Research Article

Serum Estradiol and Progesterone Profiles during Estrus, Pseudopregnancy, and Active Gestation in Steller sea lions[1]

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Abstract

While the proximate driver behind the decline of the Western stock of Steller sea lions (*Eumetopias jubatus*, >80% since 1970's) is likely multifactorial, the population reduction may have been powered by a decrease in fecundity. A harvest of Steller sea lions in the 1970's and 80's revealed a 30% reduction in the proportion of pregnant females from early (October – November) to late gestation (April – May). Identification and quantification of these reproductive failures are difficult when we lack species-specific data on endocrinology associated with discrete stages of the reproductive cycle (i.e. estrus, implantation, and gestation). We tracked changes in serum estradiol and progesterone in three adult female Steller sea lions from 2011 – 2015. In all years and most females, a discrete increase in estradiol was observed during the breeding season (June – August), indicative of estrus. Estradiol concentrations from October – May in a pregnant female compared to her corresponding values when non-pregnant did not consistently differ through gestation. An elevation in progesterone was observed in all females and all years beginning approximately in June and lasting through November. This likely results from progesterone production by the corpus luteum in both pregnant and pseudopregnant females. Serum progesterone shows promise as a diagnostic tool to identify pregnancy during month three to five (December – February) of the eight month active gestation following embryonic implantation. This study provides ranges of key hormones during estrus, embryonic diapause/pseudopregnancy, and gestation in pregnant and non-pregnant females for studying reproduction in Steller sea lions.

Introduction

Steller sea lions (*Eumetopias jubatus*) experienced significant population declines (>80%) beginning in the 1970's that led to their listing as threatened under the Endangered Species Act in 1990. In order to develop specific recovery plans, the National Marine Fisheries Service delineated Steller sea lions into the Eastern and Western distinct population segments based on differences in mitochondrial DNA [Bickham et al. 1996]. The geographic boundary between these two segments occurs at 144° W longitude or Cape Suckling, AK. By the 1990's the Eastern stock showed steadily increasing population trends, but the Western stock continued to decline and was subsequently up-listed to endangered in 1997

[Fritz et al. 2013; United States Federal Register 62:30772-30773]. Currently, the population trends of the Western stock are overall stable or increasing, but there are strong regional differences [Allen and Angliss 2012; Johnson and Fritz 2014].

Competition with commercial fisheries, changes in predation, environmental changes, anthropogenic effects, diseases and contaminants were all hypothesized as drivers behind the population decline. Several studies focused on nutritional stress [Alverson 1992; Rosen and Trites 2000; Rosen 2009; Spitz et al. 2015; Tiphaine et al. 2009] and predation [Heise et al. 2003; Horning and Mellish 2014; Maniscalco et al. 2007; Williams et al. 2004] as the proximate drivers of the decline, but low fecundity and pup production are suggested as potential sources for the continued lack of recovery [Holmes et al. 2007; Trites and Donnelly 2003].

Studies that examined the reproductive tracts of 108 sacrificed adult female Steller sea lions, [Pitcher and Calkins 1981; Pitcher et al. 1998], identified a >30% reduction in the proportion of pregnant females from early (October – November, n = 36) to late gestation (April – May, n = 34) in the 1970's and again in the 1980's. An inferential age structured model, with temporally varying vital rates, found that a steady decline in birth rates from 1976 to 2004 best fit the changes in Steller sea lion population age structure observed for the Gulf of Alaska region [Holmes et al. 2007]. This model output differed from a study assessing reproductive rates using direct observations of Steller sea lions (n=151) to estimate reproductive rates and found current rates were similar to those reported prior to the population decline [Maniscalco et al. 2010].

Despite several studies describing the reproductive biology of Steller sea lions, little data exist characterizing the physiology of the female reproductive cycle. The majority of current knowledge originates from sacrificed animals [Pitcher and Calkins 1981; Pitcher et al. 1998], demographic studies [Maniscalco et al. 2010; Parker and Maniscalco 2014], studies over a single season on non-breeding animals [Harmon 2001], or inferences from surrogate species, most commonly, the northern fur seal (*Callorhinus ursinus*) [Browne et al. 2006; Gentry 1997]. These studies have outlined seasonal windows for estrus, implantation and parturition. On average, female Steller sea lions become reproductively

mature at 4.6 years of age, pup in mid-May to mid-July [Pitcher and Calkins 1981], and spontaneously ovulate 11 days later [Parker and Maniscalco 2014]. Following copulation, if an egg is fertilized, females undergo a four-month embryonic diapause and delayed implantation, followed by an eight month active gestation [Pitcher and Calkins 1981]. Non-pregnant female otariids exhibit an obligate pseudopregnancy after ovulation for approximately four months [Boyd 1991 a], followed by an eight-month period before their next ovulation.

Reproductive failures in norther fur seals were defined as either 1) missed pregnancies (failure to ovulate, conceive or implant), or 2) failed pregnancies (implanted embryos that are reabsorbed or aborted) [Craig 1964]. Current literature has documented high ovulation or early term pregnancy rates (95% – 100%) in mature females of several pinniped species [Boyd 1985; Craig 1964; Dickie and Dawson 2003; Guinet et al. 1998; Pitcher and Calkins 1981]. In a retrospective analysis of sacrificed northern fur seals (n = 603), an estimated 11.5% of adult females failed to implant [York and Scheffer 1997]. These studies suggest that when missed pregnancies occur, they are likely infrequent and therefore not a significant contributor to reduced annual birth rates. In contrast, Boyd [1984; Boyd et al. 1999] identified implantation as a high-energy investment and suggested that nutrition and environmental conditions will likely have a significant impact on success. In other mammalian species, ~80% of pregnancy failures occurred during implantation [Cross et al. 1994] and failure rates have been shown to increase with poor environmental conditions and in physiologically compromised females [Short 1984].

Failed pregnancies, unless associated with a visibly aborted fetus, are equally as difficult to quantify as missed pregnancies. Literature on Steller sea lion abortion rates is scant and likely a result of reduced population monitoring during the late gestational period when harsh winter conditions limit access to haulouts [Palacious et al. 2011; Spraker and Bradley 1996]. Current technologies are unable to detect implantation, resorption, or factors leading to reproductive failures without sacrificing an animal. A clearer understanding of the endocrinology associated with each stage of the reproductive cycle is the first step to identifying and quantifying reproductive failures.

This present study defines and characterizes the reproductive cycle of adult, female, Steller sea lions through serum estradiol and progesterone. Specifically, we aim to 1) quantify annual estradiol and progesterone hormone ranges for pregnant and non-pregnant adult females 2) quantify changes in estradiol levels associated with copulation events following parturition, and 3) determine if hormone levels differ between pregnant and non-pregnant females.

Materials and methods

Study animals

This research was permitted by the National Marine Fisheries Service Permit No. 18534 and conducted in accordance with Alaska SeaLife Center Institutional Animal Care and Use Committee Protocol No. R12-03-02. We sampled three, permanently captive, adult female Eastern stock Steller sea lions housed at the Alaska SeaLife Center (ASLC) in Seward, Alaska. Females were either born in captivity or collected at approximately 1 month old and captive-reared at alternate facilities until April of 2011 when they were transferred to the ASLC to participate in a reproductive biology study. At the start of the project in 2011, all females were nulligravida. Females 1 and 2 were 11 years old, female 3 was 5 years old and the breeding male was 18 years old.

Female Steller sea lions were primarily maintained in an outdoor, public display exhibit exposed to natural light cycles with temporary bouts in an indoor holding pool to facilitate sample collection. Sea lion diets were comprised of Alaskan caught Pacific herring (*Clupea pallasii*), capelin (*Mallotus villosus*), walleye pollock (*Gadus chalcogrammus*), California squid (*Doryteuthis opalescens*) and pink salmon (*Oncorhynchus gorbuscha*) listed in descending order relative to each species' proportion in the diet.

From September through April, females were housed independently from the breeding male with short cohabitation bouts beginning in December to facilitate socialization prior to the breeding season. May through August, non-pregnant females were housed with the male continuously and removed only for sample collections (< 12hrs). Pregnant females were housed separate from the male until 7-16 days post-parturition; the documented time frame when 95% of wild female Steller sea lions copulate [Parker and Maniscalco 2014]. Animals were observed 24 hours a day during the breeding season to monitor

pupping and copulation events. Three pregnancies occurred within this study. Female 1 and 2 gave birth in 2013 and female 1 pupped again in 2014. Offspring were maintained on the female's milk only and weaned no later than April of the following year. In 2014, following repeated behavioral conflicts during the 2012 and 2013 breeding season, female 3 was deemed socially incompatible with the male and subsequently housed separately, but continued in the reproductive biology study as a non-pregnant female. Veterinarian assessment found her to be reproductively healthy, but was never observed copulating and she had no pregnancies in this study.

Sample Collection

Serum samples were collected via venipuncture from the caudal gluteal veins or rear flipper interdigital veins while sea lions were under general anesthesia [Heath et al. 1996; Villegas-Amtmann et al. 1996]. Blood was collected every other month from April 2011 – February 2015. Frequency of collections increased to weekly in June or July, depending on parturition events, to capture estrus, and weekly in October to look for hormonal evidence of implantation. Blood was collected into non-gel clot activator additive red top tubes (Greiner bio-one, North Carolina) and allowed to clot for no less than 30 minutes. Samples were then spun at 3500 rpm for 5 minutes on an Eppendorf 5804 centrifuge (Eppendorf, Hauppauge, NY). Serum was extracted, aliquoted and stored at -80°C until analysis. In addition to the samples collected from the main breeding females (1-3), archived samples from a 17 year old pregnant female Steller sea lion, female 4, housed at the ASLC in October 2009 – May 2010 were included into data analysis (n = 7). Due to the limited volume in these samples, only progesterone was assayed and increased the number of samples representing pregnant animals. Transabdominal ultrasounds were conducted by ASLC veterinarians during the October through May sampling events using an Sonosite 180plus with a DUP C60/5-2 MHz transducer (Sonosite INC. Bothell, WA) to detect evidence of pregnancy.

Hormone Analysis

Circulating estradiol levels were quantified using a commercially available estradiol enzyme-linked immunosorbent assay kit (ELISA; Alpco, Salem, NH). Absorbance was measured at 450 nm on a

Spectramax Plus 384 microplate reader with Softmax Pro software (Molecular Devices, Sunnyvale, CA). Alpco enumerated cross reactivity in human serum was, estradiol (100%), estriol (1.6%), estrone (1.3%), progesterone (0.1%) and cortisol (0.1%). The estradiol ELISA was validated for Steller sea lions by testing for parallelism and accuracy. Parallelism was assessed by serially diluting a sea lion serum sample with high estradiol concentration with kit provided assay buffer and assayed alongside the standard curve. Lines were parallel and the slope of the diluted sea lion sera was -0.0106 ($y = -0.0106x + 1.3754$, $R^2 = 0.92$) and the slope of the standard curve was -0.004 ($y = -0.0048 + 1.3157$, $R^2 = 0.89$). Accuracy was tested by comparing a four parameter nonlinear fit curve generated from 50 µl samples comprised of 25 µl of pooled sera (a sample from each female collected in June of 2011) and 25 µl of one of the six kit provided standard concentrations (0, 20,100, 300, 800, and 3200 ng/ml) to a standard curve generated solely from 50 µl of kit provided standards. Curves were parallel between 0 and 300 pg/ml. The slope of pooled sera plus standards was -0.21 and the slope for the standards alone was -0.31. High and low Alpco-provided controls were run in seven separate assays and resulted in an inter-assay variation of 12.85% (1272.92 ± 163.52 SD) and 12.72% (386.58 ± 49.19 SD), respectively. Intra-assay variation was calculated from 20 samples and equaled 6.04%.

Progesterone was detected using a commercially produced chemiluminescent immunoassay kit (CLIA; Diagnostic Automation, Calabasas, CA). Progesterone concentration was measured in relative light units on a Synergy 2 Multi-Mode plate reader utilizing Gen5 software (BioTek, Winooski, VT). Kit cross reactivity in human serum was reported as progesterone (100%), corticosterone (0.9%), androsterone (0.6%) and cortisone (0.2%). Parallelism and accuracy were assessed following the same protocol as described for estradiol validation with the exception of sample volumes equaling 25 µl, progesterone kit provided standards came in concentrations of 0, 0.5, 3, 10, 25, and 50 ng/ml, and pooled sera was comprised of a sample collected from each female in October – December and therefore likely to be of high progesterone concentration. Good parallelism and accuracy were demonstrated for progesterone in Steller sea lion serum. The diluted serum curve ($y = 1275.3x + 4121.6$, $R^2 = 0.99$) was parallel to the standard curve ($y = 970.56x + 127.84$, $R^2 = 0.99$) between 0.5 and 50ng/ml. Hormone

standards spiked with sea lion sera produced accurate results, where the slope of pooled sera plus kit standards was 0.728 and the slope of standards was 1.03. Diagnostic Automation provided high and low progesterone controls that were analyzed on five separate assays and resulted in an inter-assay variation of 4.77% (High Control = 11.24 ± 0.46 SD and Low Control = 2.84 ± 0.15 SD). Intra-assay variation calculated from 30 samples resulted in a mean coefficient of variation < 8.00%.

Standards, samples, and controls for each hormone assay were run in triplicate and the mean lumens (CLIA) or absorbance (ELISA) was used to calculate concentration from a four parameter nonlinear regression. Serum sample concentrations were accepted if 1) the coefficient of variation of all assay standards were <10%, 2) the standard curve's R^2 was > 95%, and 3) the coefficient of variation of the sample's mean hormone concentration was < 10%.

Statistical Analysis

The Steller sea lion reproductive cycle was partitioned into four periods to define and compare hormone concentrations between pregnant and non-pregnant females. The four periods were identified as samples collected 1) June – August, 2) October – November, 3) December – February, 4) March – May, and no samples were collected in September (Table 1). These time periods corresponding to different stages of the female Steller sea lion reproductive cycle (See Supplemental Information 1 for specific sampling dates for each female in each year). The June – August period characterizes sample concentrations from non-pregnant animals during the breeding season. Samples collected in October – November denote hormone ranges during the luteal phase in a non-pregnant female and early active gestation in a pregnant animal. The December – February time period represents a non-reproductive phase in a non-pregnant female and corresponds to the middle of active gestation in a pregnant female. Samples collected in March – May reflect the final stage of active gestation for a pregnant female and continuation of non-reproductive stage in a non-pregnant animal. Hormone concentrations from non-pregnant females are presented with the caveat that once females were identified as not pregnant via ultrasound imaging they were assumed to not have undergone fertilization, implantation or resorption.

To investigate if a reproductive endocrinological change is associated with copulation, samples collected within the discrete time frame between parturition and subsequent copulation (7-16 days post-parturition) were compared to each females' respective mean, non-pregnant values (December – May) to assess if the change was different. From 2011 – 2015 only two females (females 1 and 2) had serum collected while pregnant and non-pregnant. Using this subset of data, each females' respective estradiol and progesterone concentrations were compared when pregnant versus non-pregnant through the three identified stages of active gestation. The small sample size of the pregnancy data subset prevented robust statistical comparisons but were graphically depicted using boxplots.

Results

From 2011 – 2015, we successfully collected and analyzed 134 Steller sea lion serum samples for reproductive hormones estradiol and progesterone (See Supplemental Information 1). Transabdominal ultrasounds could identified a fetal heartbeat as early as December but reliably in January and thus female Steller sea lions were definitively recognized as pregnant or non-pregnant during the December – February sampling events. Side by side comparison of ultrasound imagery shows the differential activity in the reproductive tract in a pregnant and pseudopregnant female from October – May (Supplemental Information 2). Overall, ultrasounds were only moderately reliable in monitoring the reproductive tract prior to a detectable fetus, as gas and intestines commonly prevented viewing the small area of interest.

Estradiol Cycles: non-pregnant

Estradiol concentrations during the breeding season were highly variable for all females across study years, ranging from 14.4 to 194.7 pg/ml (mean = 68.5 pg/ml ± 44.0 SD, n = 46; Table 1). When considered annually, a distinct spike in estradiol was observed during the breeding season for all females (Fig1a). External visible signs of estrus included mucoid vaginal discharge and swollen and darkly pigmented vulvar area, but presentation was not consistent over the course of the study. From October to May, estradiol concentrations in non-pregnant animals overlapped considerably between the denoted seasons and averaged 38.3

pg/ml ± 27.2, (n = 34), 40.1 pg/ml ± 22.1 SD (n = 21) and 36.1 ± 14.3 (n = 9, Table 1), respectively.

Progesterone values during pregnancy include archived samples from a 17 year old pregnant adult female housed and sampled at the Alaska SeaLife Center from October 2009 – May 2010, n = 7.
Steller sea lion pregnancy can be confirmed via ultrasound in January and therefore all values categorized as non-pregnant prior to January assume that no fertilization occurred in those individuals.

High individual variability in estradiol concentrations was observed over the course of the study period (Fig 1a). Female 3 consistently had higher estradiol levels than the other females throughout all reproductive stages in all years. In 2014, an estrus-associated peak was observed in female 3 only, while females 1 and 2 displayed concentrations marginally above annual baseline values (Fig 1a).

During the study, female 1 had two parturition events and female 2 had one. Copulation occurred during the 7-16 days post-parturition window. Estradiol concentrations collected 7-16 days postpartum were two standard deviations greater than the mean estradiol in December – May in female 1 for both parturition events (Fig 2). Conversely, female 2's estradiol concentration did not increase above baseline values on day 7 following parturition. Female 2 gave birth to a full term still-born on May 27, 2013. Labor was extremely prolonged and the female was subsequently anesthetized to facilitate removal of the fetus.

Progesterone Cycles: non-pregnant

Mean progesterone concentrations in non-pregnant animals during the breeding season were approximately double those observed throughout the non-breeding seasons, averaging 8.9 ng/ml ± 6.5 SD (n = 46) versus 2.2 ng/ml ± 2.5 SD (n = 34) in Oct. – Nov., 5.6 ± 3.9 ng/ml (n=21) during Dec. – Jan., and 2.2 ng/ml ± 2.5 SD (n = 9) in Mar. – May (Table 1). When considered longitudinally, progesterone was elevated in all females beginning approximately in June and lasting through November (Table 1, Fig 1b). Elevated progesterone was present irrespective of pregnancy status, even if direct exposure to the male

hadn't occurred, as seen in female 3 in 2014. Annual trends in progesterone fluctuated largely among females and with reproductive status (Fig 1b).

Pregnancy and endocrinology

Pregnancy had no consistent influence on trends in estradiol concentrations from October – May in female 1 or 2 (Fig 3). Compared to estradiol, trends in progesterone concentrations had smaller interquartile ranges and differentiated between a pregnant verse non-pregnant beginning in December through May in both females (Fig 3).

Discussion

Estradiol Cycles

Estradiol concentrations observed in healthy, captive, Steller sea lions, during the reproductive season, though displaying a larger range, were similar to those reported in their wild counterparts [Harmon 2001]. Large ranges and variability in circulating estradiol during the reproductive season were also reported in captive and free-ranging California sea lions (*Zalophus californianus*) [Greig et al. 2007], northern fur seals [Browne et al. 2006] and postpartum gray seals (*Halichoerus grypus*) [Mellish and Iverson 2005]. These studies support our findings and suggest estradiol fluctuations occur frequently, but are brief in duration, therefore descriptive statistics are likely heavily influenced by variation in individual hormone production. The single ephemeral estradiol spike observed in this study occurred in June – August, a time associated with estrus and peak breeding activity. The height of each female's estrus spike varied considerably over the course of the study. This variability likely suggests elevated estradiol associated with estrus is less than 7 days in duration and weekly sample collections are too infrequent to distinguish the apex of the estrus peak in Steller sea lions. In Antarctic fur seals (*Arctoephalus gazelle*), daily serum samples were required to clearly track fluctuations in estradiol following parturition [Boyd 1991 b]. Therefore, studies utilizing fecal or daily serum samples may better define the duration of elevated estradiol associated with peak sexual receptivity in the Steller sea lion. Though seen in northern fur seals [Browne et al. 2006; Kiyota et al. 1999], when we considered our data longitudinally, we did not

detect an increase in estradiol believed to be a precursor to the reactivation of a delayed embryo [Daniel 1974], this is possibly a result of our sampling frequency.

Similar to reproductive hormone studies in other mammals [Boyd 1991 b; Carter et al. 1986; Wielebnowski and Brown 1998], we observed a correlation between increased estradiol and copulation. For the majority of samples collected following parturition, estradiol levels were elevated relative to each their corresponding baseline concentrations for each individual. The one case where this was not observed was for female 2 following traumatic birthing. The serum sample collected during the anesthesia event necessary to remove the stillborn, revealed a serum cortisol concentration of 49.22 µg/ml, 3.5 times greater than female 2's mean baseline cortisol concentration. Traumatic births have been linked to disrupted subsequent reproductive receptivity or the estrus cycle as previously suggested in other species [Gee et al. 1990; Moberg 1991]. Though female 2 was observed copulating 13 days postpartum, estrus was not detected via serum estradiol in 2013 nor did this female become pregnant during the remainder of the study.

Progesterone Cycles

Circulating progesterone concentrations observed in the captive female Steller sea lions were similar to progesterone ranges reported in free ranging Steller's [Harmon 2001], California sea lions [Greig et al. 2007], female northern fur seals [Browne et al. 2006] and South American fur seals *(Arctocephalus australis)* [Katz et al. 2013] during comparable seasons. Though circulating progesterone ranges overlapped between the delineated stages of the annual reproductive cycle, an approximate two-fold increase in the mean progesterone in pregnant and non-pregnant females was observed during October – November when compared to concentrations in non-pregnant females from December – May. This could be a result of an individual(s) that were pregnant during the October – November sampling but experienced a reproductive failure prior to ultrasound identification of a fetus and thus were misclassified as non-pregnant. Alternatively, the increased mean progesterone observed during October – November in both non-pregnant and pregnant females is likely the result of pseudopregnancy. Pseudopregnancy has been documented in several species of pinnipeds (hooded seals (*Crystophora cristata*): [Noonan and

Ronald 1989]; harbor seals (*Phoca vitulina*): [Reijnders 1990]; harp seals (*Phoca groenlandica*): [Renouf et al. 1994]. During pseudopregnancy the corpus luteum produces progesterone for a length of time approximately equal to embryonic diapause, making pregnant and non-pregnant conspecifics endocrinologically indistinguishable via traditional sex steroids [Daniel 1974; Reijnders 1990]. While the corpus luteum of non-pregnant females regresses, pregnant individuals experience a continual input of progesterone from the corpus luteum during gestation [Ishinazaka et al. 2001; Ishinazaka et al. 2002]. In our study, evidence of pseudopregnancy was indisputably observed in Female 3 in 2014. Although, female 3 was never exposed to the breeding male, her October – November sampling revealed elevated progesterone levels similar to those displayed by Female 1 and 2 (Fig. 1b).

Pregnancy and endocrinology

The differential production of progesterone has been utilized to identify pregnant individuals in a variety of taxa including Galapagos sea lions (*Zalophus wollebaeki*): [Villegas-Amntmann et al. 2009], New Zealand fur seals (*Arctocephalus forsteri*): [McKenzie et al. 2005], Reindeer (*Rangifer tarandus*): [Ropstad et al. 1999], domestic goat (*Capra aegagrus hircus*): [Gonzalez et al. 2004], and Friesian horses (*Friesian*): [Sevinga et al. 1999]. Based on our data from three females spanning four pregnancies, serum progesterone shows promise as a diagnostic tool to identify pregnancy during the three to five month window (December – February) of the eight month active gestation following implantation in Steller sea lions. Following this period, progesterone in pregnant animals decreases as parturition nears and thus distinguishing pregnant animals from non-pregnant during months six to eight of active gestation (March – May) using progesterone may not be possible, although additional data is needed. Repeat sampling for pregnancy hormones from the middle of gestation through parturition could aid in quantifying abortion rate for known individuals. While approximately 80% of failed pregnancies have been reported to occur during the implantation stage in many species [Cross et al. 1994; King 1991; Roberts et al. 1990; Roberts et al. 1992], for Steller sea lions, nearly all failed pregnancies occur later in gestation [Pitcher et al. 1998]. Boyd et al. [1999] further highlighted that environmental factors and nutrition have the greatest influence on successful pinniped reproduction, and implantation represents a commitment to high energy

investment into offspring [Boyd 1984]. Therefore, developing methodologies to identify pregnancies and reproductive failures prior to January could significantly assist the continuing investigation into drivers of population trends for the Western stock of the Steller sea lion population.

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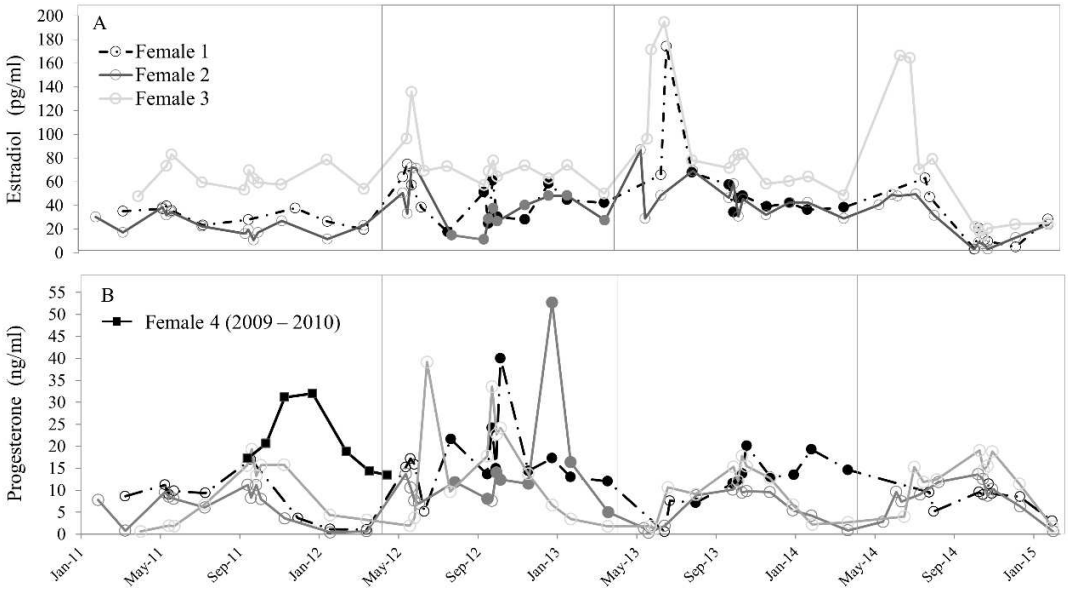

Figure 1

Fig 1. Serum estradiol (pg/ml; A) and progesterone (ng/ml; B) concentrations for three captive adult female Steller sea lions. Filled circles denote samples collected from a pregnant sea lion and unfilled from a nonpregnant female. Each panel represents a single reproductive cycle beginning in May. Panel B includes archived samples collected from a 17 year old pregnant adult female from October 2009 – May 2010. These values are overlaid on the 2011-2012 annual cycle for easy comparison to the primary breeding sea lions in this study

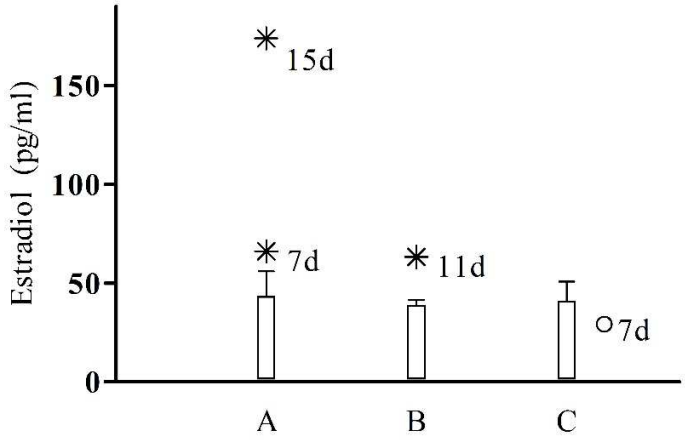

Figure 2

Fig 2. Mean serum estradiol concentration (pg/ml) + SD during the non-reproductive season (Dec. – May) of female 1's first pregnancy (A), female 1's second pregnancy (B), and female 2's first pregnancy (C). For pregnancy A and B estradiol concentrations collected in days following parturition were outside females corresponding mean (*). For pregnancy C the estradiol concentration collected postpartum was within female 2's corresponding mean (O).

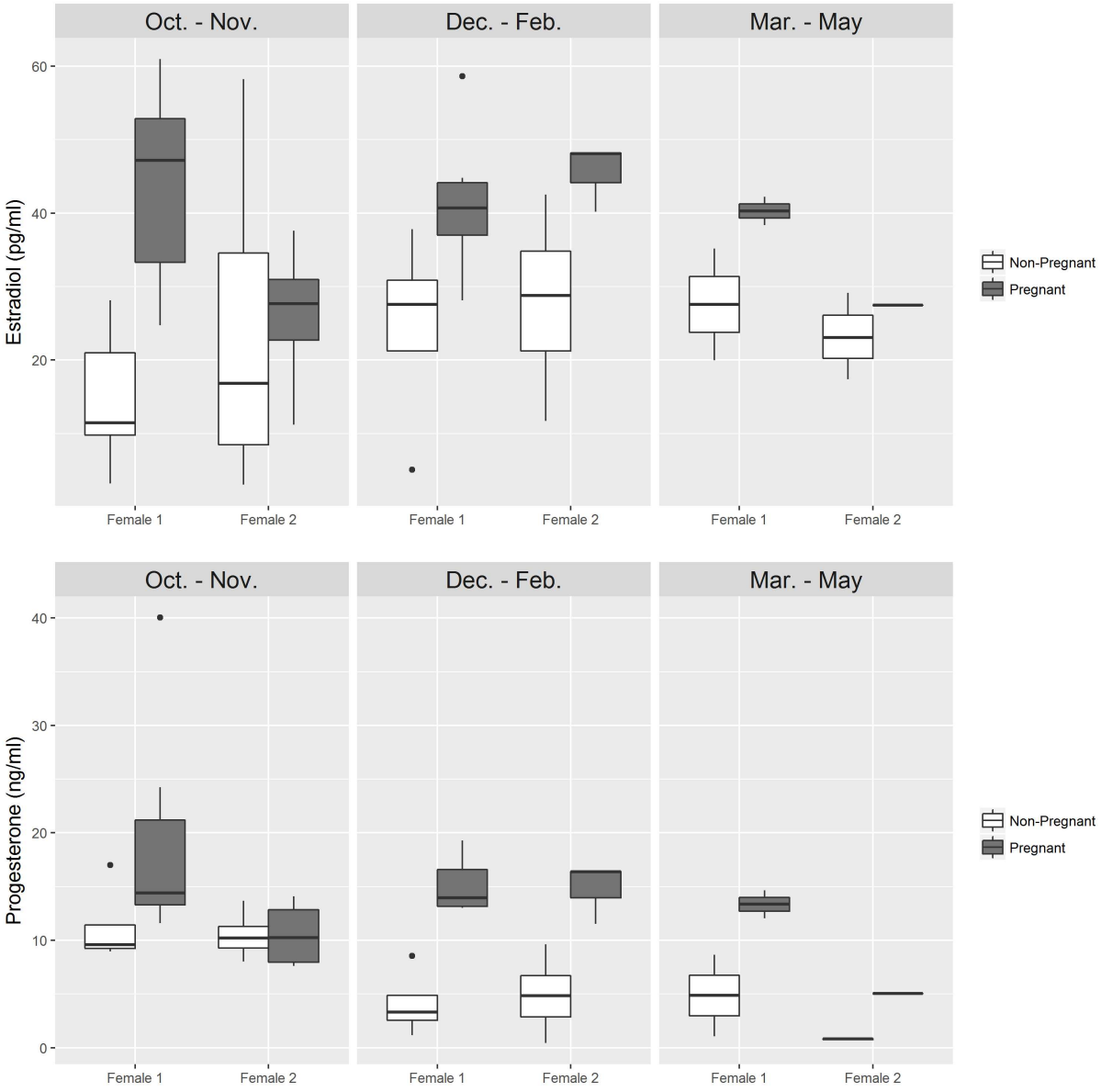

Figure 3

Fig 3. Box plots of the distribution of median and 95% CI of serum estradiol (pg/ml) and progesterone (ng/ml) concentration for female 1 and female 2 from Oct. – May when pregnant and non-pregnant.

Table 1. The range, mean ± SD of serum estradiol (pg/ml) and progesterone (ng/ml) concentrations and corresponding sample size (n) of three female Steller sea lions partitioned by season of the reproductive cycle from 2011 – 2015.

	Estradiol (pg/ml)						Progesterone (n			
	Pregnant			Non-Pregnant			Pregnant			
	n	range	mean	*n*	range	mean	*n*	range	mean	*n*
June – Aug.				46	14.9 – 194.7	68.5 ± 44.0				46
Oct. – Nov.	12	11.1 – 60.9	38.1 ± 14.9	34	3.0 – 83.6	38.3 ± 27.2	14	7.6 – 40.0	16.4 ± 8.2	34
Dec. – Feb.	9	28.1 – 58.6	42.8 ± 8.6	21	5.0 – 78.7	40.1 ± 22.1	11	11.5 – 52.7	21.3 ± 12.3	21
Mar. - May	3	27.4 – 42.1	36.0 ± 7.6	9	17.3 – 53.9	36.1 ± 14.3	6	5.0 – 18.7	13.0 ± 4.5	9