Characterizing estrus by trans-abdominal ultrasounds, fecal estrone-3-glucuronide, and vaginal cytology in the Steller sea lion (*Eumetopias jubatus*).

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Abstract

The ability to monitor the estrus cycle in wild and captive marine species is important for identifying reproductive failures, ensuring a successful breeding program, and monitoring animal welfare. Minimally invasive sampling methods to monitor estrus in captive populations have been developed, but results suggest these tools can be species-specific in their precision and accuracy. Therefore, the minimally invasive sampling methods of trans-abdominal ultrasounds, a fecal steroid analysis (estrone-3-glucuronide, E1G), and vaginal cytology, were evaluated for their efficacy to characterize and monitor estrus in a captive breeding population of Steller sea lions (*Eumetopias jubatus*). Three adult females were sampled over five breeding seasons, resulting in six estrus profiles characterized by trans-abdominal ultrasounds, five by fecal E1G, and four by vaginal cytology. Animals were trained to allow trans-abdominal ultrasounds, fecal samples, and vaginal swabs to be collected approximately daily. Of the 76 trans-abdominal ultrasound sessions attempted, 8 successfully visualized both ovaries. From these scans, the chronology of ovarian changes during proestrus and estrus was estimated. The time from the detection of developing follicles to the identification of a dominate follicle occurred in 2-5 days

and a corpus hemorrhagicum formed approximately 4 days later. However, because visualization of the ovaries was prevented by the gastrointestinal system in 88% of scans, this tool was overall unreliable for monitoring changes associated with estrus. To detect fine scale physiological changes associated with estrus, we analyzed changes in fecal E1G (n = 62) and vaginal cytology (n = 157) 15 days before and after each female's single copulation event (Day = 0). Changes in fecal E1G had the highest accuracy at detecting Day = 0. Fecal E1G increased leading up to estrus, peaked at Day = 0, and then declined. Although we did observe the characteristic increase in superficial cells associated with impending estrus, the type of cell which peaked closest to Day = 0 was intermediate. The uncertainty around the peak in intermediate cells, indicating estrus, was greater than the uncertainty associated with detecting estrus from fecal E1G. Collectively, these results suggest that changes in fecal E1G and vaginal cytology are viable tools to detect estrus in Steller sea lions, but require daily sampling to detect gradual changes, limiting their applicability to studies of wild populations.

1. Introduction

Pinnipeds exhibit a high degree of annual synchronicity in their breeding and pupping season, a mechanism likely developed to optimize offspring survival by timing parturition and subsequent breeding with favorable environmental conditions [1]. Females of these species quickly transition between pupping and breeding. This reproductive strategy is enabled by rapid hormonal changes that initiate proestrus and estrus following parturition. Our understanding of the timing of estrus in pinnipeds is primarily derived from observations of mounting and breeding behavior. These studies show that while the time from parturition to fertilization varies among pinnipeds [1], the duration of estrus--the period of sexual receptivity and fertility— is short in duration and is likely less than two days [2].

Understanding the physiological and endocrinological changes associated with proestrus and estrus can be important for the conservation and management of many wild and captive marine mammal populations. In captive populations, monitoring the onset of estrus is critical for successful breeding management. Such information can be used for timing artificial insemination [3,4], for deciding on when cohabitation of males and females is optimal to enable or prevent breeding [5], or for determining the age of maturation or senescence in females [6]. In free-ranging populations, monitoring estrus can be useful for assessing demographic factors associated with natality such as age of maturation, timing of senescence [7,8] and cases of reproductive failures due to failed ovulation or conception [9].

The Steller sea lion (*Eumetopias jubatus*), the largest of the otariid pinnipeds, exhibits an annual synchronous breeding and pupping period. Beginning approximately in May and continuing June, pregnant females return to terrestrial rookeries to give birth, with approximately

90% of pups being born within a 25-day period [8,10]. Seven to sixteen days following parturition, females spontaneously ovulate and copulate with a single male [11,12]. If an egg is fertilized, females go through a 4-month embryonic diapause with implantation occurring in September-October followed by an 8-month long active gestation [8]. Female otariids that do not conceive display a 4-month obligate pseudopregnancy [1], followed by an 8-month period before their next ovulation. Steller sea lions are currently listed as endangered throughout parts of their range in Alaska (United States Federal Register 62:30772-30773). While low pup production has been suggested as a potential factor contributing to the lack of recovery of these populations [13,14], the physiology and endocrinology associated with discrete stages of the estrus cycle are poorly understood in this species.

The establishment of a captive Steller sea lion breeding population (Alaska SeaLife Center, Seward, AK) provided the opportunity to explore the reproductive endocrinology of this species on a fine-temporal scale in controlled conditions. From this, one study on breeding females found elevated circulating estrogen occurred briefly during the breeding season, but weekly blood draws were too infrequent to determine the exact timing of estrus using this method [5]. In Antarctic fur seals (*Arctoephalus gazelle*), daily serum samples to quantify circulating estradiol were needed to clearly detect ovulation [15]. Profiles of serum estrogen or progesterone hormones are commonly used to identify a window for estrus in other species [16,17]. However, for large semi-aquatic marine mammals, the logistical and safety challenges associated with capture hinder collecting the serial blood draws needed to clearly detect ovulation [15]. Since this tool is limited in its ability to consistently capture the onset and duration of estrus for both wild and captive populations [5], an exploration of alternative sampling methods is needed to

82 better describe the physiology associated with estrus in Steller sea lions, and to assess the
83 applicability of these methods to various management concerns.
84 Less invasive samples (i.e feces, hair, whiskers, saliva, blowhole exhale, vaginal swabs) that
85 minimize or eliminate the need for chemical or physical restraint are becoming increasingly
86 popular to track female reproductive cycles in both wild populations and those held in zoos and
87 aquaria [18-20]. Ultrasound imaging, patterns in vaginal cytology, and fecal hormones are three
88 minimally invasive sampling methodologies that have been used to track estrus and detect
89 ovulation in a variety of taxa [4, 21-24] and may provide insights into the Steller sea lion estrus
90 cycle. While ultrasound imaging detects real-time structural changes in ovaries [4], it is a
91 challenging methodology requiring consistent visualization and measurement of small structure
92 [24], whereas vaginal cytology and fecal hormones reflect the ancillary signature of estrogen
93 released by developing follicles. For example, estrogen, released by developing follicles and the
94 corpus luteum, is positively correlated with increased cornification of vaginal epithelial cells
95 [25,26] but there is species specific variability in the reliability of this method. A vaginal smear
96 with > 85% superficial cells marked estrus in several terrestrial species [27-29], but the timing
97 and presentation of cytological changes did not consistently repeat across marine mammals (e.g.
98 walrus, *Odobenus rosmarus*, [30]; northern fur seals, *Callorhinus ursinus*, [31]; bottlenose
99 dolphin, *Tursiops truncatus*, [26]).
100 Alternatively, fecal estrogens have been positively correlated with circulating estrogen levels
101 [29,32] and the non-invasive nature of fecal sample collection is especially attractive when
102 working with captive and wild populations that are potentially dangerous, are prone to stress, or
103 where frequent blood samples are not possible. Unlike some hormones, estrogens undergo
104 relatively little steroid metabolism during the passage through the intestinal tract [33] making it a

practical marker to track ovarian activity. Since there is a significant time lag in fecal hormones due to gastrointestinal transit and ultimate evacuation in feces, fecal hormones do not reflect real-time endocrine activity. Fecal hormones reflect pooled endocrine activity from hours to days before excretion [34]. In free-ranging pinnipeds, fecal samples can be collected from terrestrial haulouts, such samples have been extensively used for dietary studies, but are a significantly less explored tool for tracking reproductive physiology in marine mammals [19].

Here we aimed to provide the first baseline data characterizing the physiological signals of estrus from minimally invasive samples for Steller sea lions. To do this, we explored the use of three minimally invasive sampling methods: 1) trans-abdominal ultrasound, 2) a fecal estrogen metabolite analysis, and 3) vaginal cytology to characterize and detect estrus in the Steller sea lion.

2. Methods

2.1 Study Animals

This work was permitted by the National Marine Fisheries Service Permit No. 18534 and conducted in accordance with the Alaska SeaLife Center Institutional Animal Care and Use Committee No. R12-03-02. Three permanently captive adult female Eastern stock Steller sea lions housed at the Alaska SeaLife Center (ASLC; Seward, AK) were sampled from 2012 – 2016 as part of a captive breeding program. Animals were housed in an outdoor, public display exhibit with natural environmental and light conditions and were fed a mixed diet of fish and squid. Sea lions were monitored 24 hours a day by either onsite staff or through overnight video recordings during the breeding season (May –August) to observe pupping and copulation events [5].

The captive breeding population included females 004 and 005 born in 2000, and female 011 born in 2003 (Table 1). Additionally, the breeding male during the 2012 – 2014 breeding seasons was male 001 (born 1993) and male 007 (born 2009) was the breeding male during the 2015-2016 season. Four viable pregnancies and one fetal death occurred during the study. Female 004 successfully pupped in 2013, 2014, 2016 and 2017. Female 011 birthed a full-term pup that died due to dystocia and impinged umbilical cord in 2015. She was primiparous, and the fetus was rear-flippers first in the birth canal.

2.2 Sample Collection - Trans-abdominal Ultrasound

Trans-abdominal ultrasounds to monitor the onset of estrus were initiated at ASLC staff veterinarian discretion, typically at the beginning of June or near parturition for pregnant females. Sessions were attempted weekly, and increased up to thrice daily when follicular activity was identified using an Ibex Pro ultrasound with a CL 3.8 5-2.5MHz 60-mm curved linear array transducer (E.I. Medical Imaging, Loveland, CO). All trans-abdominal ultrasound scans were conducted during routine training sessions. Females were trained using operant conditioning to station in ventral recumbency and allow each ovary to be examined for signs of activity. A combination of isopropyl alcohol followed by a generous dollop of ultrasound coupling gel (Echophonic Ultrasound Gel, Kruuse International, Newark, NJ) was massaged into the fur coat to enhance image resolution. Animals' fur coats were not clipped for the study. The ultrasound probe was fed through a three-foot semi-rigid pvc pipe to allow the operator to manipulate the transducer from a distance for human protection. Ovaries were identified by locating each kidney as a major landmark, and then proceeding caudally and slightly ventrally until the ovary was identified.

To quantify the efficacy of this method, each scanning session was classified as a "success" or "failure". A successful scanning session met the following conditions: 1) the animal allowed a thorough examination of the anatomic region of interest, and 2) both ovaries were visualized with enough clarity to characterize follicular activity. Therefore, an unsuccessful scanning session resulting from an inability to visualize both ovaries, may still have included useable information of follicular activity in the opposing ovary. During ultrasound sessions we sought to 1) identify the presence of follicles, 2) track changes in follicle size (regression or growth), 3) note on which side of the animal the dominant follicle occurred, and 4) observe ovarian structures that confirm ovulation has occurred. Ovulation (estrus) was believed to have occurred between the date the last large follicle was noted, and the subsequent date in which the follicle was absent and anechoic, irregularly shaped post-ovulation corpus hemorrhagicum (CH) was observed. The CH was then monitored until an irregular hyperechoic corpus luteum (CL) formed. Since transabdominal ultrasounds were used to conduct real-time monitoring of developing follicles, the determination of the date of ovulation did not require the observation of copulation events.

Intermittent trans-abdominal ultrasound imaging was also done outside of the breeding season (September – April) under general anesthesia during ASLC routine health assessments on our study females. This provided a framework for using trans-abdominal ultrasound imaging to monitor follicular activity and estrus, and enabled us to provide the first characterization of the previously undescribed ovarian structures during proestrus and anestrus for Steller sea lions (Sup Fig. 1).

2.3 Sample Collection - Fecal Estrone-3-Glucuronide

Scat samples were opportunistically collected at a minimum of once a week and up to daily May through August. Because females were commonly housed together, each sea lion was assigned a color of non-toxic glitter (Creatology, China) and daily fed < 4 gm encased in a pharmaceutical grade BSE and GMO free gel capsule (Capsule Connection LLC, Prescott, AZ) placed in a frozen fish to allow identification of individual females scat samples. Fecal samples were collected and stored in plastic storage bags at -20 F until steroid extraction.

To extract hormone steroids, scat samples were thawed and made homogenous through manual mixing. Approximately 15 ml of scat was placed in 30 ml glass vials, weighed and freeze-dried for 72 hours on a Labconco Freezone 6 (Labconco, Kansas City, MO). Freeze-dried samples were individually sifted through a 1mm stainless steel sieve to remove any foreign materials including prey hard parts. Steroid extraction was completed following Arbor Assays published Steroid Solid Extraction protocol honed for fecal hormone immunoassays (Arbor Assays, Ann Arbor, MI), in which all samples were diluted 1:10 with buffer provided in the kit prior to assaying.

Fecal Estrone-3-Glucuronide (E1G) was quantified using a commercially available estradiol enzyme-linked immunosorbent assay kit (Arbor Assays; Ann Arbor, MI). Absorbance was measured at 450 nm on a Spectramax Plus 384 microplate reader with Softmax Pro software (Molecular Devices, Sunnyvale, CA). The E1G enzyme immunoassay kit was validated for Steller sea lions by testing a pooled fecal extract of samples collected in June for parallelism and accuracy. Parallelism was tested by comparing a curve generated from the serially diluted pooled Steller sea lion fecal extract with kit provided buffer to the standard curve from kit-provided standards (0, 15.62, 31.25, 62.5, 125, 250, 500, and 1000 pg/ml). Curves were parallel between 0 and 250 pg/ml, the slope of pooled fecal extract plus standards was -0.0026 (y = -0.0026x +

197 0.7855, R^2 = 0.94) and the standards slope was -0.003 (y = -0.003 + 0.8307, R^2 = 0.92).
198 Accuracy was tested by comparing the standard curve (line equation above) to a curve generated
199 from 25 µl of pooled fecal extract and 25 µl of one of the eight kit provided standard
200 concentrations. Curves were parallel between 125 and 700 pg/ml, and slopes were similar where
201 the slope of pooled fecal extract was -0.0005. A sea lion fecal sample of high and low E1G
202 concentration was assayed in five separate plates and resulted in an inter-assay variation of
203 5.06% (21.35 ± 1.08 SD) and 12.72% (6.52 ± 1.48 SD), respectively. Intra-assay variation was
204 4.65% (n = 15).
205 E1G kit standards and fecal samples were run in triplicate and the mean absorbance was used
206 to calculate E1G concentration from a four parameter nonlinear regression. Raw E1G
207 concentrations were corrected for the hormone extraction process and are reported as the amount
208 of E1G per mg of solid feces (Arbor Assays, Ann Arbor, MI). Sample concentrations were
209 accepted if 1) the coefficient of variation of all assay standards were <10%, 2) the standard
210 curve's R^2 was > 95%, and 3) the coefficient of variation around the sample's mean hormone
211 concentration was < 10%.
212
213 *2.4 Sample Collection - Vaginal Cytology*
214 Vaginal swabs were collected under operant conditioning at minimum twice a week, and up
215 to daily, from May through August. For pregnant females, daily vaginal swab collections were
216 not initiated until approximately 3 days postpartum and continued until 15 days after copulation.
217 Vaginal cytology was collected using a sterile cotton swab (Fisher Scientific, Waltham, MA)
218 pre-moistened with phosphate-buffered saline (Amresco, Solon, OH) and inserted 10 – 16 cm
219 into the vaginal tract and rotated twice before removal. Each swab was rolled in two parallel

tracks on a clean microscope slide (VWR, West Chester, PA) and fixed immediately with a water-soluble spray fixative (BD, Sparks, MD). Slides were stained following the modified Papanicolaou (PAP) staining procedure as described by Gupta et al. [35]. Approximately, two hundred randomly selected cells per slide were counted; classified by morphology, and presented as percent parabasal, intermediate, or superficial cells. Additionally, cells were classified per stain coloration as basophilic, acidophilic, or keratinized as previously described by Durrant et al. [23].

2.5 Data Analysis

Steller sea lion breeding is primarily female driven and copulation events occur largely once, with a single male in a breeding season [11]. Therefore, we assumed the day copulation occurred was concurrent to estrus. Over the course of our study, there were five observed copulation events from two females that had associated fecal samples; female 004 in 2012, 2013, 2014, 2016, and female 011 in 2016 (Table 1). There were four observed copulation events with associated vaginal cytology samples from three females; female 004 in 2014 and 2016, female 005 in 2015, and female 011 in 2016 (Table 1). From these events, we assessed changes in fecal estrogen levels and vaginal cytology in samples collected from 15 days before to 15 days after the day copulation was observed. Female Steller sea lions mate on average 11 days post parturition [12], so a 15 day pre/post window of analysis provided a conservative temporal range for detecting changes associated with estrus.

We predicted that fecal estrogen would increase up to the day of copulation, reflective of ovarian activity and impending estrus, and decline following the copulation event. For the vaginal cytology samples, as seen in northern fur seals, we predicted that that there would be a

clear increase in the proportion of intermediate cells up to day of observed copulation and that superficial cells would begin to increase 6-8 days prior to observed copulation [31]. We also predicted we would see a shift from the majority of cells being acidophilic to the majority being basophilic, 6-8 days prior to estrus as observed in a highly controlled terrestrial model [27].

To assess these predictions, generalized linear models (GLM) were fit using R [36]. Response variables for the separate models included: (1) ΔE1G, (2) proportion of intermediate cells, and (3) proportion of superficial cells. Days from copulation (-15 to 15, Copulation = Day 0) was included as the predictor variable in all models. To account for individual variation in average E1G concentrations, and for repeatedly sampling some females in multiple years, we used the difference in sample concentration from the same female's mean concentration for the 15 day pre and post window in that year (ΔE1G) in analyses. Since cytology data was proportional, we could not calculate the differences between samples and individuals' means within years to account for inter-individual differences, and multiple samples from the same individuals. Instead, animal ID was included in analyses of vaginal cytology to account for these factors. The fit GLMs were then analyzed using a continuous segment analysis package in R [36,37] that defines any break-points where the linear relationships changed direction. Once the break point was estimated, the slopes for the linear regressions before and after the break were estimated. Significance was assessed from the 95% CI of the slope estimates. To test our predictions of changes in cell pH, a linear model was fit comparing the relationship between the difference in the number of acidophilic and basophilic cells present in samples, and the day from observed copulation. For this analysis, we used an iterative piecewise method for breakpoint detection instead of the segmented continuous approach, as we predicted the chromic shift would

represent an instantaneous shift in the relative abundance, and not a change in the direction of the slope of the relationship over time [27].

3. Results

3.1 Characterization of ovarian structures via trans-abdominal ultrasound

The Steller sea lion ovary was located caudally to the kidney and was an oval to round structure measuring between 1.5 - 2.25 cm in width and 2 – 4 cm in length in our adult Steller sea lions (Sup_Figure 1). During anestrus, the ovaries were small, oval shaped, and had a homogenous echogenicity which frequently lacked distinct margins (Sup_Figure 1a). The ovarian artery was generally not noticeable during anestrus. Multiple small follicles of 1-3mm diameter, a slightly more "plump" quality to the ovary, and a mildly increased hyperechogenic ventral membrane (germinal epithelium) were all intermittently noted in our study group during proestrus (Sup_Figure 1b). Ultimately, a single follicle became dominant, and increased in size and volume while the other follicles regressed in size (Sup_Figure 1c). During this time, the pulsatile ovarian artery was somewhat prominent resembling a follicle, but did not vary and remained just a few millimeters in diameter. At the time of ovulation, the dominant follicle ruptured, and left an irregularly shaped, primarily anechoic to hypoechoic CH made of blood, like a scab, in its place (Sup_Figure 1d). This stage rapidly changed in appearance from hypoechoic to hyperechoic and became smaller over several days until it transformed into an irregular mottled hyperechoic CL (Sup_Figure 1e). Once the CL was present, the ovary lost its round and prominent appearance and become less distinct from the surrounding soft tissues.

3.2 Monitoring Estrus: Trans-abdominal ultrasound

A total of 76 trans-abdominal ultrasound sessions were attempted over six breeding seasons and three females (Table 1). Of the total scanning effort, eight (10%) met our criteria for success. Of the failed scans, 67 (98%) were due to gastrointestinal tract ingesta or intraluminal gas artifacts preventing adequate visualization of the ovaries. The remaining 2% of unsuccessful scans were due to a lack of animal participation.

Successful scans were able to track follicular activity through the formation of a CL in two of the six estrus events (Table 2). Data from these events suggest that the time from the detection of follicles to that of a CH can range from 8-9 days, and a CL is detectable by at least 11-16 days following ovulation (Table 2). The maximum observed diameter of a dominant follicle equaled 1.57 cm in female 004. We did not have successful sequential scans and thus were unable to detect the exact date of follicle rupture via this method.

Ovulation did not consistently alternate between the left and right ovaries across our study animals. Female 005 ovulated from alternating ovaries in 2014 and 2015, female 011 ovulated from her right ovary in 2015 and 2016, and female 004 ovulated from her left ovary in two sequential seasons (2014-2015). The position of the ovaries became more ventral in multiparous females, presumably due to the weight of the developing fetus on the suspensory ligament.

3.3 Detecting Estrus: Fecal Estrone-3-glucuronide

Over the 30 day sampling period (15 day before and after an observed copulation event), female 004's fecal E1G values ranged from 3.03 – 21.37 pg/ml (mean = 9.13 ± 4.53 SD, n = 47) while female 011's values ranged from 3.57 – 12.32 pg/ml (mean = 7.06 ± 2.94 SD, n = 15). The general linear model showed fecal E1G significantly increased up to the day of copulation, with a breakpoint identified at day -0.06 (± 2.99, 95% CI). Following the copulation event, fecal E1G

concentrations significantly declined (Figure 1, Table 3). Fecal E1G concentrations three days around copulation averaged 11.32 ± 4.82 pg/ml SD (n = 18).

3.4 Detecting Estrus: Vaginal Cytology

The proportion of intermediate cells significantly increased during the 15 days prior to copulation, peaked 0.68 days after copulation (± 3.68, 95% CI), then significantly declined following this peak (Table 3, Figure 2a). Over this four day window before and after a copulation event, the proportion of intermediate cells averaged 0.63 ± 0.19 SD (n = 16). Among females, 011 had a significantly higher proportion of intermediate cells on average relative to 005 and 004, and 004 had a greater proportion on average relative to 005. The maximum proportions of intermediate cells observed for each individual during our sampling window were 0.725 (004 year 1), 0.840 (004 year 2), 0.645 (005), and 0.875 (011).

The proportion of superficial cells also changed relative to copulation events. The breakpoint for superficial cells was identified as -8.99 (± 1.4, 95% CI) days prior to copulation (Table 3). The slope before the breakpoint was not significant, but superficial cells significantly increased in prevalence starting at this breakpoint and through the end of our sampling window (Table 3, Figure 2b). Female 011 had a significantly lower proportion of superficial cells on average, relative to 004 and 005. The maximum proportions of superficial cells observed for each individual during our sampling window were 1.00 (004 year 1), 0.792 (004 year 2), 0.991 (005), and 0.497 (011). We did not detect a significant chromic shift prior to estrus ($R^2 = 0.11$, $F = 2.26$, $p = 0.08$).

4. Discussion

While discrete phases of the estrus cycle (i.e. follicular, luteal) have been tracked using trans-abdominal ultrasound in several cetacean species (Atlantic bottlenose dolphin, [38,39]; Killer Whales, [3]; Pacific white-sided dolphin, *Lagenorhynchus obliquidens*, [4]; Beluga, *Delphinapterus leucas*, [22], this method has been less successful in otariids [40]. For Steller sea lions, our results suggest trans-abdominal ultrasound was not a consistently reliable method for monitoring proestrus and the onset of estrus due to the common interference of the ingesta within the colon and intraluminal gas artifact frequently present in our sea lions. This may be a product of the regular meals and relatively sedentary lifestyle of sea lions held in human care when compared to the meal regularity and activity level of their wild counterparts. Despite our animals being operantly conditioned to participate, comprehensive exams were not tolerated in every session. While additional training may have increased each animal's tolerance for a longer examination, veterinary authorized efforts to alter the time of day, fasted versus unfasted status, and the use of mild laxatives seemed to have no effect on visibility. While we were able to intermittently identify some follicular development, the presence of CH, and subsequent CL formation; we caution relying on trans-abdominal ultrasound as the sole tool to monitor follicular activity and identify estrus in a captive setting for this species.

During our study, there were only two cases where both real-time monitoring (trans-abdominal ultrasound) and retrospective detection of estrus (vaginal cytology or fecal E1G) were conducted from the same animal during the same breeding season. In 2014, all three techniques monitored and detected estrus within the same window. However, in 2015 female 005 was observed copulating with the breeding male early in the summer, but follicular development was observed from trans-abdominal ultrasound approximately 2 months later (Table 1). The male that copulated with female 005 in this case was 6 years old, and the copulation event occurred

356 immediately following his first introduction to a breeding-age female. In the wild, male Steller
357 sea lions typically become sexually mature at 6 years, however most are not competitive to hold
358 territories and gain reproductive success until they are 9-13 years old [7,41] . In a captive setting,
359 a young sexually mature male may be introduced into a breeding context, but studies have shown
360 inexperienced males are less adept at detecting when a female is receptive [42]. In our study,
361 following his first year of breeding, male 007 was observed copulating with both female 004 and
362 011 in 2016, both of which became pregnant, suggesting that date of copulation (Day = 0) in
363 these cases was an appropriate behavioral indicator for estrus as is observed in the wild [11].
364 These findings suggest that in captive settings, and in cases with young breeding individuals in
365 particular, utilizing multiple sampling methods to monitor a females' estrus cycle may be
366 necessary to accurately time exposure or cohabitation for breeding.
367 We found increases in the concentration of E1G in feces leading up to estrus, which suggests
368 fecal E1G may be a plausible non-invasive proxy for monitoring circulating estrogen levels.
369 Additionally, we found that the peak change in fecal E1G occurred on the same day as
370 copulation with an uncertainty of ± 3 days. This temporal uncertainty may reflect variation in
371 individuals' digestion rates and the associated pooling of excreted hormone. Pinnipeds have
372 displayed rapid digestion rates as was observed in a sample of 13 elephant seals (*Mirounga*
373 *angustirostris*), 4 California sea lions (*Zalophus californianus*), and 3 harbor seals (*Phoca*
374 *vitulina)* that passed a dye marked meal on average in 5 hrs or less [43]. However, a study
375 examining prey skeletal recovery rates in fecal samples from two juvenile female Steller sea
376 lions reported initial defecation time following a feed ranged from 2 hours to 2.3 days, and
377 varied with activity level and with prey species [44]. Even with the uncertainty, our results
378 suggest monitoring E1G in feces could be useful in resident programs where acquiring serial scat

samples from the same individual is possible, but its use may be limited in studies of free-ranging populations.

We found the vaginal cytology of the Steller sea lions in this study did exhibit the expected increased cornification of epithelial cells through copulation associated with estrogen release [25], but lacked a defined peak at copulation followed by a precipitous decline. In black-footed ferrets, peak superficial cells typically comprised 90% of a sample during estrus then declined in prevalence 4 -10 days post copulation [28]. Similarly, in giant pandas, superficial cells rose above 50% by day -7, increased steadily to 86% on the day of ovulation (day = 0) and then declined [27]. In our study, superficial cells did increase steadily through the breeding season and up through the day of copulation, reaching similar maximum percentages, but there was no clear peak identified, or sharp decline in percentage post-copulation. Alternatively, we found that intermediate cells were a better predictor of estrus and peaked on the day of copulation, followed by a significant decline. This pattern of increases in intermediate and, to a lesser extent, superficial cells associated with estrus was also reported in the northern fur seal [31] and Pacific walrus [30]. As observed in the Pacific walrus [30], we did not detect a chromatic shift as found to occur prior to ovulation in vaginal cycles of the giant panda [27]. Why changes in intermediate cells are a better predictor of estrus in marine mammals versus superficial cells in terrestrial mammals remains unknown, but represents an area for future investigation. These results suggest vaginal cytology is a limited tool that can still be useful for identifying general cycling patterns but is not suitable for identifying the exact timing of estrus. Similar to the fecal E1G tool, monitoring endocrine activity via changes in vaginal cytology would require almost daily sampling during proestrus to identify gradual changes, and therefore is likely not suitable for field applications.

5. Conclusions

This study has contributed to understanding marine mammal reproductive physiology, and to our knowledge, we present the first investigation into the utility of using fecal estrogen as a monitoring tool for estrus in pinnipeds. Our results indicate that across the non-invasive sampling methods explored, changes in fecal E1G and vaginal cytology can both be used to identify estrus for Steller sea lions in a residential breeding program, and that the estimated date of estrus from fecal E1G is less uncertain relative to cytology estimates. Trans-abdominal ultrasounds were overall unreliable as a standalone tool, but did provide intermittent snapshots of ovarian activity and impending estrus, which can be useful in identifying a general timeframe to expose females to or cohabitate with males for breeding, particularly if paired with other methods of estrus detection or endocrine monitoring.

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References

[1] Boyd IL. 1991. Environmental and physiological factors controlling the reproductive cycles of pinnipeds. Can J Zool. 1991;69: 1135-1148.

[2] Boness DJ. Estrus and Estrous Behavior. In: Perrin WF, Würsig B, Thewissen JG, editors. Encyclopedia of marine mammals. Academic Press; 2009, p. 392-395.

[3] Robeck TR, Steinman KJ, Gearhart S, Reidarson TR, McBain JF, Monfort SL. 2004. Reproductive Physiology and Development of Artificial Insemination in technology in Killer Whales (*Orcinus orca*). Biology of Reproduction 71(2): 650-660.

[4] Robeck TR, Steinman KJ, Greenwell M, Ramierz K, Van Bonn W, Toshioka M, Katsumata E, Dalton L, Osborn S, and O'Brien JK. 2009. Seasonality, estrous cycle characterization, estrus synchronization, semen cryopreservation, and artificial insemination in the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*). Reproduction 138(2):391-405 doi: 10.1530/REP-08-0528

[5] Sattler R, Polasek L. 2017. Serum estradiol and progesterone profiles during estrus, pseudopregnancy and active gestation in Steller sea lions. Zoo Biology 36(5):323-331.

[6] Larson S. 2007. Reproductive hormone levels within captive female northern fur seals (*Callorhinus ursinus*) with and without chemical contraceptives. Aquatic Mammals 195-201.

[7] Perlov AS. 1971. The onset of sexual maturity in sea-lions. All-Union Research Institute of Marine Fisheries and Oceanography Proc. 80:174-189.

[8] Pitcher KW, Calkins DG. 1981. Reproductive biology of Steller sea lions in the Gulf of Alaska. Journal of Mammalogy 62:599-605.

[9] Craig, A. M. (1964). Histology of reproduction and the estrus cycle in the female fur seal, *Callohinus ursinus*. Journal of the Fisheries Research Board of Canada, 21, 773–811.

[10] Pitcher KW, Burkanov VN, Calkins DG, LeBoeuf BJ, Mamaev EG, Merrick RL, Pendleton GW. 2001. Spatial and temporal variation in the timing of births of Steller sea lions. Journal of Mammalogy 82:1047–1053.

[11] Mathisen, OA, Baade RT, Lopp, RJ. 1962. Breeding habits, growth and stomach contents of the Steller sea lion in Alaska. Journal of Mammalogy *43*(4), 469-477.

[12] Parker P, Maniscalco JM. 2014. A long-term study reveals multiple reproductive behavior strategies among territorial adult male Steller sea lions (*Eumetopias jubatus*). Canadian Journal of Zoology 92:405-415.

[13] Holmes, E. E., Fritz, L. W., York, A. E., & Sweeney, K. (2007). Age-structured modeling reveals long-term declines in the natality of western Steller sea lions. Ecological Applications, 17(8), 2214–2232.

[14] Trites, A. W., & Donnelly, C. P. (2003). The decline of Steller sea lions (*Eumetopias jubatus*) in Alaska: A review of the nutritional stress hypothesis. Mammal Review, 33(1), 3–28.

[15] Boyd, I. L. 1991. Changes in plasma progesterone and prolactin concentrations during the annual cycle and the role of prolactin in the maintenance of lactation and luteal development in the Antarctic fur seal (*Arctocephalus gazelle*). Journal of Reproduction and Fertility, 91, 637–647.

[16] Berkeley EV, Kirkpatrick JF, Schaffer NE, Bryant WM, Threlfall WR. 1997. Serum and fecal steroid analysis of ovulation, pregnancy, and parturition in the Black Rhinoceros (*Diceros bicornis*). Zoo Biology 16:121-132.

[17] Greig DJ, Kendall ML, Ruthishauser M, Frances M, Gulland D, Williams RM, Atkinson S. 2007. Seasonal changes in circulating progesterone and estrogen concentrations in the California sea lion (*zalophus californianus*). Journal of Mammalogy 88(1):67-72.

[18] Schwarzenberger F. 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. International Zoo Yearbook. 41:52-74.

[19] Amaral RS. 2010. Use of alternative matrices to monitor steroid hormones in aquatic mammals: a review. Aquatic Mammals 36(2):162-171.

[20] Kersey DC, Dehnhard M. 2014. The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. General and Comparative Endocrinology 203:296-306.

[21] Robeck TR, Schneyer AL, McBain JF, Dalton LM, Walsh MT, Czekala NM, Kraemer DC. 1993. Analysis of urinary immunoreactive steroid metabolites and gonadotropins for characterization of the estrous cycle, breeding period, and seasonal estrous activity of captive killer whales (*Orcinus orca*). Zoo Biology 12(2):173-187.

[22] Steinman KJ, O'Brien JK, Monfort SL, Robeck TR. 2012. Characterization of the estrous cycle in female beluga (*Delphinapterus leucas*) using urinary endocrine monitoring and transabdominal ultrasound: Evidence of facultative induced ovulation. General and comparative endocrinology 175(3):389-397.

[23] Durrant BS, Ravida N, Spady T, Cheng A. 2006. New technologies for the study of carnivore reproduction. Theriogenology 66:1729-1736.

[24] Curry E, Browning LJ, Reinhart P, Roth TL. 2017. Integrating trans-abdominal ultrasonography with fecal steroid metabolite monitoring to accurately diagnose pregnancy and predict the timing of parturition in the red panda (*Ailurus fulgens styani*). Zoo Biology 369:193-200.

[25] Goldman JM, Murr AS, Cooper RL. 2007. The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. Birth Defects Research (Part B) 80:84-97.

[26] Herrera JA, Sanchez R, Bernal JA, Lopez A, Rivera JG, Guzman A, Avalos A. 2015. Reproductive activity after induced anestrus using altrenogest in *Tursiops truncates* females in captivity in marine environment. Revista de la Facultad de Medicinia Veterinaria y de Zootecnia 62(2): 16-22.

[27] Durrant BS, Olson MA, Amodeo D, Anderson A, Russ KD, Campos-Morales R, Gual-Sill F, and Garza JR. 2003. Vaginal cytology and vulvar swelling as indicators of impending estrus and ovulation in the giant panda (*Ailuropoda melanoleuca*). Zoo Biology 22:313-321.

[28] Williams ES, Throne ET, Kwiatkowski DR, Lutz K, Anderson SL. 1992. Comparative vaginal cytology of the estrous cycle of the black-footed ferrets (*Mustela nigripes*), Siberian polecats (*M. eversmanni*), and domestic ferrets (*M. putorius furo*). Journal of Veterinarian Diagnostic Investigation Jan; 4(1):38-44.

[29] Valdespino C, Asa CS, Bauman JE. 2002. Estrous cycles, copulation and pregnancy in the fennec fox (*Vulpes zerda*). Journal of Mammalogy 83(1):99-109.

[30] Kinoshita K, Kiwata M, Kuwano R, Sato N, Tanaka T, Nagata M, Taira H, Kusunoki H. 2012. Temporal association of serum progesterone concentrations and vaginal cytology in walruses (*Odobenus rosmarus*). Theriogenology 77:933-939.

[31] Kiyota M, Yamaguchi Y, Nishikawa F, Kohyama K. Cytological changes in vaginal smear and epithelium associated with reproductive cycle in Northern fur seal, *Callorhinus ursinus*. 1999. Bulletin National. Research Institute of Far Seas Fisheries 36:17-25.

[32] Ziegler TE, Wittwer DJ, Snowdon CT. 1993. Circulating and excreted hormones during the ovarian cycle in the cotton-top tamarin, *Sauinus Oedipus*. American Journal of Primatology 31:55-65.

[33] Brown JL, Wasser SK, Wildt DE, Graham LH. 1994. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biology of Reproduction 51:776-786.

[34] Whitten PL, Brockman DK, Stravisky RC. 1998. Recent advances in noninvasive techniques to monitor hormone-behavior interactions. Yearbook Physical Anthropology. 41:1-23.

[35] Gupta S, Chachra KL, Bhadola P, Sodhani P. 2010. Modified Papanicolaou staining protocol with minimum alcohol use: a cost-cutting measure for resource-limited settings. Cytopathology 21:229-233.

527 [36] Crawley Michael J. 2007. The R Book. Wiley Publishing 1st ed.

528 [37] Muggeo VMR. 2003. Estimating regression models with unknown break-points. Statistics in
529 Medicine 22:3055-3071.

530 [38] Brooks FM. 2001. Sonographic imaging of the reproductive tract of the female bottlenose
531 dolphin, *Tursips trucatus aduncas*. Reproduction 121:419-428.

532 [39] Biancani B, Da Dalt L, Lacave G, Romagnoli S, Gabai G. 2009. Measuring fecal
533 progestogens as a tool to monitor reproductive activity in captive female bottlenose dolphins
534 (Tursiops truncatus). Theriogenoogy 72(9): 1282-1292.

535 [40] Adams GP, Testa JW, Goertz CE, Ream RR, Sterling JT. 2007. Ultrasonographic
536 characterization of reproductive anatomy and early embryonic detection in the northern fur seal
537 (*Callorhinus ursinus*) in the field. Marine Mammal Science 23(2): 445-452.

538 [41] Thorsteinson FV, Lensink CJ. 1962. Biological observations of Steller sea lions taken
539 during an experimental harvest. The Journal of Wildlife Management 26(4):353-359.

540 [42] Ziegler TE, Snowdon CT. 2000. Preparental hormone levels and parenting experience in
541 male cotton-top tamarins (*Saguinus Oedipus*). Hormones and Behavior 38(3):159-167.

542 [43] Helm RC. 1984. Rate of digestion in three species of pinnipeds. Canadian Journal of
543 Zoology 62:1751-1756.

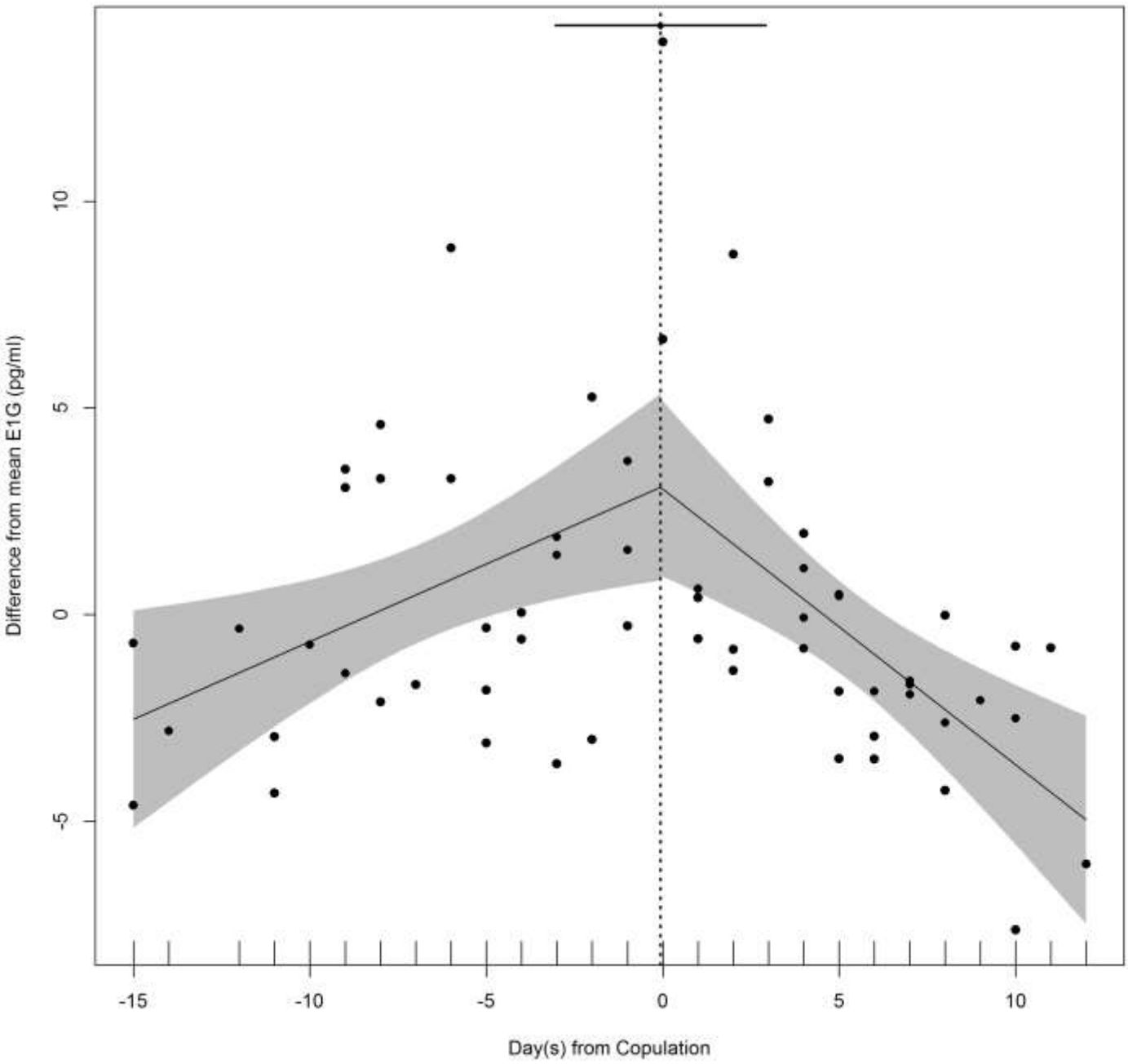
544 [44] Tolliet DJ, Wong M, Winship AJ, Rosen DAS, Trites AW. 2003. Quantifying errors
545 associated with using prey skeletal structures from fecal samples to determine the diet of Steller
546 sea lion (*Eumetopias jubatus*). Marine Mammal Science 19(4):724-744.

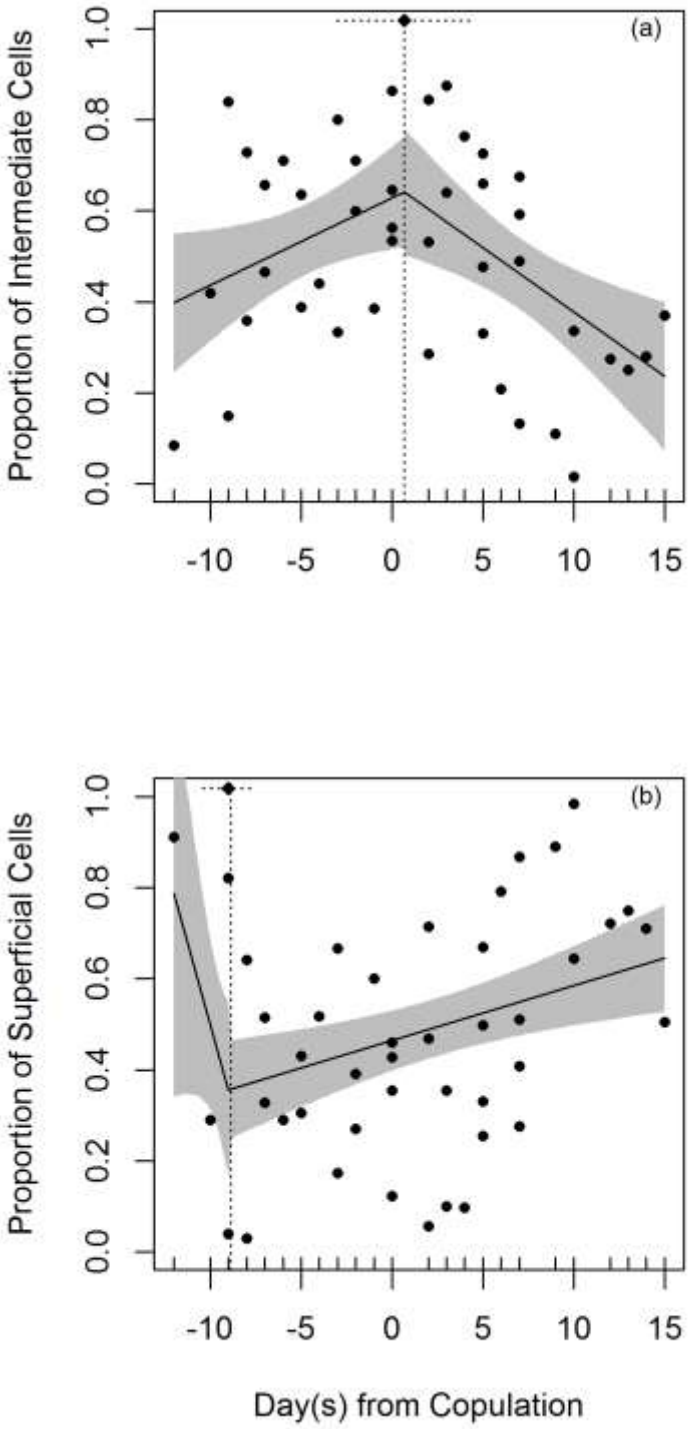
Supplementary Figure 1. Representative trans-abdominal ultrasound images of an adult female Steller sea lions ovaries transitioning from A) Anestrus, B) Proestrus (Follicular Development), C) Proestrus (Formation of a dominant follicle), D) Estrus (Corpus Hemorrhagicum), and E) Diestrus (Corpus Luteum). Panel A shows a dormant ovary during anestrus which is very difficult to differentiate from surrounding tissues, approximately one month before follicular development. Panel B displays the development of multiple round hypoechoic follicles on the left ovary in early proestrus. Panel C shows the presences of a single round anechoic dominant follicle on the dorsal aspect of the associated ovary in late proestrus. Panel D displays an irregularly shaped, primarily anechoic to hypoechoic corpus hemorrhagicum within the dorsal aspect of the ovary at the site of the ruptured follicle. Panel E shows an irregular mottled hyperechoic corpus luteum and a more oval shaped ovary that is more difficult to differentiate from surrounding tissues. Ultrasound images were collected using an Ibex Pro with a CL 3.8 5-2.5MHz 60-mm curved linear array transducer (E.I. Medical Imaging, Loveland, CO) with a permanent 1cm by 1cm grid overlay. The images presented here were selected as the best representative images of each stage of the estrus cycle on a single female.. All panels depict the left ovary .

Figure 1. The change in adult female Steller sea lion (*Eumetopias jubatus*) fecal estrone concentration (E1G) before and after a copulation event (Day = 0). To account for variation in fecal E1G concentration between females over multiple years, each individual data point was differenced from the respective female's mean E1G (pg/ml) calculated for that year. Breakpoints (black dashed vertical line), identify the day relative to estrus where the relationship's slope

shifted (95% CI: black horizontal line around breakpoint). Slope of regression before and after the breakpoint are represented as black solid lines with 95% confidence interval shaded.

Figure 2. Changes in vaginal cytology, (a) proportion of intermediate cells and (b) proportion of superficial cells, relative to observed copulation (Day = 0). Breakpoints (black dashed vertical line), identify the day relative to estrus where the relationship's slope shifted (95% CI: black dashed horizontal line around breakpoint). Slope of regression before and after the breakpoint are represented as black solid lines with 95% confidence interval shaded.


Difference from mean E1G (pg/ml)
10
5
0
-5
-15
-10
-5
0
5
10
Day(s) from Copulation


(a)
Proportion of Intermediate Cells
0.0
0.2
0.4
0.6
0.8
1.0
-10
-5
0
5
10
15

(b)
Proportion of Superficial Cells
0.0
0.2
0.4
0.6
0.8
1.0
-10
-5
0
5
10
15
Day(s) from Copulation

Table 1. For the detection of estrus using a fecal steroid and vaginal cytology: the date range of sample collections (15 days pre and post the day copulation was observed) for three female Steller sea lions (ID), and the number of each type of sample collected during that window by year. For transabdominal ultrasounds to monitor follicular activity and estrus: the window during which scans were collected and the number of sessions in that window by year. The day of parturition is listed for animals that were pregnant at the start of the reproductive season and the day copulation was observed.

Female ID	Sampling Year	Day of Parturition	Day of Copulation	Fecal and Vaginal Swab Sampling Window	Fecal Samples (n)	Vaginal Cytology Samples‡ (n)	Trans-abdominal Ultrasound Sampling Window	Trans-abdominal Ultrasound Sessions (n)
004	2013	Jun-20	Jul-2	Jun-17 : Jul-17	13	--		--
004	2016	Jul-1	Jul-13	Jun-28 : Jul-28	18	13		
011	2016	Jun-24*	Jul-8	Jun-23 : Jul-23	15	11		
004	2014	Jul-20	Aug-1	Jul-17 : Aug-16	11	7	Aug-1 : Oct 30	6
004	2012		May-24	May-9 : Jun-8	5	--		--
005	2015		Jun-10	May-26 : Jun-25	--	12	Aug 4 : Aug 19	10
004	2015				--	--	Jul 13 : Aug 6	18
005	2014				--	--	Jun 12 : Jul 1	7
011	2015				--	--	Jun 26 : Aug 24	29

**indicates the pup was born naturally but was dead at birth*

‡ *vaginal cytology samples were all collected post parturition for cases where females were pregnant*

Table 2. The efficacy of transabdominal ultrasounds to identify (yes or no) defined phases of ovarian activity; Follicle Development (FD), Dominant Follicle (DF), Corpus Hemorrhagicum (CH), and Corpus Luteum (CL) for three Steller sea lions by year (Year – ID). For each defined phase, if the preceding developmental phase was detected (y) then, the days from that phase were listed to provide a relative chronology of ovarian activity through the onset of estrus. The initial date of detection (m/dd) and the Day(s) from the previous phase reflect the earliest each morphological change was detected and thus the days between successive phase may be an overestimation as events may have occurred before our ability to detect it.

	FD		DF		CH		CL	
Year - ID	Detection	Date	Detection	Day(s)	Detection	Day(s)	Detection	Day(s)
2014 – 005	y	6/12	y	4	y	4	y	11
2014 – 004	y	8/12	n	-	n	-	y	-
2015 – 005	y	8/06	y	2	n	-	y	-
2015 – 004	y	7/13	y	5	y	4	y	16
2015 – 011	y	6/26	n	-	n	-	y	-
2016 – 011	y	7/01	y	2	n	-	y	-

Table 3. Results of the continuous segment analysis to assess the change in relative fecal E1G (pg/ml), and iterative piecewise analysis of the proportion of intermediate vaginal cells and proportion of superficial vaginal cells relative to the day(s) from copulation (-15 to 15, copulation = Day 0) in three adult female Steller sea lions. This analysis identified breakpoints (95% CI) in the relationships, and quantified the slope of each linear regression before and after the identified breakpoints. Relationships were determined to be significant if the lower and upper 95% confidence intervals did not include zero.

	Break Point	95% CI lower	95% CI upp	Slope		95% CI lower	95% CI upp	Sig
				Before	0.375	0.089	0.661	Yes
ΔE1G (pg/ml)	-0.06	-3.05	2.92	After	-0.667	-1.007	-0.328	Yes
				Before	0.019	0.001	0.038	Yes
Intermediate	0.68	-3.01	4.39	After	-0.028	-0.047	-0.010	Yes
				Before	-0.144	-0.318	0.030	No
Superficial	-8.99	-10.41	-7.59	After	0.012	0.004	0.020	Yes