates were of high quality with percent sperm motility, viability, intact acrosomes and normal morphology > 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 d post-collection, 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; PPM; 94; KR: 3.5; Table 1). The post-thaw TM and KR were significantly ( $P < 0.001$ ) lower than that obtained for the undiluted ejaculate and liquid-stored semen.

## Artificial Inseminations

Artificial insemination was performed during 8 cycles over 2-yr period. Eleven inseminations were performed during the first cycle attempted; after this, an average of  $2.6 \pm 1.1$  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 - 70 cm, and the distance remained relatively constant among females.

The overall conception rate (conceptions/attempts x 100; 3/8) was 38 %. Using liquid stored semen, conceptions were achieved (2/3) 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 prior to ovulation because additional fresh or frozen-thawed semen was unavailable (Table 2). In another artificial insemination trial endocrine analysis revealed that insemination had been conducted 72 h after the pre-ovulatory LH surge. Of the three conceptions (2 male, 1 female offspring), two females delivered live calves at 552 and 554 d, respectively. The third animal, Female 2, died from an unrelated cause with a normal 129-d, 136-gm, 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, artificial insemination is potentially the most effective and practical assisted reproductive technology (ART) available for managing genetics of these populations [14, 26, 27, 28, 29]. 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-term systematic evaluations of female reproductive physiology performed in the present study were 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 as twice-daily samples were analyzed in the present study. While elevated iEC data can be used to confirm that an animal is in estrus, 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 (iEC and LH) and ovulation (58 and 38 h, respectively), we used less frozen or liquid-stored semen since inseminations were commenced only after the peak iEC had been detected. Efforts are ongoing to develop a more rapid semi-quantitative LH assay that can be used poolside to further improve our ability to predict ovulation, as opposed to the current test that has a 3-d turnaround time.

Altrenogest has been used effectively in several species to regulate the estrous cycle without reducing fertility (horses [30, 31, 32]; pigs [33, 34]). Long term (> 1 year) use of altrenogest has been used to suppress estrus without affecting subsequent fertility in the killer whale [6] and Pacific white-sided dolphin (Robeck, unpublished data). Similarly, both animals in 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 (post-withdrawal) that mimic a normal follicle phase (post-luteal regression). However, in killer whales, altrenogest caused a delay or suppression of follicular growth during and for a protracted period after hormone withdrawal that was longer (25 d) than the interval from normal luteal regression to peak iEC excretion (13 d). A protracted interval between hormonal withdrawal and subsequent ovulation was also observed in bottlenose and pacific white-sided dolphins [7]. While 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 post altrenogest cycles. However, they all exhibited a subsequent luteal phase, which provided presumptive evidence of ovulation. However, this was probably due to an inadequate 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.

Semen characteristics presented in this study represent the only information to date 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 because some ejaculate was invariably lost during the technically difficult collection procedure. Anecdotal reports from animal staff having observed killer whales 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 results were highly

correlated with the fluorescent stain FITC-PSA [38]. In addition to the canine, it has recently been validated for use in bottlenose dolphins [12]. Spermac was also 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 post-thaw acrosomal status in the killer whale.

Similar to dolphin sperm stored at 4°C [12], killer whale sperm maintained over 90% of its original SMI for up to 3 d post collection 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 d before significant reduction in fertility is observed [40]. Susceptibility to aging appears to be dependent on male, or on ejaculate quality prior to storage [40, 41], and can be affected by the storage temperature (<5°C vs. 18 to 24°C; [42]). Based on our success using semen from one male, it appears that fertility of killer whale spermatozoa stored in Biladyl at 4°C will allow for once daily intrauterine inseminations with semen for up to at least 3 d post-ejaculation.

The simple method described for killer whale semen cryopreservation resulted in adequate progressive motility 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 (i.e., semen extender, cooling rates, holding temperatures) and cryopreservation (i.e., cryodiluent, freezing and thawing rates) methods with the aim of enhancing in vitro sperm quality parameters.

Due to the difficulties encountered when attempting to cannulate the cervix of small ruminants and companion animals, intrauterine insemination in small ruminants and companion animals has usually been accomplished using laparoscopy (e.g. ovine and caprine: [43]; canine and feline: [44]). However, non-surgical endoscopic methods have been reported in the canine [45]. In addition, endoscopic methods have recently been used in the equine to reduce the total number of sperm necessary for fertilization as compared to traditional non-surgical vaginal cervical cannulation methods [46, 47, 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 intra-cervical 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. Traditionally in bovine, the minimum number of cryopreserved sperm required for a successful AI is approximately 10 times greater than when using fresh sperm [50]. However, recent reports have shown that intrauterine deposition of spermatozoa, either at the mid-body or uterine horn can dramatically reduce the numbers of spermatozoa required for conception (e.g. bovine: [51]; and equine: [52]).

In killer whales, the ability to perform deep cornual inseminations should lend itself to reduced numbers of sperm required per insemination and thus 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 mean time from initial follicle development ( $>0.4$  mm) to ovulation was  $12.0 \pm 1.3$  d. In our 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  $<2.0$  cm in diameter that were followed for 20 d prior to ovulation. These preliminary data suggest that follicular growth and recruitment occurs over a greater period of time in killer whales than dolphins. Another difference between the two species was size of the preovulatory follicle, which was 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 (CA) in the bottlenose dolphin, ovulation and pregnancy occurred in the left ovary and left uterine horn, respectively, more than 68% of the time [54, 55]. In support of this, 83% of ovulations occurred on the left ovary in the Indo-Pacific bottlenose dolphin [9]. In this study, developing follicles and ovulations were observed on the R and L ovaries of 4 killer whales. However, definition of the symmetry of ovulation in killer whales is not yet

possible, since 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 and preovulatory follicles are similar in size to 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 a result of impending ovulation. In killer whales, the follicles are located on the stromal surface and the change in shape between exams may be due to 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 examination on both the ipsilateral and contralateral ovary. But in contrast to the bottlenose dolphin, secondary follicles did not regress and were still present up to 10 d post-ovulation. We hypothesize that the secondary follicle observed at the time of ovulation were destined to become primary follicles during the subsequent estrous cycle. As reported in this and other studies [3, 4, 1], killer whales often experience prolonged anestrus. We theorize that prolonged anestrus intervals may be predicted by the absence of a secondary follicle during the previous peri-ovulatory interval. Despite using a 3.5 MHz probe, insufficient resolution due to the presence of 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 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. While overall conception rate following AI was only 38%, success rate was hampered by failures encountered during the method development stage of the study. Since 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 (>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 the maximum size for a POF. Thus, our protocol initially included daily inseminations (11 total insemination, Table 2) using ovulation or follicle disappearance [9] as our endpoint for discontinuing inseminations. However, it soon became apparent that the follicular growth was slower in killer whales compared to bottlenose dolphins, and that endocrine data was essential for determining whether or not we were, in fact, visualizing a functional follicle. Despite achieving an initial pregnancy, we subsequently relied more heavily on peak urinary iEC measures to fine-tune insemination timing. Despite these improvements, two subsequent inseminations (Female 4 and 2) were unfortunately stopped prior to ovulation because fresh or frozen semen were unavailable.

Genome resource banking (GRB) 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 GRB. The widespread use of this technology in

captive killer whale populations will depend, in part, upon the ability to collect and cryopreserve semen from additional males located at other facilities, and the training of females for sample collection, ultrasound, and AI procedures. Overall, we have demonstrated the potential of utilizing systematic approaches to improving our understanding of the reproductive biology of the killer whale, information that we believe was essential for ensuring the first ever successful conception, resulting in offspring using artificial insemination in any cetacean species.

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## 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 orca*) 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 Amer Food Anim Prac* 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; 66.
7. Robeck TR, Jensen E, Brook F, Rouke N, Rayner C, Kinoshita R. Preliminary investigations into ovulation manipulation techniques in delphinids. *Proc Amer Assoc Zoo Anim Vet and Intl Assoc Aquat Anim Med* 2000; 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 Wild 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*. AZA Marine Mammal Taxon Advisory Group, Silver Springs, MD; 2000: pp. 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*. Academic Press San Diego CA; 1990: pp. 447-460.
12. Robeck TR, O'Brien JK. Effect of cryopreservation methods and pre-cryopreservation storage on bottlenose dolphin (*Tursiops truncates*) spermatozoa. *Biol Reprod* published 7 January 2004; 10.1095/biolreprod.103.025304.
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,S.L.; Wemmer,C.; Kepler,T.H.; Bush,M.; Brown,J.L.; Wildt,D.E.  
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 Endo* 1984; 101:41-9.
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; 37:838-44.
20. M<sup>c</sup>Geehan L, Li X, Jackintell L, Huang S, Wang A, Czekala NM. Hormonal and behavioral correlates of estrus in captive giant pandas. *Zoo Biol* 2002; 21:449-66.
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 1294-99.
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 Intl 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 post-thaw 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. Equiservice, Cross Plains, Wisconsin; 1992: pp. 25.
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 ungulates: 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 University Press, Cambridge; 2003: pp.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 oestrous cycle in mares with altrenogest. Journal Reproduction Fertility 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. Chap 11: Reproduction. In: Dierauf, L, Gulland, F (eds.), *CRC Handbook in Marine Mammal Medicine*, Second edition. CRC Press, Boca Raton, FL; 2002: pp. 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 Dom 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 Fert* 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. *New Zeal J of Agri Res* 1984; 27:173-177.
43. Evans G, Maxwell WMC. *Salamon's Artificial Insemination of Sheep and Goats*. Butterworths, Sydney 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 Non-surgical 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 non-sorted 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 Wild Med* 1994; 25:321-336.
50. Shannon P. Factors affecting semen preservation and conception rates in cattle. *J Reprod Fert* 1978; 54:519–527.
51. Hunter RHF. New breeding opportunities with deep corneal insemination: exploiting modern sperm technologies in cattle. *Reprod Dom 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 utero-tubal 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 non-human primates. *Reprod Fertil Develop* 2003; 15:1-9.
- Ohsumi, S. Comparison of maturity and accumulation rate of corpora albicantia between the left and right ovaries in cetacea. *Tokyo Whale Research Institute* 1964; 18: 123-149.
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*. Equiservice, Cross Plains, Wisconsin; 1986: pp. 142-149.

## Figure Legends

Fig. 1. Three typical cycles from Female 4 characterized by urinary concentrations of luteinizing hormone (LH), estrogen conjugates (iEC) and pregnandiol-3-glucuronide (iPdG). Note the obvious LH surge in two of the three cycles; iPdG was not measured beyond day 165.

Fig. 2. Mean killer whale estrous cycle components including urinary concentrations LH, iEC and iPdG. Note peak LH approximately 0.5 d following the iEC peak.

Fig. 3. altrenogest treatment (black bar) in two killer whales and the effects on urinary concentrations of iPdG, iEC and LH. The top panels shows results from Female 2, whereas the bottom two are from Female 1. Note the lack of an LH surge in the top two panels. Also note that at the start of all three treatments the animals excreted elevated iPdG, representative of circulating progesterone.

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.

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



Table 1. Characteristics of Killer Whale Ejaculates used for Artificial Insemination

Parameter	Neat Ejaculate (n = 13)	Liquid-stored semen PTr (12-24 h post-collection, n = 5) <sup>a</sup>	Frozen-stored (0 h post-thaw, n = 8) <sup>b</sup>
Semen characteristics			
Volume (ml)	13.9 ± 11.5	41.1 ± 5.1 <sup>c</sup>	39.9 ± 9.9 <sup>c</sup>
Sperm concentration (x 10 <sup>7</sup> ml <sup>-1</sup> )	98.8 ± 69.7	8.8 ± 75.9	5.4 ± 5.2
Total spermatozoa per ejaculate (x 10 <sup>7</sup> ml <sup>-1</sup> )	1315 ± 1379.1 <sup>1</sup>	262.5 ± 181.2 <sup>d,1,2</sup>	88.0 ± 88.3 <sup>d,2</sup>
Sperm characteristics			
Total Motility	90.5 ± 4.7 <sup>1</sup>	87.3 ± 6.6 <sup>1</sup>	50.3 ± 5.0 <sup>2</sup>
Percent progressive motility	94.2 ± 3.8	95.0 ± 0.0	94.0 ± 4.3
Kinetic rating (0-5) <sup>e</sup>	4.6 ± 0.4 <sup>1</sup>	4.4 ± 0.4 <sup>1</sup>	3.5 ± 0.4 <sup>2</sup>
Sperm motility index <sup>f</sup>	391.3 ± 49.7	92.2 ± 5.9 <sup>g</sup>	46.2 ± 12.9 <sup>g</sup>
Viability (%)	89.5 ± 6.7	94.3 ± 4.0 <sup>h</sup>	90.6 ± 8.7 <sup>h</sup>
Intact Acrosomes (%)	87.4 ± 10.6	----	----
Morphologically normal (%)	88.4 ± 4.8	----	----

Values are means ± SD

<sup>a</sup>PTr: Post Transport: A total of 10 inseminations were performed using 5 ejaculates; ejaculates (n=?) were held for ≤ 3 d at 4°C.

<sup>b</sup>A total of 8 ejaculates were cryopreserved and used during 20 inseminations.

<sup>c</sup>Final volume of insemination dose

<sup>d</sup>Total Progressively motile sperm

<sup>e</sup>Kinetic rating of spermatozoa graded subjectively:0 = no movement, 5 = rapid forward progression

<sup>f</sup>Sperm motility index = (total motility x percent progressive motility) x kinetic rating

<sup>g</sup>Mean percent of Neat SMI for each sample or %SMI

<sup>h</sup>Mean percent of Neat percent Live or Percent of original sample.

<sup>1,2</sup>Data in same row with different superscript are significantly different (P < 0.001)

**Table 2.** Killer whale artificial insemination data

Animal	IPE <sup>a</sup>	SOD <sup>b</sup>	SS <sup>c</sup>	TPMS (x 10 <sup>7</sup> ) <sup>d</sup>	LHO <sup>e</sup> (h)	LHI <sup>f</sup> (h)	PFC <sup>g</sup> (cm)	IO <sup>h</sup> (h)	Conception (Cn)
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 <sup>j</sup>	Uterine	F	67.5	-60	-36	11.3	-96	No
Female 2	2 <sup>j</sup> 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 Cn (± SD)	3.5 ± 4.4	---	---	85.0 ± 29.6	-12 ± 18	6 ± 25	10.6 ± 2.8	-6 ± 12	---
Mean NC <sup>k</sup> (± SD)	2.0 ± 1.1	---	---	46.3 ± 32.9	-56 ± 30	26 ± 47	9.7 ± 2.2	-48 ± 41	---

<sup>a</sup>IPE: Inseminations per estrus: The total number and frequency of inseminations per estrus period.

<sup>b</sup>SOD: Site of Deposition: Site where semen was deposited during insemination.

<sup>c</sup>SS: Semen Storage: Method of storage for semen used closest to ovulation. L: Liquid state- slowly diluted in BF5F extender v/v, cooled and transported via equitainer to location of insemination. F: frozen (cryopreserved).

<sup>d</sup>TPMS/dose: Total progressively motile sperm per insemination dose

<sup>e</sup>LHO: Luteinizing hormone (LH) Peak to Ovulation: The estimated time between the LH surge and ovulation. Ovulation determined by the disappearance of the follicle from one exam to the next. The time between exams was divided to approximate when ovulation occurred.

<sup>f</sup>LHI: LH peak in relation to the farthest temporally located insemination that was closest to ovulation.

<sup>g</sup>PFC: Preovulatory Follicle Circumference

<sup>h</sup>IO: Insemination to Ovulation: Closest temporally (h) related insemination to ovulation

<sup>j</sup>Total numbers of inseminations were limited by the availability of semen. Thus, inseminations were stopped prior to ovulation.

<sup>k</sup>NC: no conception

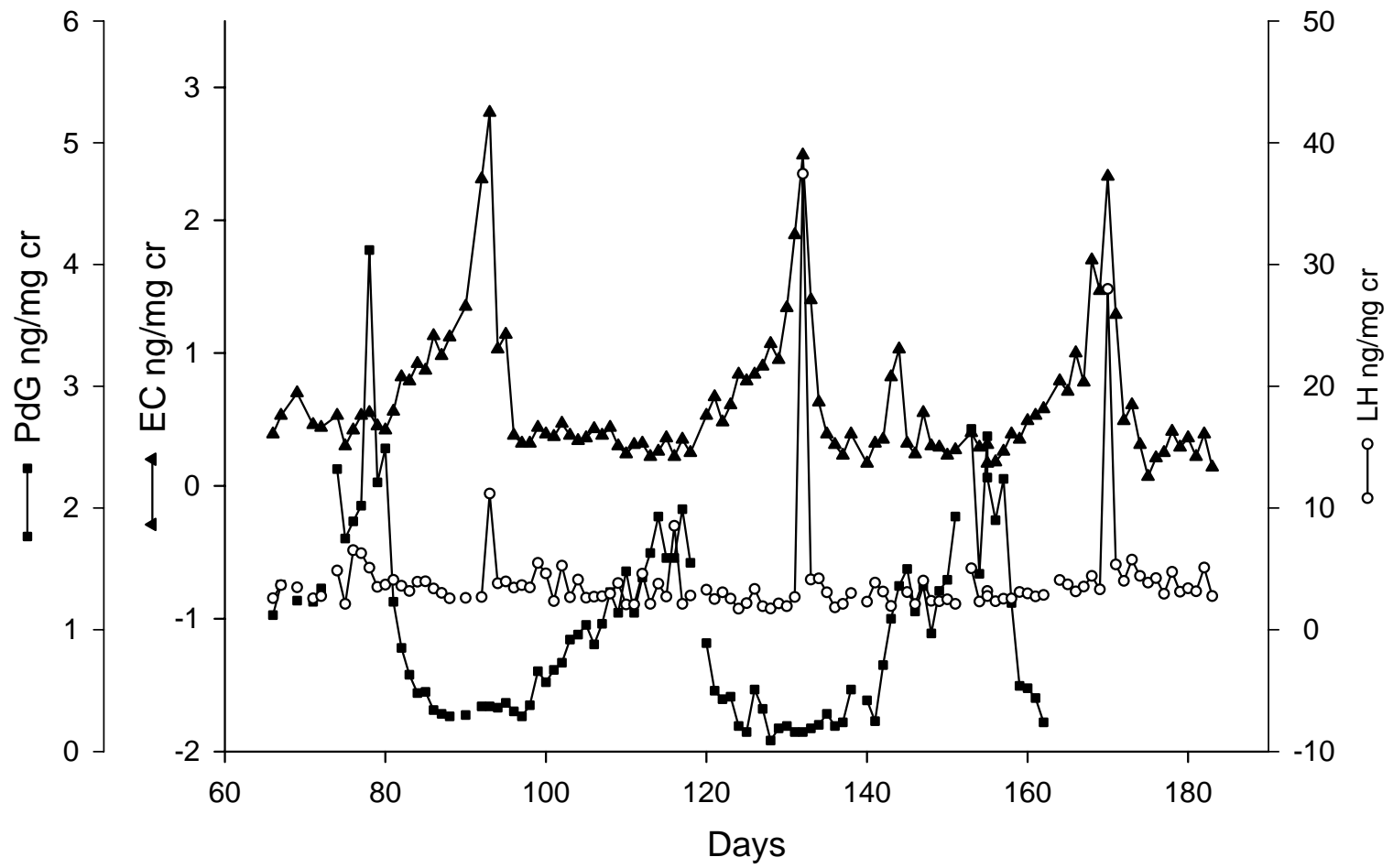


FIG. 1

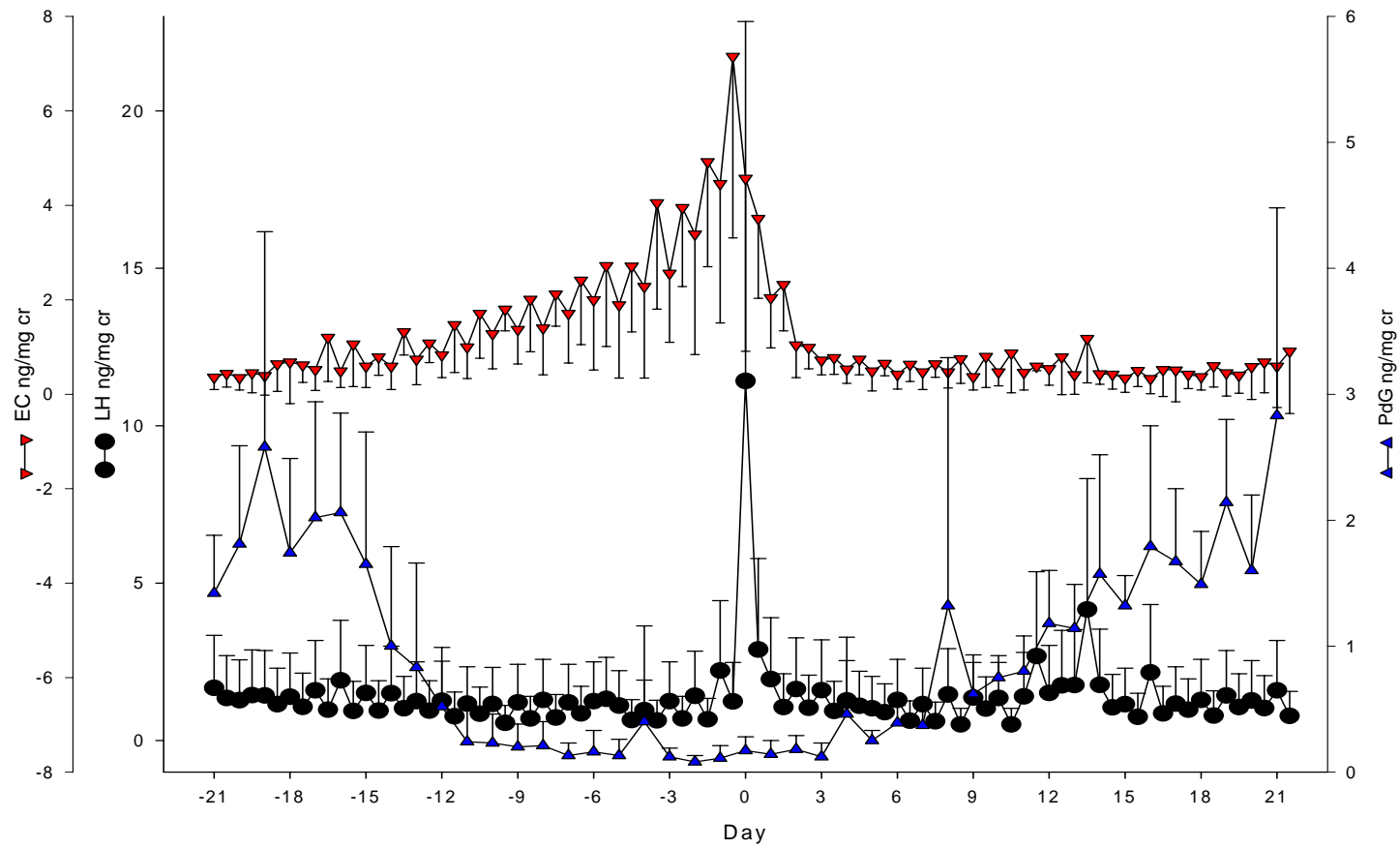


FIG. 2

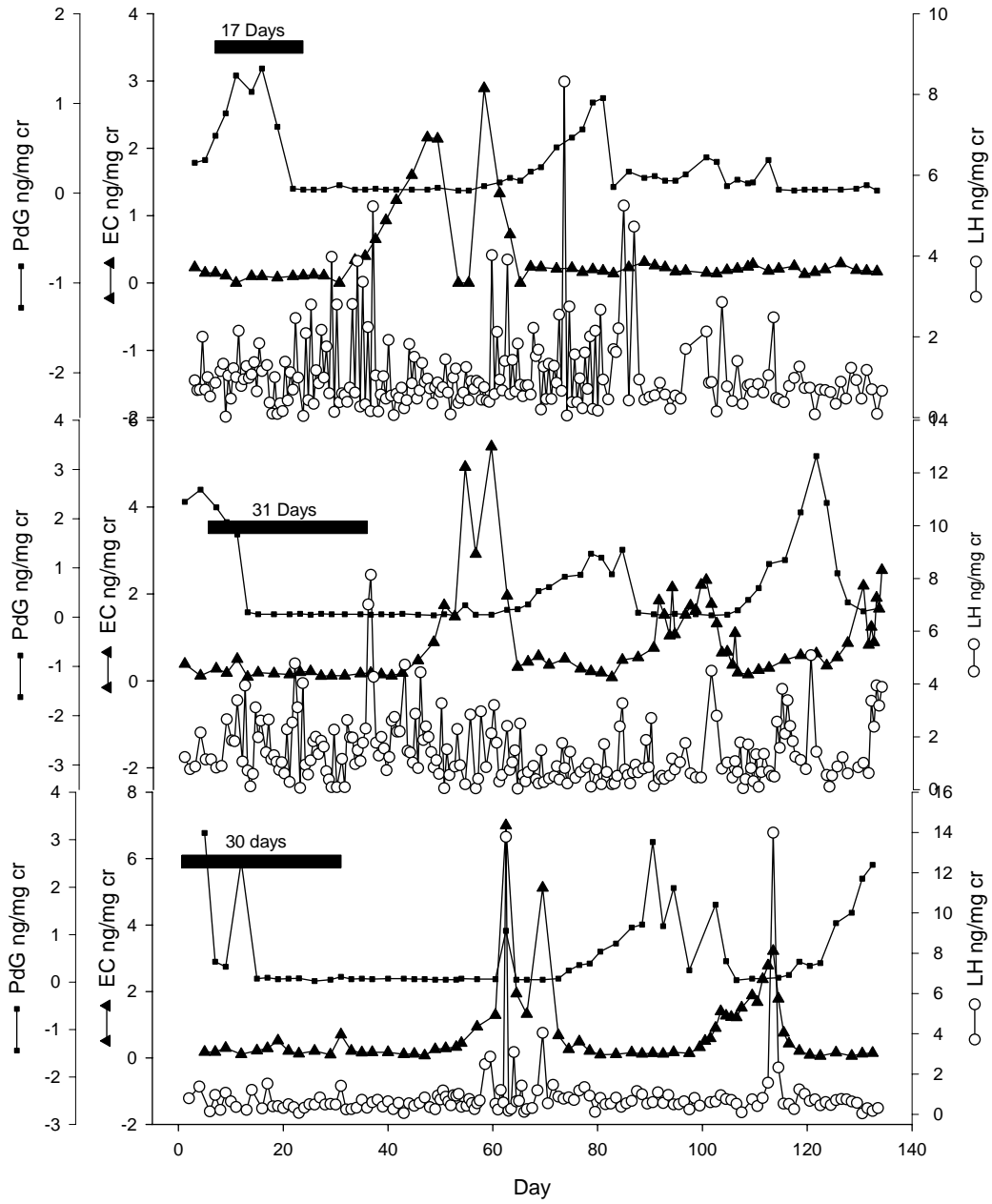


FIG. 3



Fig 4.

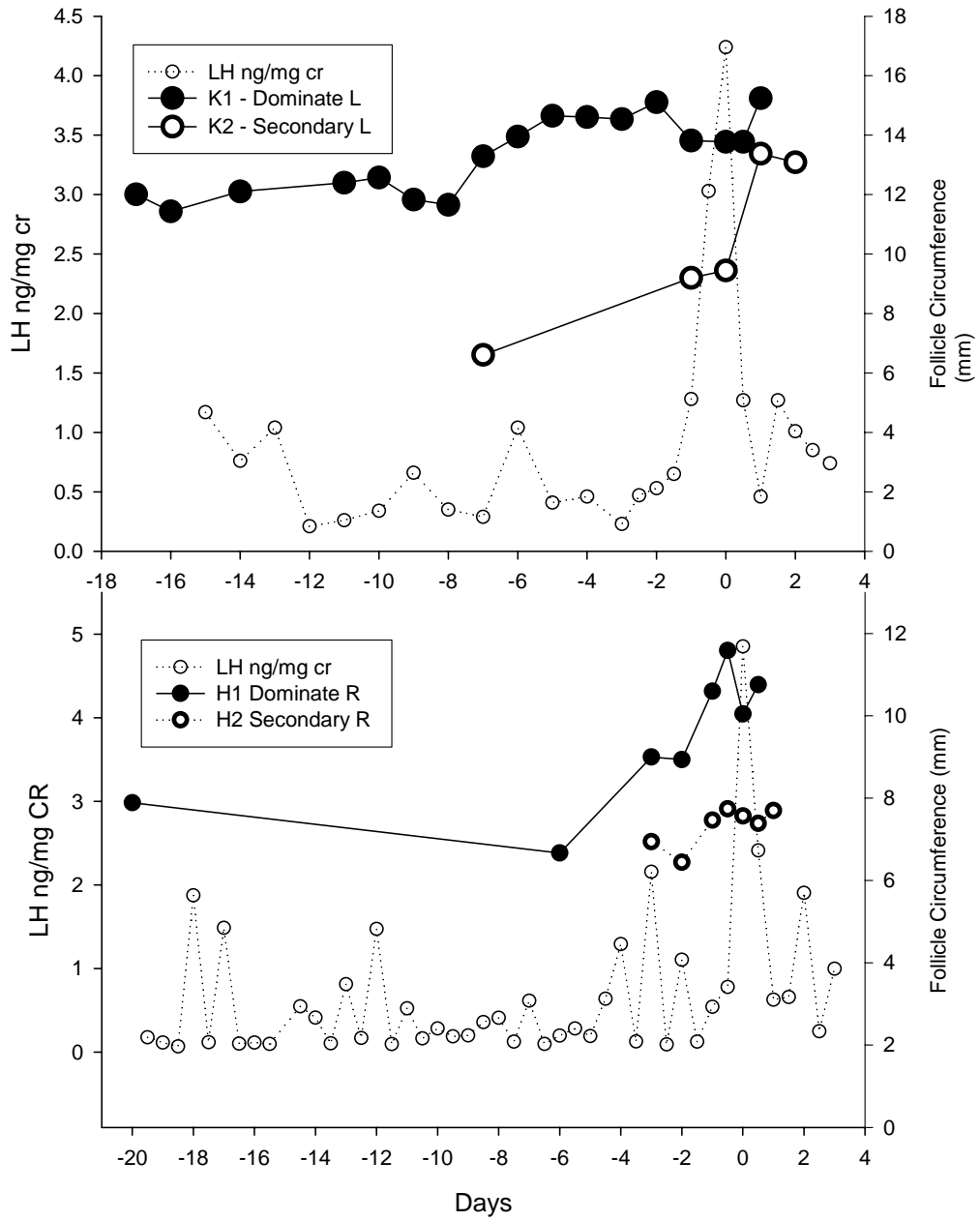


Fig 5.