Lay Summary

Many species of fresh and saltwater dolphins and porpoises are facing increasing pressure for food and habitats due to human activities. One of the primary aspects that can be affected by such activities is reproduction. The bottlenose dolphin has successfully bred in zoos and aquariums for at least 70 years, and they can be trained for several voluntary behaviors that enable medical examinations. Therefore, they represent a unique resource for research and understanding of normal dolphin reproduction. In this research, voluntary ultrasound exams and urine samples from 15 female dolphins were used to describe changes in their ovaries. The resulting descriptions and comparisons provide insight into the unique ovarian physiology of bottlenose dolphins and into the differences between animals after treatment. This information adds to the body of knowledge which one day may be used for developing advanced reproductive techniques to help preserve endangered species of dolphin.
Follicular and Hormonal Changes after Estrous Synchronization in Bottlenose Dolphins

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Short title: Follicular growth post altrenogest in dolphins

Keywords: reproduction, cetaceans, endocrinology, bottlenose dolphin, folliculogenesis

Word count of full article: 9677
Abstract

To gain more knowledge about the influence of hormone regulation on follicle development, ovarian ultrasounds were performed, and urinary hormone profiles were determined in ovulating and non-ovulating female bottlenose dolphins (n = 15) following estrus synchronization with altrenogest. Ovarian ultrasounds were conducted daily, post synchronization to describe follicular recruitment in relationship to the endocrine profile. Follicle sizes were grouped into very small (VSM), small (SM), medium (MD) and large (LG). In ovulating females, two follicular waves were identified, and follicular deviation towards establishing a dominant follicle only occurred during the second wave. For non-ovulating females, only the first wave was observed. For all urinary hormones, the non-ovulating group presented significantly lower concentrations of follicle stimulating hormone (uFSH), luteinizing hormone (uLH), estrone conjugates (uE1-C) and estriol (uE3) but similar progestagen and cortisol concentrations compared to the ovulating group. Concentrations of uE1-C and uE3 and numbers of MD and LG follicles significantly (P < 0.05) increased, while uFSH concentrations significantly (P < 0.05) decreased as ovulation approached. Urinary LH significantly increased concurrently with increasing numbers of LG follicles and decreasing numbers of SM follicles. The characterization of follicular development and its relationship with hormone assessments complements our understanding of follicular recruitment post-synchronization in bottlenose dolphins and provides new information concerning differences between ovulating and non-ovulating females in response to an estrous synchronization protocol.

Lay Summary
Many species of fresh and saltwater dolphins and porpoises are facing increasing pressure for food and habitats due to human activities. One of the primary aspects that can be affected by such activities is reproduction. The bottlenose dolphin has successfully bred in zoos and aquariums for at least 70 years, and they can be trained for several voluntary behaviors that enable medical examinations. Therefore, they represent a unique resource for research and understanding of normal dolphin reproduction. In this research, voluntary ultrasound exams and urine samples from 15 female dolphins were used to describe changes in their ovaries. The resulting descriptions and comparisons provide insight into the unique ovarian physiology of bottlenose dolphins and into the differences between animals after treatment. This information adds to the body of knowledge which one day may be used for developing advanced reproductive techniques to help preserve endangered species of dolphin.

Introduction

The use of serial ultrasonography combined with simultaneous endocrine profiling has resulted in a better understanding of follicular wave dynamics in domestic animals (Ginther et al., 1989, Bergfelt et al., 1991, Ginther and Kot, 1994, Rubianes and Menchaca, 2003, Jaiswal et al., 2004). This knowledge has facilitated the improvement of methods used for estrous cycle manipulation for natural or assisted breeding programs, and it can be integrated into species’ reproductive health assessment program in order to help diagnose abnormalities in reproductive function, determination of pregnancy, and detection of senescence.

Cetaceans under human care can be trained for a variety of behaviors that help facilitate veterinary care, and among these behaviors is transabdominal ultrasound. Although this tool has been previously used to describe ovarian activity in bottlenose dolphins, *Tursiops*
truncatus (Brook, 1997, Robeck et al., 1998, Robeck et al., 2005, Muraco et al., 2009), the relationships between follicular wave dynamics and profiles of commonly monitored reproductive hormones have not been adequately described for this species.

Bottlenose dolphin females are classified as facultatively polyestrous with seasonal trends and cycle length of 33 days (range of 31-36; (Robeck et al., 2018). However, the irregularity of their cyclicity makes the reliability of monitoring natural estrous cycles for timed natural or artificial breeding difficult and, as a result, a synchronization methodology using progestagen suppression of follicular activity has been developed. The use of oral altrenogest or medroxyprogesterone acetate is common in dolphin facilities for temporary suppression of ovarian activity (Robeck et al., 2005, Robeck et al., 2013, Katsumata, 2010). Altrenogest is a synthetic progesterone analog and has been widely used in horse reproduction for more than 45 years (Squires et al., 1979, Allen et al., 1980). In addition, estrus synchronization or suppression with the use of oral altrenogest has been achieved in domestic pigs (Kraeling et al., 1981), domestic cats (Stewart et al., 2010) and other exotic (Micheletti et al., 2015, Crosier et al., 2017, Herrick, 2019) and cetacean species (Robeck et al., 2004, Robeck et al., 2005, Robeck et al., 2009, Robeck et al., 2010).

For bottlenose dolphins, estrous synchronization with altrenogest results in ovulation approximately 20 days post withdrawal (Robeck et al., 2005). Although this is the primary synchronization protocol currently used for bottlenose dolphins, its effectiveness is a major limitation in that only approximately 50% of animals placed on the hormone will respond to it by ovulating (Robeck et al., 2013).

Quantitative measurement of luteinizing hormone (LH) in bottlenose dolphins was first reported using radioimmunoassay (RIA) of sera (Schneyer et al., 1985, Yoshioka et al.,
1986). This method was validated for use in urine to help characterize the estrous cycle of the killer whale (Robeck et al., 1993, Robeck et al., 2005) and a different LH specific RIAs were also used to determine serum concentrations of the hormone for the Yangtze finless porpoise (Neophocaena phocaenoides asiaeorientalis).

Measurement of delphinid urinary estrogen conjugates by EIA was first validated in killer whales and later applied to bottlenose dolphins using an antibody with high cross-reactivity to estrone conjugates (uE1-C), i.e. estrone-3-glucuronide and estrone-3-sulfate (E1-S) (Robeck et al., 2004, Robeck et al., 2005). As with many other mammalian species, estradiol (E2) is assumed to be the major bioactive estrogen in delphinids with serum circulating concentrations during estrus increasing ~10-fold from baseline to preovulatory concentrations in the bottlenose dolphin (Yoshioka et al., 1986). Estriol (E3) is considered a less bioactive form of estrogen with increased concentrations typically associated with placental aromatase activity (Ryan, 1982).

Follicle stimulating hormone (FSH) is a relatively conserved pituitary peptide associated with follicular recruitment in mammalian species (reviewed by (Suresh et al., 2011). In cetaceans, serum FSH was first analyzed using RIA in random samples of bottlenose dolphins (Schneyer et al., 1985). Work in killer whales demonstrated a bimodal pattern of FSH in the follicular phase in killer whale urine samples (Walker et al., 1988, Robeck et al., 1993). In free ranging animals, RIA has been used to analyze circulating FSH concentrations in Yangtze finless porpoise (Hao et al., 2007).

Plasma progesterone concentrations were first validated and measured in bottlenose dolphins following ovulation induction in 1975 using a competitive protein binding assay (Richkind and Ridgway, 1975). Using RIA, (Sawyer-Steffian et al., 1983) measured
circulating progesterone in bottlenose dolphin serum for two years to establish baseline and
reference for this species. RIA was first used to measure urinary progestagens in the false
killer whale (*Pseudorca crassidens*) and the killer whale (Robeck et al., 2004, Robeck et al.,
1994). Enzyme immunoassays have also been validated for quantifying urinary progestagens
in bottlenose dolphins, Pacific White-sided dolphin (*Lagenorhynchus obliquidens*), and
beluga (*Delphinapterus leucas*) (Steinman et al., 2012, Robeck et al., 2005, Robeck et al.,
2009).

Cortisol concentrations have been recently described during normal pregnancies in the
bottlenose dolphin and killer whale (Steinman et al., 2016, Robeck et al., 2017), and changes
in concentrations have become an important indicator of relative stress and well-being in
captive and wild marine mammal species (Fair et al., 2014, Nabi et al., 2021, Serres et al.,
2020, Steinman and Robeck, 2021). Given the potential relationship of cortisol measurements
to stress and reproductive suppression (Moberg, 1985, Dobson and Smith, 1995, Breen and
Karsch, 2006), no direct hormone analysis has been studied within a controlled group of
cetaceans that have or have not responded to estrus synchronization protocols. In killer
whales, evidence suggests that estrus causes a transient increase in cortisol in females that
have conceived naturally (Robeck et al., 2017).

Despite all the aforementioned data on follicular growth, estrogens, LH and FSH in
cetaceans, no research has been published that describes the temporal association between
profiles of these hormones and follicular wave development, including follicular recruitment
and growth until the establishment of follicular dominance and ovulation. The overall goal of
this research was to describe the ovarian and endocrine response to an exogenous progestagen
synchronization protocol in an effort to understand differences between bottlenose dolphin
females that do or do not respond to treatment by ovulating. The specific objectives were to:

(i) describe follicular recruitment, development and primary follicular deviation in female bottlenose dolphins that ovulate post altrenogest treatment; (ii) describe and compare excretory profiles of urinary progestagens, uE1-C, E3, FSH, LH and cortisol in females that do and do not respond by ovulating post altrenogest withdrawal; and (iii) provide biologic validation of hormonal activity by correlating concentrations with follicular dynamics during the estrous cycle.

Materials and Methods

Ethics of experimentation

All samples were collected as part of routine husbandry procedures for bottlenose dolphins. All procedures described within were reviewed and approved by each institution’s Animal Care and Use Committee and were performed in accordance with the Animal Welfare Act for the care of marine mammals.

1. Animals

Bottlenose dolphin females (n = 15) with a mean age of 17 years (median: 15.5, range 7 to 30 years) and weighing at least 180 kg were located at Discovery Cove, Orlando (n = 9), Dolphin Research Center (n = 2), and SeaWorld San Diego (n = 4). Samples were taken during spring and fall of 2011, fall of 2012, fall of 2013, and winter of 2014. Animals from SeaWorld were housed in pools containing \( \geq 850 \text{ m}^3 \) or natural processed salt water (ambient temperatures 14 – 28 °C). Animals from Dolphin Research Center were housed in various
natural saltwater interconnected enclosures ranging in size from 300 to 600 m³ (ambient
temperature of 10 – 30 °C). Dolphins were fed approximately 4 to 5 % of their body weight
per day, a diet of frozen-thawed whole fish (herring, *Clupea harengus*, capelin, *Mallotus
villosus*, and Columbia River smelt, *Thaleichthys pacificus*).

2. *Synchronization of ovulation and artificial insemination*

   Females didn’t have access to males for at least one month prior to study. To control
the day of ovulation for AI scheduling purposes, estrus was synchronized using the synthetic
progestin, altrenogest (Regu-Mate®, Intervet Inc., Millsboro, Delaware 19966, USA). All
female dolphins were administered altrenogest (0.044 mg/kg po) once daily for 20 days
(O’Brien and Robeck, 2006, Robeck et al., 2013). The drug was given orally by injection
directly into the coelomic cavity of a fish just prior to feeding. Serum progesterone was
determined at the start of altrenogest to ensure that none of the females had ovulated.

   Artificial inseminations (n = 11) were performed on all females that developed a
preovulatory follicle as previously described (Robeck et al., 2013). Briefly, animals were
initially administered diazepam (0.1-0.2 mg/kg po; Abbott Lab, Chicago, USA) one hour
prior to the procedure. Animals were then placed in lateral recumbency on 10.2 cm thick
closed cell foam pads. Vital signs were monitored, and animals were kept wet throughout the
procedure. A flexible endoscope (9-11 mm in diameter and 120-250 cm long, specifics
depend on availability in each facility) was then introduced into the cranial vagina and
insufflated using air to improve cervical visualization. After passing the cervix, semen was
deposited into uterine body and/or uterine horn(s) through a catheter (5 or 7fr, 190 – 400 cm
long, Cook Vet Supplies, QLD, Australia) placed in the working channel of the endoscope.
3. **Ovarian ultrasonography**

Trans-abdominal ultrasonography was used to detail follicular activity following altrenogest synchronized cycles and to confirm pregnancy as previously described for bottlenose dolphins (Brook, 2001, Robeck et al., 2005). Ultrasonographic examinations (n = 230) were performed using either an Aloka 900 machine (Corimetrics Medical, Charlotte, NC), GE Logibook™ Book, (GE LogiqGE Medical Systems, Milwaukee, WI) or a SonoSite 180 Plus (SonoSite, Inc, Bothell, WA) all with a 3.5-4.0 MHz transducer (wide footprint convex linear probe). For examinations, the animals were trained to station in lateral recumbency adjacent to the edge of the pool. The ovaries were located trans-abdominally using a previously described technique (Brook, 2001). Animals were examined once on the last day of altrenogest (day 0), every other day until day 10 post-altrenogest and then daily until ovulation. For the exam, each ovary was scanned several times using similar methods during each exam and the images were stored in three second cineloops for retrospective analysis on the machine or using OsiriX MD software (Pixmeo Labs, Geneva, Switzerland).

Follicle diameter (maximum diameter) of each follicle greater than 3 mm was determined, retrospectively, by examining the cineloops one frame at a time. Individual follicle sizes and approximate locations from each daily exam were represented on an ovarian map that facilitated the tracking of individual follicles from one exam to the next. A follicular wave was defined as the synchronous emergence of follicles 3 mm in diameter that, as the wave progressed, at least one of the cohort of follicles continued to grow while the others regressed at variables intervals. The largest and second largest follicles that emerged (determined retrospectively as any follicle 2 mm or greater in diameter in the remaining group
that continued to separate itself in size from the others for at least four days in a row) from the cohort of growing follicles were defined as F1 and F2 (Manjunatha et al., 2012). For analysis, the day relative to the day prior to ovulation (DPO) when the F1 separated itself from the remaining cohort was termed the day of deviance (DOD). The DOD was considered the point at which the preovulatory follicle established dominance over the remaining cohort (Burns et al., 2005). Follicles observed daily included growing, non-growing, and atretic follicles and were retrospectively grouped by ovulatory or non-ovulatory ovarian side, actual ovarian side (Left or Right) and size as follows: very small (VSM: 3 to 6 mm); small (SM: >6 to 10 mm); medium (MD: >10 to 16mm); and large (LG: >16mm; Fig. 1).

For a retrospective comparison of ovulatory and non-ovulatory cycles, the estimated DOD for ovulatory cycles was overlaid onto the mean day post-altrenogest at which ovulation occurred. For example, ovulation occurred at mean 23 days post-altrenogest with the DOD approximately 10 days prior, and therefore, follicular and hormone data from all non-ovulating cycles were collected until 23 days post-altrenogest, with 13 days post-altrenogest becoming the pseudo day of deviance and 23 days post-altrenogest represented the pseudo day of ovulation. For ease of discussion and comparisons between this non-ovulating and the ovulation group, the non-ovulating female groups were then aligned, analyzed, and discussed based on these estimated DOD and days prior to ovulation, despite the fact that ovulation did not occur.

2. Hormone analysis

Urine samples were collected from unrestrained animals as previously described (Lenzi, 2000, Robeck et al., 2009). Daily urine collections were performed from day one to nine after
altrenogest withdrawal, twice a day urine collection (8-12h apart) started on the 10th day until a dominant follicle was identified by ultrasonography, after which, urine collection was increased to three times a day (6 to 8 h apart) until ovulation. Each sample was split in two, 2 mL aliquots, one was stored at 4 °C and analyzed in batches every 2-3 days for EC and creatinine. The other aliquot was stored at -80 °C and analyzed at further date for cortisol, E3, LH, FSH, Pg and re-analysis of EC. During the periovulatory period, a third aliquot of the urine samples was used to identify the LH curve using a semi-quantitative LH test kit (Witness Synbiotics Corp., Kansas City, MO, USA) as previously described (Muraco et al., 2009, Robeck et al., 2009).

2.1 Creatinine assay

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

2.2 Estrone conjugates (uE1-C)

Urinary estrone conjugates were measured by single antibody, direct enzyme immunoassay (EIA) as previously described and validated for use with bottlenose dolphin urine (Robeck et al., 2005, Robeck et al., 2013). Briefly, neat urine samples (0.050 – 0.0025 ml) and standards (estrone-B-glucuronide, range 200 – 0.79 pg/well, Sigma-Aldrich, St. Louis, MO) were added to a microtiter plate coated with antisera (1:20,000, R522-2, C. Munro). An enzyme conjugate label (horse-radish peroxidase [HRP], 1:250,000, C. Munro)
was added to all wells and plates were incubated for two hours at room temperature and then
washed before the addition of tetramethylbenzidine (TMB) in phosphate citrate buffer
(Sigma-Aldrich) and a final incubation on a plate shaker (400 rpm, MidSci, Valley Park,
MO). A stopping solution was then added (0.6 M H$_2$SO$_4$) and the optical density was
measured at 450 nm with a 655 nm reference filter (Model 680, BioRad, Hercules, CA).
Assay sensitivity was 3.9 pg/well and intra-assay variation was <10% and inter-assay
variation was 10.1 % and 11.9 % (n = 71) at 30% and 70% binding, respectively.

2.3 Urinary Estriol (uE3)

The uE3 concentrations were measured using a single antibody, direct EIA as
previously described (Hedriana et al., 2001). This EIA was previously validated for bottlenose
dolphin serum (Steinman et al., 2016). Neat urine (0.05 to 0.025 ml) and standard (4000 to
15.6 pg/well) were added to a 96 well microtiter plate coated with a polyclonal antibody
(1:10,000, R4835, C. Munro) along with an E3-HRP (1:50,000). Following a 2 hour
incubation at room temperature, 0.1 ml substrate solution (azino-bis-3ethyl benzthiazoline-6-
sulfonic acid [ABTS] in citrate buffer, Sigma Aldrich) was added and the plate incubated for
another 20-30 min on a plate shaker. The plate was read at 405 nm with a 540 nm reference
filter. Reported cross-reactivity for antibody R4835 was specific for estriol with other steroids
and metabolites structurally similar to estriol showing <0.1% cross-reaction (Hedriana et al.,
2001). However, Steinman et al. (2016) demonstrated that estradiol had a ~67% cross-
reactivity with this antibody. Assay sensitivity was 15.6 pg/well and intra-assay variation was
<10%. Parallel displacement of a pool of urine compared to the standard curve was
demonstrated ($r = 0.999$, $P < 0.05$) and an accuracy test plotting the concentrations of a pool
of urine spiked with known concentrations of standard against non-spiked pool yielded a
recovery of 89.4 ± 11.3% (linear regression, y = 0.89x – 14.66, r² = 0.999). Samples were run
in duplicate and if the CV between the replicates was >10%, the sample was repeated. Inter-
assay coefficients of variation for two quality controls binding at 30 and 70% were 6.7 and
8.7% respectively (n = 28).

2.4 Urinary Luteinizing hormone (uLH)

A double antibody LH EIA has been previously described and validated for bottlenose
dolphin urine (Graham et al. 2002; Robeck et al. 2005). Briefly, microtiter plates pre-treated
with a goat anti-mouse IgG (X12-1EA, Arbor Assays, Ann Arbor, MI) were blocked with a
0.02M Trizma buffer (Sigma Aldrich) and incubated at room temperature overnight or up to
three weeks. Plates were emptied, washed (0.02% Tween solution, Sigma Aldrich), and neat
urine (0.05 to 0.001 ml) and standards (bovine LH NIH-LH-B10, AFP-5551B, National
Institute of Health, Bethesda, MD, USA) were added to wells along with 0.1 ml of antibody
(monoclonal anti-bovine LH, LH 518-B7, 1:600,000, J. Roser, UC Davis, CA, USA) and
incubated at room temperature overnight. Biotinylated LH (1:1,500,000) was then added and
after another incubation (four hours, room temperature), plates were washed and streptavidin
peroxidase solution (1 µl in 24 ml assay buffer, Roche Diagnostics, Manheim, Germany) was
added. Following 40 min incubation, TMB substrate was added prior to a final incubation (45
to 60 minutes). A stopping solution was then added (0.6 M H₂SO₄) and the optical density
was measured at 450 nm with a 655nm reference filter. Intra-assay variation was <10% and
inter-assay variation was 13.3% and 12% at 30% and 60% binding (n = 35), respectively.
2.5 Urinary Follicle Stimulating Hormone (uFSH)

Analysis of urine samples for FSH was performed at the Endocrine Research Laboratory at the Smithsonian Conservation Biology Institute using an in-house I-125 RIA described previously (Brown et al., 2004). Briefly, 0.1ml neat urine and ovine FSH standards (250 – 0.495 ng/ml oFSH NIADDK-oFSH-17, National Institute of Health) were added to glass tubes. A rabbit anti-ovine FSH antibody (1:25,000) was then added to all tubes and the assay incubated overnight at room temperature. The next day, 0.1ml I-125 labeled oFSH (~20,000 cpm/tube,) was added to all tubes and incubated overnight. Finally, 1ml of a second antibody, sheep anti-rabbit gamma globulin (1:300 in phosphate buffered saline containing 5% polyethylene glycol) was added to all tubes, incubated for 1 hour. Tubes were then centrifuged (3000g for 25 minutes) and the supernatant decanted. Remaining pellets in the tubes were counted in a gamma counter (Iso Data 20/20 Series, GMI Inc., Ramsey, MN).

Assay sensitivity was 0.5 ng/ml (Brown et al., 2004) and a serially diluted pool of urine demonstrated parallelism to the standard curve (r = 0.998). Intra-assay variation was <10% and inter-assay variation was 11.6 and 7.5% (n = 5) for two controls binding at 45 and 75%, respectively.

2.6 Urinary Progestagens (uPg)

Urinary Pg were measured by single antibody, direct EIA as previously described and validated for bottlenose dolphin urine (Robeck et al. 2005). Briefly, neat urine samples (0.050 – 0.01 ml) and standards (progesterone, range 200 – 0.79 pg/well, Sigma-Aldrich, St. Louis, MO) were added to a microtiter plate coated with a monoclonal antibody (1:10,000, Cl-425, C. Munro). An enzyme conjugate label (HRP, 1:40,000, C. Munro) was added to all wells and
plates were incubated for two hours at room temperature and then washed before the addition of ABTS substrate and a final incubation on a plate shaker for 45-60 minutes. The optical density was measured at 405 nm with a 540 nm reference filter. Intra-assay variation was <10% and inter-assay variation was 10 % and 14 % (n = 71) at 20% and 65% binding, respectively.

2.7 Urinary Cortisol (uCort)

Urinary Cort concentrations were measured using a single antibody, direct EIA as previously described (Munro and Lasley, 1988) and described in detail for use with bottlenose dolphin sera (Steinman et al., 2016). Antibody cross-reactivity and sensitivity information are described in detail elsewhere (Steinman et al., 2016). Briefly, dilutions of neat urine containing 0.005 – 0.001 ml of sample and standards (range 1000 – 3.9 pg/well, Sigma-Aldrich, St. Louis, MO) were added to a microtiter plate coated with a monoclonal antibody (1:10,600, R4861, C. Munro). An enzyme conjugate label (HRP, 1:33,000, C. Munro) was added to all wells and plates were incubated for one hour at room temperature and then washed before the addition of ABTS substrate and a final incubation on a plate shaker for 15 minutes. The optical density was measured at 405 nm with a 540 nm reference filter. Parallel displacement of a pool of urine compared to the standard curve was demonstrated (r = 0.998, P < 0.05) and an accuracy test plotting the concentrations of a pool of urine spiked with known concentrations of standard against non-spiked pool yielded a recovery of 105.42 ± 8.96% (linear regression, y = 1x + 4.78, r² = 0.999). Inter-assay coefficients of variation (CV) for two quality controls binding at 30 and 70% were 7.3 and 9.5% respectively (n = 19).
5. **Statistical analysis**

Unless otherwise stated all statistical analyses were done using STATA (Stata Corp., Version 14, College Station, TX). For analyses, all daily observations (follicle data and urinary hormone concentrations) were aligned as either the day post altrenogest or day prior to ovulation (DPO). For follicular wave analysis, a maximum likelihood (ML) linear mixed model (LMM) regression analysis (West et al., 2014) with animal id as the repeated measures random variable was run separately for total numbers of follicles within each size group (VSM, SM, MD, LG) for each day post-altrenogest as the dependent variable against the main effects of ovary (ovulating and non-ovulating ovary), rate of increase in follicular numbers within each group day post-altrenogest, the size of the developing pre-ovulatory follicle (POF) and interactions. In addition, a restricted maximum likelihood LMM was also performed with total follicular numbers from both ovaries within each size category DPO as the dependent variable against the independent factorial variables pre and post DOD (coded as 0 for pre and 1 for post DOD) and ovulation (coded 0 for ovulating females and 1 for non-ovulating females) and their interactions with the random variable animal id. Multiple pairwise ML LMM, with animal id as the random variable, were conducted to determine if follicle numbers within each size category correlated against the other categories (eg., VSM vs SM follicles) before or after DOD while accounting for the repeated measures characteristics of the data. If the analysis indicated a significant effect, then a relative comparison of goodness of fit between each pair was conducted to determine marginal ($mR^2$) and conditional r-squared ($cR^2$) using a mixed model analysis (Nakagawa and Schielzeth, 2013) with the MuMLN package (lme4) (Bates et al., 2014). A similar analysis for pairwise correlation using LMM with $mR^2$ and $cR^2$ was used to determine if any significant
correlations (P ≤ 0.05) existed between urinary hormones and follicles size across all time points prior to ovulation (only within ovulating females), and within urinary hormones. Finally, bivariate pairwise correlation (two-tailed) was used to determine if any significant relationships existed between age of animal and peak number of follicles within each group associated with each individual animal during each estrus event and the size of the POF, respectively.

Marginal means from each group were compared using pairwise comparisons with a Sidak correction factor. All final mixed models were checked for normality using quantile plots of the standard residuals. If quantile-quantile plots of standardized residuals exhibited non-normal distribution then data were transformed as recommended by the Shapiro-Wilk test (Ladder command, STATA) until residuals were normalized.

Results

Of the 19 (within 15 animals) estrus synchronization attempts in this study, 11 (58%) resulted in ovulation with 4 (36%) becoming pregnant after AI. A total of 230 ovarian ultrasound exams (Fig. 1) were conducted over these 19 cycles (average: 14.77 and 8.5 exams per cycle for ovulating and non-ovulating females, respectively). For one female, due to poor resolution of her ovaries during ultrasound exams, only POF determination was possible. This was believed to be due to her large subcutaneous fat layer (>2.8 cm) which attenuated the image quality (Robeck, 1996).

1. Follicular growth characteristics in animals that ovulated
For animals that ovulated, we observed a relatively large cohort (> five) of very small follicles with two waves of small follicles detected, and the dominant follicle was identified from the second wave prior to ovulation. The two waves of small follicles began on day 18 and 11 prior to ovulation and peaked on days 15 and 7, respectively (Fig. 2B). During the second wave of small follicles, only one follicle was identified as having separated itself from the remaining cohort of follicles. In all cases, this follicle was the dominant follicle (F1) that eventually ovulated. The day of deviance (DOD) was established with a mean of 9.7 d (± 0.74 d) prior to ovulation and a mean size of 7.1 ± 0.4 mm in diameter. From the DOD, the F1 follicle grew at a rate of 1.3 ± 0.2 mm/day reaching a mean size of 18.4 ± 0.6 mm at 12 to 24 hours prior to ovulation. A steady mean daily growth rate was observed until the day prior to ovulation, when it doubled to 24 mm/day. When excluding the F1 from the total follicular counts, no significant differences were detected between total follicle numbers on contra or ipsilateral (to the POF) ovaries in the VSM (P = 0.31), SM (P = 0.79), MD (P = 0.43) or LG (no follicles) groups, therefore, for all further analysis, follicle numbers from both ovaries within each group were combined to represent total follicle numbers (T).

A significant, negative effect (β2 = - 3.14, P = 0.008) was detected between the size of the F1 follicle and total VSM numbers (Fig. 1). VSM follicles were influenced by DPO (P < 0.001) and had significantly different (P = 0.04) total numbers before and after dominance (DOD) was established. In addition, VSM follicle numbers had a linear relationship with DPO (P < 0.001) where they increased at a rate of 0.49 follicle/DPO until day of deviation after which they decreased linearly (P < 0.001) at a rate of 0.56 follicle/DPO (Fig. 2A). Overall SM follicles had no relationship with DPO (P = 0.477), but total SM follicle numbers were reduced (P = 0.01) before versus after dominance was established (Fig. 2B). MD follicles
were associated with DPO ($P < 0.001$), but total follicle numbers were not different ($P = 0.71$) when DPO was included as a factor in the analysis. A positive association ($P < 0.001$) was detected between numbers of MD follicles and DPO after dominance was established but not before ($P = 0.39$). As expected, when simply comparing follicle numbers before versus after DOD (without controlling for DPO), MD follicles were greater ($P = 0.0001$) after DOD (Fig. 2C). Large follicle numbers increased with DPO ($R^2 = 0.25$, $P < 0.001$, Fig. 2C). Although no significant relationship was detected between follicular size groups, there appeared to be an initial wave of follicles that occurred prior to the emergence of the F1 follicle or DOD (Fig. 2A, B, C). This wave peaked prior to regression at DPO of 17 for VSM, at DPO of 15 for SM and at DPO of 14 for MD follicles.

After dominance was established, significant correlations existed between total (t) VSM and tSM ($mR^2 = 0.011$, $cR^2 = 0.66$, $P = 0.03$) follicles, tVSM and tLG ($mR^2 = 0.03$, $cR^2 = 0.60$, $P = 0.001$) follicles, tSM and tLG ($mR^2 = -0.02$, $cR^2 = 0.59$, $P = 0.05$) and tMD and tLG ($mR^2 = -0.15$, $cR^2 = 0.42$, $P < 0.0001$) follicles. These correlations can be visualized (Fig. 2A, B, C) and represented the second or final follicular wave prior to ovulation. During this final wave, peak VSM numbers occurred on day 9, peak small follicle numbers on day 7, and medium number of follicles on day 4 prior to ovulation. As age increased, so did the peak number of small ($r = 0.67$, $P = 0.03$) and medium follicles ($r = 0.63$, $P = 0.049$), but no significant correlation was detected between age and very small ($r = 0.31$, $P = 0.38$) or the size of the POF ($r = -0.29$, $P = 0.39$). No difference was detected in F1 growth rates between animals that did (n = 4) or did not conceive (n = 7, $R^2 = 0.10$, $P = 0.33$). However, animals that produced a term calf (n = 3) had a shorter period of time from peak VSM to ovulation ($8 \pm 0.7$ d versus $10.4 \pm 0.5$ d, $P = 0.02$) and an increased F1 growth rate ($1.8 \pm 0.2$ mm day
versus $1.1 \pm 0.2$ mm/day, $P = 0.043$) compared to ones that did not produce a live calf, a group that included those that failed to conceive ($n = 7$) and one that was pregnant but experienced an early embryonic loss previously defined as < day 120 post ovulation (O'Brien and Robeck, 2012).

2. **Follicular patterns in ovulating vs. non-ovulating female dolphins**

Within all follicle size groups, non-ovulatory females had significantly reduced mean numbers of follicles observed post-altrenogest treatment (Table 1). This also held true when follicular growth patterns were partitioned between pre and post day of deviance (Table 1). Initially, the pattern of follicular development for animals that did not ovulate appeared similar to ovulators with an initial development of VSM that peaked on day 14 versus day 17 DPO (Fig. 2A). This peak was then followed by SM follicles that peaked at day 12 DPO, with the second peak on 8 DPO. Similarly, MD follicles peaked on Day 7 but regressed quickly, without the formation of an obvious F1 follicle by Day 6 (Fig. 2C). The similarities in follicular patterns within the VSM and SM follicle cohorts between the non-ovulatory female group and the ovulatory female group was largely attributed to a subset of females ($n = 3$) within the non-ovulatory group designated as initially responding (to synchronization protocol), non-ovulatory females.

The initially responding non-ovulating females (IR) had several characteristics that differentiated themselves from the remaining non-ovulatory, non-responding females ($n = 4$) including: 1) VSM and SM follicle groups were significantly reduced ($P < 0.05$) for the non-responding, non-ovulatory females compared to the initial responder females before and after the estimated day of deviance (Table 1 and 2); 2) Initial responding females had mean ovarian
VSM and SM follicle counts that were either similar (VSM) or significantly greater than (SM) ovulating females prior to the DOD (Table 1).

3. Urinary endocrine patterns in ovulating vs. non-ovulating female dolphins

Because no significant differences (P < 0.05) were detected in urinary hormone concentrations among non-ovulating, initial responding females or non-responding female groups, the data were combined for comparisons against the ovulatory group. Across all days prior to ovulation (or post-altrenogest for non-ovulators), uE1-C, uE3, uFSH and uLH were significantly (P < 0.05) increased, but uPg and uCort were not different between females that ovulated compared to anovulatory females (Table 2). For uE1-C, uFSH and uLH the difference between the two groups was almost exclusively due to changes that were observed in hormone concentrations after DOD (Table 2). Within ovulating females, uE1-C, uE3 and uLH all had significantly (P < 0.05) increased mean concentrations post DOD (Table 2). Mean uFSH and uE3 were significantly higher in ovulating females compared to anovulatory females for all comparisons pre and post DOD, while no differences were detected within or between any group comparisons in mean uPg and uCort concentrations (Table 2).

4. Urinary endocrine concentrations and follicle groups in ovulating female dolphins across day prior to ovulation

LMM regression demonstrated that uE1-C concentrations significantly increased (P < 0.001) across all days prior to ovulation, with significant relationships detected with tVSM (P = 0.027), tMD (P < 0.001) and tLG (P < 0.001) as follicles increased in numbers (Fig. 3). No significant effect was detected between uE1-C concentrations and TSM. Urinary E3
significantly increased (P < 0.001) across all days prior to ovulation however no significant
association (P > 0.05) with any follicle group was detected. No significant relationship (P >
0.05) was detected between uFSH and DPO, tVSM, tSM, tMD or tLG follicles across all days
prior to ovulation (Fig. 3). Urinary LH only significantly changed (increased) with increasing
numbers of tLG follicles (P < 0.001). Significant, mildly marginal (mR^2 ≥ 0.1) correlations
were detected between tMD follicles and uE1-C, tLG follicles and uE1-C, tLG follicles and
uLH, uE1-C and uE3, and uE1-C and uLH (Table 3). LMM analysis of these significant
relationships pre and post DOD indicated that tMD (P = 0.01) and tLG (P = 0.001) only
varied significantly with uE1-C after dominance (Table 4). In addition, uE3 was only
correlated with uE1-C and in a similar fashion before and after dominance was established.
As expected, uLH was significantly correlated with both tLG and uE1-C but only after DOD.
All significant marginal correlations with uFSH were questionable (mR^2 < 0.1), but they
included both uE1-C and uPg before DOD and LH after DOD (Table 4).

**Discussion**

In the present study, 15 female bottlenose dolphins were examined, and 18 estrus
synchronization treatment responses were analyzed using ovarian ultrasound imaging and
urinary hormone assays. The results of these analyses have provided the first comparison of
both hormonal and follicular changes between ovulating and non-ovulating post-altrenogest
treated females. We also have provided the first detailed description of follicular cohort
growth patterns including the identification of two follicular waves and the mean day for F1
deviation from the remaining follicular cohort from post synchronization to ovulation. Finally,
we described relationships between multiple urinary reproductive hormones and changes observed in ovarian follicular activity.

A total of 58% of females placed on altrenogest responded to the treatment with a post-withdrawal ovulation. This is in line with a previous report in bottlenose dolphins whereby only 48% (13 of 27) of the females responded to altrenogest treatment (Robeck et al., 2005). A 30% response (17 of 57) has also been observed in the Pacific white-sided dolphin (Lagenorhyncus obliquidens), a closely related delphinid (Robeck et al., 2009).

Although it has been hypothesized that seasonal ovarian sensitivity plays a role in the lack of response to exogenous progestagen synchronization in bottlenose dolphins, no conclusive, supportive evidence has emerged (Robeck et al., 2018).

The urinary hormone concentration comparisons between these two groups, ovulators and non-ovulators, demonstrated that ovulating females had significantly greater mean concentrations of urinary FSH, LH, and urinary estrogens (uE1-C, uE3), but no differences were detected in urinary Pg or cortisol. In an attempt to pinpoint the time when these hormone production differences occurred, possibly leading to a theory for this varied response, the relative changes in concentrations before and after the day of deviance (DOD) of the dominant follicle were evaluated. It was found that although FSH, the hormone commonly associated with follicular recruitment, was greater across both periods, it only significantly increased after the DOD. Similarly, uE1-C, primarily representative of circulating E1 and E2, and uLH had significantly greater concentrations only after DOD. Both uFSH and uE3 had greater concentrations pre and post DOD in ovulating dolphins compared to those that did not ovulate.
The changes in uE1-C and uLH primarily after DOD were not unexpected because their production is stimulated via the well documented hypothalamic-pituitary-ovarian (follicular) axis (Mariana, 1991), a physiologic feedback loop that, as demonstrated within and elsewhere, clearly applies to bottlenose dolphins (Robeck et al., 2005, Robeck et al., 2013). However, the role of E3 within this loop is unknown. Although uE3 significantly increased post DOD compared to anovulatory females pre and post DOD, it differed from uE1-C in that its concentrations only gradually increased towards ovulation and did not result in an obvious per-ovulatory surge. Estriol is considered the least bioactive estrogen and is of primary importance during pregnancy (Kuiper et al., 1997). Bioactivity of E3 has been well described in primates, where it is believed to be produced via 16-hydroxylation of fetal dehydroepiandrosterone sulfate (Challis et al., 2000, Peter et al., 1994, Troisi et al., 2003). In bottlenose dolphins, it has been identified as the dominant (in concentration) estrogen during early pregnancy (Steinman et al., 2016), and its presence in significantly greater concentrations in ovulating females immediately post-altrenogest may indicate that it also plays a role in follicular recruitment. However, although not reported by the manufacturer, Steinman et al. (2016) discovered the E3 antibody used in this study also has a high cross-reactivity with E2 (66.5%). As a result, it cannot be ruled out that the observations of E3 activity reported herein were solely due to E3 and not influenced by E2 concentrations. Regardless, no correlations between uE3 concentrations and follicle growth similar to a study where follicular fluid concentrations of E3 were evaluated in humans (Plaino et al., 2000). Interestingly, both uE3 (across the entire post-altrenogest DPO period) and uE1C (only before DOD) had significant positive correlations with uFSH indicating a possible stimulatory role for these estrogens. Therefore, although E3’s role in the bottlenose dolphin estrous cycle
appears to be independent of follicular growth, it may work in concert with E1 and E2 to provide the classic feedback stimulation of FSH production during early follicular recruitment.

FSH was significantly increased in ovulating females compared to non-ovulating dolphins prior to and after DOD. Current understanding of FSH’s role in follicular recruitment in other species support the theory that the lack of gonadotropin releasing hormone (GnRH) production was a primary reason for the lack of a secondary follicular wave that we observed (Richards, 1979, Baird and McNeilly, 1981, Turzillo and Fortune, 1990, Bergfelt and Ginther, 1985). Despite this observation, no significant relationship was found between follicle size and uFSH concentrations. However, a significant positive correlation between uFSH and uE1-C was detected, but only prior to the deviation that coincided with the strongest (mR² = 0.14) marginal correlation (as compared any other follicle group except large) between VSM and uE1-C. The correlation between very small follicles and uE1-C, combined with a simultaneous uE1-C correlation with uFSH and the absence of a correlation between uE1-C and small follicles, provides us with indirect evidence that uFSH concentrations in dolphins may reflect biologic activity and, in conjunction with its indirect effect on estrogen production, its primary function in bottlenose dolphins is for follicular recruitment.

Although stress has been known to centrally inhibit or reduce GnRH release from the hypothalamus (Dobson and Smith, 1995, Kalantaridou et al., 2010), urinary cortisol did not differ between the two groups, and therefore, stress most likely had negligible influence on the different responses. Finally, genetic seasonality, which has been postulated to occur in bottlenose dolphins (Schroeder and Keller, 1989), maybe the most likely explanation. This is
based on non-latitudinally correlated seasonal changes in birth dates in wild bottlenose
dolphins in the Gulf of Mexico that appear to coincide with seasonal food availability and not
changes in the photoperiod (Urian et al., 1996). Because the animals in our study were first-
or second-generation captive born females, whose lineages would be from founders located in
multiple oceanic regions, it is not known if this played a role in their response to the treatment
with altrenogest.

Urinary FSH appeared to be associated with two waves of small follicles with
concentrations peaking around day 14 and day 10 prior to ovulation. These hormonal peaks
were also followed by growth in the total numbers of small follicles, with both small follicle
numbers and uFSH concentrations simultaneously decreasing as the dominant follicle reached
1.12 cm in size and six days prior to ovulation. These changes are similar to what has been
observed in other mono-ovular species (equine and bovine), where, after follicular deviation,
FSH is typically depressed from the follicular production of inhibin (Bergfelt et al., 1991,
Adams et al., 1993, Rubianes and Menchaca, 2003). In these species and, like the findings of
this study (Fig. 2), small follicles stop developing and undergo regression over a few days
after dominance has been established (Rubianes and Menchaca, 2003, Adams et al., 1992).

In addition to these two possible waves of uFSH, we also observed a third increase
which peaked one day prior to the LH surge and then dropped inversely as the LH surge
occurred and two days prior to ovulation. This is similar to what has been previously reported
for killer whale uFSH whereby a third peak of uFSH occurs just prior to LH peak (Robeck et
al., 1993). Generally, in humans and domestic species, FSH is progressively depressed
through combination of estrogen modulated inhibin production, suppression of activin A, and
differential receptor expression within follicles, as the dominant follicle approaches ovulation
In delphinids, due to small number of medium and large follicles which are producing estrogens, this uncoupling of FSH and LH may not occur until just prior to ovulation when threshold levels of estrogen reach sufficient concentrations to exert its described effect within these species. For this study, we were unable to validate a urinary inhibin or Activin A assays and thus future work with serial serum samples may be the only way to define or detect when or if this feedback mechanism functions within cetaceans.

Constant presence (from day 22 to 1 day prior to ovulation) of very small follicles (3-6 mm, Fig. 2) was observed. Overall, in ovulating females, very small size follicles progressed to small follicles (6-10 mm) continually up until ovulation. Studies in other domestic animals have also observed the presence of small follicles during throughout inter-ovulatory periods (Ginther and Pierson, 1984, Pierson and Ginther, 1984). For example in mares, Ginther and Pierson (1984) observed follicles that were 2 mm in diameter for 10 days after ovulation and in cattle they were observed for 10 days prior to ovulation (Pierson and Ginther, 1984). In non-ovulating bottlenose dolphins, after DOD, approximately half the number of very small follicles compared to ovulating females. And after ovulation, there were only one to two small follicles in the non-ovulating female group. Medium size (10-16 mm) follicles most often appeared approximately 5 days before ovulation, and the dominant follicle transitioned quickly (a few days) to a large size follicle (>16 mm), similar to what has been observed in cows (Pierson and Ginther, 1984).

Within this study, it was observed an initial post-altrenogest follicular wave did not culminate in the formation of a dominant follicle, similar to cattle that can have 2 to 3 follicular waves per cycle (Ginther et al., 1989, Adams et al., 1992, Burns et al., 2005).
Follicles in the first wave regressed after reaching a medium size (between 6.1 and 10.0 mm).

Conversely, during the second follicular wave initiated approximately 11 days prior to ovulation, before the F1 follicle could be identified. In the bottlenose dolphins, the period from altrenogest discontinuation to ovulation (~20 days) and the length of time where the F1 follicle can be identified prior to ovulation (~10 days) allows veterinarians and animal care managers the opportunity to observe the growth of the preovulatory follicle and to take action prior to the onset of the impending estrous cycle, i.e., physical separation of the female from a male in the same group to prevent copulation, preparation for timing of artificial insemination or moving the female to a group where breeding can occur.

In mares, a wave of follicles starts to develop before the previous ovulation (22 days) and growth continuously until reaching approximately 10 mm in size during the subsequent luteal phase before regressing (Pierson and Ginther, 1987). This study only analyzed follicular growth prior to post-synchronized ovulation in female dolphins. Nonetheless, the mean number of very small follicles started to increase two days before ovulation for the animals that ovulated. In addition, a follicular wave of up to 10 mm in diameter peaked around day 6 post altrenogest (16 days prior to ovulation) and declined until day 14 (8 days prior to ovulation). For bottlenose dolphins that ovulated, a follicular wave of the very small follicles increased until the day of deviation (9.7 days prior to ovulation) and significantly decreased after that. An inverse correlation between predicted very small follicles and preovulatory follicle size was observed. This is supported by research in mares where after deviation, the dominate follicle increases in size while the remaining follicles regress (Ginther et al., 2018).

In the present study, follicular dominance was established at 9.7 d prior to ovulation and from this point forward, the dominant follicle grew at a linear rate of 1.3 mm/day until
ovulation at a mean size of 18.4 mm. This pattern of growth for the F1 follicle is similar to what has been described for other small delphinids including the Indo-pacific bottlenose dolphin (Tursiops truncatus aduncus) and the Pacific white sided dolphin. For the Indo-pacific bottlenose dolphin a 1.25 mm/day linear growth rate has been observed from day -12 until ovulation (Brook, 1997). In Pacific white-sided dolphins, dominance is established when the follicle reaches 0.8 cm in diameter 6 days prior to ovulation for a linear growth rate of 0.12 cm/day (Robeck et al., 2009). For the largest delphinid, the killer whale, follicular growth rates of 0.98 cm/day with a mean POF size of 3.6 cm have been reported (Robeck et al., 2004). Interestingly, the growth rate of the F1 observed in the small cetaceans mentioned above is essentially the same as what has been observed in cattle (1.2 mm/day), a species with an approximately 10-day shorter estrous cycle length (Ginther et al., 1989).

Within non-ovulating females, urinary FSH and LH were reduced compared ovulating females across, prior to and after DOD. This phenomenon of reduced gonadotropin concentrations that results in inadequate follicular recruitment and/or follicular growth toward deviance is also observed in mares during the transition phase from autumn into winter anestrus (Aurich, 2011). Although it is not known the exact mechanism in bottlenose dolphins, ovulation and calving occur in bi-seasonal peaks during the spring and fall with a large amount of individual variation, whereby some animals cycle throughout the year while others have more pronounced periods of anestrus (Robeck et al., 2018). In horses, seasonality is controlled by the photo period (Freedman et al., 1979), but it has yet to be determined what, if any, environmental cue(s) regulate(s) individual seasonal ovarian activity in the bottlenose dolphin (Robeck et al., 2018). Besides differences in gonadotropin concentrations, non-ovulating females also had reduced concentrations of both uE1-C and uE3. It was assumed
that these low concentrations could be explained by the reduced number of follicles of adequate size required to produce these steroid hormones.

Overall, this is the first detailed description of follicular and hormonal activity post-synchronization in the bottlenose dolphins. The evidence presented herein supports the theory that dolphins experience two follicular waves, and it is during the second wave that the ovulatory follicle deviates from its cohorts and increases in size until ovulation. It was also observed a differential secretion of reproductive associated hormones between ovulating and non-ovulating animals. Future research to determine if this pattern of follicular growth is also present during spontaneously cycling animals is warranted.

Conflicts of Interest

The authors have no competing interests and SeaWorld Parks and Entertainment affiliations did not influence or alter our adherence to any Reproductive, Fertility and Development policies.

Declaration of Funding

This project was funded by SeaWorld Parks and Entertainment, Inc. Funder also provided support in form of salary for authors GM, KS, TS, JO, MD and TR.

Author Contribution Statement

GAM conceived the study, collected data, wrote the paper; PC, TLS and MRD collected data. KJS conceived the study, wrote paper, did laboratory assessment, analyzed
data. JKO conceived and designed study, wrote paper. TRR conceived study, wrote paper, analyzed data, designed study. All authors approve the final submission of this manuscript.

Acknowledgements

We thank Angela Ho, Amanda McDonnell and Jaqueline Posy from Species Preservation Laboratory for their help curating and processing urine samples. We thank Kym Folkemer, Jesse Pottebaum, Kelly Flaherty Clark, and dolphin trainers from Discovery Cove for their continue support and training of animals. We thank Brad Andrews and Christopher Dold for their support of this project. This is a SeaWorld Parks and Entertainment Technical Contribution Number 2022-3.
Tables

Table 1. Developmental comparisons of very small follicles (VSM), small follicles (SM) and medium follicles (MD) from both ovaries between animals that ovulated (n = 11) and ones that did not ovulate (n = 7) before and after day of deviance (DOD).1

Table 2. Comparisons of urinary estrone conjugate (E1-C), estriol (uE3), follicle stimulating hormone (uFSH), luteinizing hormone (uLH), progestagens (uPg) and cortisol (uCort) concentrations (ng/mg creatinine) between animals that ovulated (n = 11) and ones that did not ovulate (n = 8) before and after day of deviance (DOD).1

Table 3. Follicle and urinary hormones pairwise correlations post-altrenogest treatment and across day prior to ovulation in bottlenose dolphin as represented by pairwise linear mixed model analysis with marginal and conditional correlation coefficients (mR², cR²), respectively. Bolded values indicate a mild (r ≥ 0.1) or greater marginal correlation.

Table 4. Follicle and urinary hormones pairwise correlations post-altrenogest treatment before (B) a dominant follicle was identified (DOD) and after dominance was established (A) until ovulation in bottlenose dolphin as represented by pairwise linear mixed model analysis with marginal and conditional correlation coefficients (mR², cR²), respectively. Bolded values indicate a mild (r ≥ 0.1) or greater marginal correlation.
Figure Legends

Figure 1. A) Follicle visualization in an animal (not used in the study) with thick blubber layer to demonstrate how it affects the quality of the image. B) Very small follicles (3 – 6 mm). C) Small Follicles (6.1 – 10 mm). D) Medium size follicles (10.1 – 16 mm). E) Large size follicle (> 16 mm).

Figure 2. Daily follicular counts from both ovaries within each size group from ovulators (black or white) and non-ovulators (red). A) Cohort of very small follicles (3 – 6 mm) with peak at day 17 prior to ovulation. B) For ovulators, wave peak for small follicles (6.1 – 10 mm) occurred on days 15 and 7 prior to ovulation. C) Medium size follicles (10.1 – 16 mm) peaked on day 14 followed by a second wave that commenced on day 8 prior to ovulation, and developed into the large follicle (> 16 mm).

Figure 3. Top graph: daily measurements of urinary hormones. Estrone conjugate concentrations (red triangles) increased significantly (P < 0.001) daily until day of ovulation. Estriol concentrations (purple diamonds) significantly increased until 3 days prior to ovulation. LH concentrations (blue circles) only increased prior to ovulation. Bottom Graph: daily measurements of urinary FSH and ovarian follicles. No significant relationship was found between FSH and follicular sizes across all days prior to ovulation.
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117x181mm (300 x 300 DPI)
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Table 1. Developmental comparisons of very small follicles (VSM), small follicles (SM) and medium follicles (MD) from both ovaries between animals that ovulated (n = 11) and ones that did not ovulate (n = 7) before and after day of deviance (DOD). Data are presents total follicle numbers (marginal means (95% CI)).

<table>
<thead>
<tr>
<th></th>
<th>VSM: 3 to 6 mm</th>
<th>SM: 6 to 10 mm</th>
<th>MD: 10 to16 mm</th>
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<tr>
<td>Post-regumate to Ovulation</td>
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<tr>
<td>Ovulatory (A)</td>
<td>8.8 (6.0 to 11.5)</td>
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<td>Non-ovulatory (B)</td>
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<td>*Mean comparisons</td>
<td>B &lt; A</td>
<td>B &lt; A</td>
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<td>Prior to DOD (A)</td>
<td>10.7 (6.7 to 14.8)</td>
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<td>After DOD (B)</td>
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<td>Prior to DOD (C)</td>
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<td>After DOD (D)</td>
<td>1.4 (0 to 3.9)</td>
<td>0.12 (0.0 to 0.42)</td>
<td>0.55 (0.43 to 0.69)</td>
</tr>
<tr>
<td>*Mean comparisons</td>
<td>C &amp; D &lt; A &amp; B</td>
<td>D &lt; B</td>
<td>A &amp; C &amp; D &lt; B</td>
</tr>
<tr>
<td>Initial Responders (n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior to DOD (A)</td>
<td>5.17 (1.78 to 10.32)</td>
<td>1.39 (0.94 to 1.84)</td>
<td>0.69 (0.62 to 0.76)</td>
</tr>
<tr>
<td>After DOD (B)</td>
<td>5.22 (3.63 to 7.11)</td>
<td>1.02 (0.65 to 1.40)</td>
<td>0.77 (0.71 to 0.84)</td>
</tr>
<tr>
<td>Non-responders (n = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior to DOD (C)</td>
<td>0.33 (0.15 to 2.34)</td>
<td>0.32 (0 to 0.68)</td>
<td>0.71 (0.67 to 0.76)</td>
</tr>
<tr>
<td>After DOD (D)</td>
<td>0.34 (0.04 to 0.95)</td>
<td>0 (0 to 0.34)</td>
<td>0.79 (0.71 to 0.87)</td>
</tr>
<tr>
<td>*Mean comparisons</td>
<td>D &amp; C &lt; B &amp; A</td>
<td>D &lt; B &amp; A; C &lt; A</td>
<td></td>
</tr>
</tbody>
</table>

1DOD for animals that did not ovulate was determined by overlaying the DOD (Day 10 prior to ovulation) and the mean days post-regumate that animals ovulated (day 23 post regumate).

The F1 follicle was removed from the comparison.

*Only means within each group (values prior to the mean comparison) within each column that were significantly different (P ≤ 0.05), with Sidak correction, are listed.
Table 2. Comparisons of urinary estrone conjugate (E1-C), estriol (uE3), follicle stimulating hormone (uFSH), luteinizing hormone (uLH), progestagens (uPg) and cortisol (uCort) concentrations (ng/mg creatinine) between animals that ovulated (n = 11) and ones that did not ovulate (n = 8) before and after day of deviance (DOD). Data are presented as marginal Marginal mean hormone concentrations (95% CI)

<table>
<thead>
<tr>
<th></th>
<th>E1-C</th>
<th>uE3</th>
<th>uFSH</th>
<th>uLH</th>
<th>uPg</th>
<th>uCort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovulators</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall (A)</td>
<td>1.4 (1.1 to 1.7)</td>
<td>3.9 (2.6 to 5.7)</td>
<td>10.8 (8.3 to 14.0)</td>
<td>4.6 (3.4 to 6.0)</td>
<td>0.45 (0.40 to 0.53)</td>
<td>16.5 (14.0 to 19.2)</td>
</tr>
<tr>
<td>Prior to DOD (B)</td>
<td>0.9 (0.7 to 1.0)</td>
<td>3.3 (2.3 to 4.9)</td>
<td>9.6 (7.3 to 12.6)</td>
<td>3.1 (1.8 to 4.7)</td>
<td>0.45 (0.37 to 0.56)</td>
<td>15.5 (12.7 to 18.5)</td>
</tr>
<tr>
<td>After DOD (C)</td>
<td>2.0 (1.6 to 2.5)</td>
<td>4.5 (3.0 to 6.6)</td>
<td>11.8 (9.0 to 15.4)</td>
<td>6.4 (4.5 to 8.6)</td>
<td>0.46 (0.41 to 0.51)</td>
<td>17.4 (14.6 to 20.3)</td>
</tr>
<tr>
<td>Non-ovulators</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall (D)</td>
<td>0.8 (0.6 to 0.9)</td>
<td>1.3 (0.8 to 2.0)</td>
<td>6.9 (5.2 to 9.1)</td>
<td>2.3 (1.8 to 2.8)</td>
<td>0.50 (0.36 to 0.69)</td>
<td>17.3 (15.3 to 19.4)</td>
</tr>
<tr>
<td>Prior to DOD (E)</td>
<td>0.8 (0.6 to 0.9)</td>
<td>1.4 (0.9 to 2.1)</td>
<td>7.0 (5.2 to 9.5)</td>
<td>3.7 (2.8 to 4.6)</td>
<td>0.50 (0.31 to 0.81)</td>
<td>17.9 (15.7 to 20.3)</td>
</tr>
<tr>
<td>After DOD (F)</td>
<td>0.8 (0.6 to 1.0)</td>
<td>1.2 (0.8 to 1.9)</td>
<td>6.8 (5.1 to 9.0)</td>
<td>1.3 (0.7 to 1.9)</td>
<td>0.50 (0.39 to 0.64)</td>
<td>16.7 (14.6 to 19.0)</td>
</tr>
</tbody>
</table>

Mean Comparisons*

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovulators</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>D &lt; A; E, F &amp; B &lt; C</td>
<td>D &lt; A; E, F &amp; B &lt; C</td>
<td>D &lt; A; E, F &amp; B &lt; C</td>
<td>D &lt; A; E, F &amp; B &lt; C</td>
<td>D &lt; A; F &lt; B &amp; E &lt; C</td>
<td></td>
</tr>
<tr>
<td>Non-ovulators</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>D &lt; A; E, F &amp; B &lt; C</td>
<td>D &lt; A; E, F &amp; B &lt; C</td>
<td>D &lt; A; E, F &amp; B &lt; C</td>
<td>D &lt; A; E, F &amp; B &lt; C</td>
<td>D &lt; A; F &lt; B &amp; E &lt; C</td>
<td></td>
</tr>
</tbody>
</table>

1DOD for animals that did not ovulate was determined by overlaying the DOD (Day 10 prior to ovulation) and the mean days post-regumate that animals ovulated (day 23 post-regumate).

2Overall: hormone concentration from day of regumate withdrawal until ovulation.

*Only means within each column that were significantly different (P ≤ 0.05), with Sidak correction, are listed.
Table 3. Follicle and urinary hormones pairwise correlations post-altrenogest treatment and across day prior to ovulation in bottlenose dolphin as represented by pairwise linear mixed model analysis with marginal and conditional correlation coefficients (mR², cR²), respectively. Bolded values indicate a mild (r ≥ 0.1) or greater marginal correlation. P values are provided within parentheses.

<table>
<thead>
<tr>
<th></th>
<th>tSM</th>
<th>tMD</th>
<th>tLG</th>
<th>E1-C</th>
<th>E3</th>
<th>Pg</th>
<th>FSH</th>
<th>LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>tVSM: 3 to 6 mm</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.04, 0.51; (0.025)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>tSM: &gt;6 to 10 mm</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>-0.004, -0.51; (0.03)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>tMD: &gt;10 to 16 mm</td>
<td>-0.04, 0.18; (0.017)</td>
<td>NS</td>
<td>NS</td>
<td>0.18, 0.27; (&lt; 0.0001)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>tLG: &gt;16 mm</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.20, 0.57; (&lt; 0.0001)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>E1-C</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.31*, 0.50; (&lt; 0.0001)</td>
<td>NS</td>
<td>0.03, 0.33; (0.0005)</td>
<td>0.11, 0.34; (&lt; 0.0001)</td>
<td>0.39*, 0.83 (&lt; 0.0001)</td>
</tr>
<tr>
<td>E3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.02, 0.31; (0.004)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Pg</td>
<td>0.05, 0.43; (0.0003)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.05, 0.43; (0.001)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>FSH</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.05, 0.43; (0.001)</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

LH: luteinizing hormone. tVSM, Total very small follicles; tSM, Total small follicles; tMD, Total medium follicles; tLG, Total large follicles; E1-C, Estrone conjugates; E3, Estriol; Pg, Progestagens; FSH, Follicle Stimulating Hormone

*Denotes a moderate marginal correlation (r ≥ 0.30). NS = not significant (P > 0.05).
Table 4. Follicle and urinary hormones pairwise correlations post-altrenogest treatment before (B) a dominant follicle was identified (DOD) and after dominance was established (A) until ovulation in bottlenose dolphin as represented by pairwise linear mixed model analysis with marginal and conditional correlation coefficients (mR², cR²), respectively. Bolded values indicate a mild ($r \geq 0.1$) or greater marginal correlation.

<table>
<thead>
<tr>
<th>DOD</th>
<th>tLG</th>
<th>E1-C</th>
<th>E3</th>
<th>Pg</th>
<th>FSH</th>
<th>LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>tVSM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>NA</td>
<td>0.14, 0.41; (0.016)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>-0.07, -0.57; (0.014)</td>
</tr>
<tr>
<td>A</td>
<td>-0.03, -0.51; (0.015)</td>
<td>0.06, 0.62; (0.002)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
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<tr>
<td>tSM</td>
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<tr>
<td>B</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>A</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>-0.01, -0.49; (0.01)</td>
<td>NS</td>
<td>-0.03, -0.50; (0.015)</td>
</tr>
<tr>
<td>tMD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>NA</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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</tr>
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<td>NS</td>
<td>0.08, 0.15; (0.01)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>*tLG</td>
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<td>B</td>
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</tr>
<tr>
<td>A</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>-0.01, -0.49; (0.01)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>E1-C</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>B</td>
<td>NA</td>
<td>0.20, 0.57; (&lt; 0.0001)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.37, 0.84; (&lt; 0.0001)</td>
</tr>
<tr>
<td>A</td>
<td>0.30, 0.63; (&lt; 0.0001)</td>
<td>0.05, 0.56; (0.03)</td>
<td>0.06, 0.61; (0.0001)</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td></td>
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<td>B</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>A</td>
<td>0.26, 0.46; (&lt; 0.0001)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Pg</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>NA</td>
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<td>FSH</td>
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<td></td>
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<tr>
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<td>NS</td>
<td>NS</td>
</tr>
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<td>NS</td>
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</tr>
</tbody>
</table>

NA = not applicable because no large follicles were present, PrDOD: prior to dominance, aDOD: after dominance established, Total very small follicles: tVSM; 3 to 6 mm, total small follicles: tSM; > 6 to 10mm, total medium follicles: tMD; >10 to 16 mm, total large follicles: tLG; > 16 mm, estrone conjugates: E1-C, Estriol: E3, Progestagens: Pg, Follicle Stimulating Hormone: FSH, Luteinizing hormone: LH.

*No large follicles were present before DOD, so the row was omitted.