Title

Longitudinal patterns in progesterone metabolites in pregnant and non-pregnant Steller sea lions (Eumetopias jubatus)

Journal

General and Comparative Endocrinology, 326(J. Reprod. Fertil. 91 2 1991)

ISSN

0016-6480

Authors

Legacki, Erin
Sattler, Renae
Conley, Alan

Publication Date

2022-09-01

DOI

10.1016/j.ygcen.2022.114069

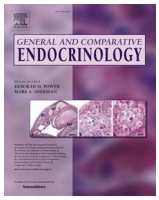
Peer reviewed

General and Comparative Endocrinology 326 (2022) 114069

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Longitudinal patterns in progesterone metabolites in pregnant and non-pregnant Steller sea lions (*Eumetopias jubatus*)

Erin Legacki [a,b,*], Renae Sattler [c,d], Alan Conley [a]

[a] School of Veterinary Medicine, University of California Davis, Davis, CA 95616, United States
[b] National Cold Water Marine Aquaculture Center, USDA, Franklin, ME 04469, United States
[c] Alaska Department of Fish and Game, Palmer, AK 99645, United States
[d] Alaska SeaLife Center, 301 Railway Avenue, Seward, AK 99664, United States

ARTICLE INFO

Keywords:
Steller sea lion
Pregnanes
Gestation
Pinniped
Reproduction

ABSTRACT

Similar to the several pinniped and a few terrestrial carnivore species, the Steller sea lion has a seasonal synchronized mating scheme enabled by a female reproductive cycle that includes embryonic diapause, delayed implantation, and pseudopregnancy (a state in which the corpus luteum produces progesterone for approximately as long as in pregnant females). Due to this, circulating systemic progesterone concentrations cannot be used to differentiate pregnant and nonpregnant females during early gestation. With the use of advanced measurement technologies such as liquid chromatography tandem mass spectrometry (LC-MS/MS) additional steroid hormones are measurable which can provide additional information on the endocrine pathways throughout gestation. Our objectives were to further characterize endocrine patterns in female Steller sea lion pregnancy by 1) quantifying longitudinal profiles of hormone metabolites in pregnant and non-pregnant female sera, and 2) evaluating hormone profiles to identify pregnant animals within the early stage of gestation. Three gestation stages were delineated based on what is believed to be the period of implantation (September-October): EARLY (August– November), MID (December-February), and LATE (March to May). Five steroids, Progesterone (P_4), 5α-dihydroprogesterone (DHP), 17αOH-progesterone (17OHP), 20αOH-progesterone (20OHP), and androstenedione (A_4), were detected in both pregnant and non-pregnant animals. A significant difference in P_4 concentrations was measured between EARLY and MID gestation ($p \leq 0.01$) in both pregnant and non-pregnant animals. During MID gestation there was a significant difference ($p \leq 0.05$) between pregnant and non-pregnant animals in all pregnanes measured. Significant patterns of correlation between P_4 and 17OHP and between P_4 and DHP were detected during EARLY and MID gestation in non-pregnant animals. While those significant correlations also exist in EARLY pregnant animals, this pattern was lost by MID gestation. This loss of correlation suggests a potential shift in progesterone metabolism from ovarian to alternative tissue (e.g. fetal gonads or adrenal glands) by MID gestation in Steller sea lions. We were unable to identifying a steroid hormone biomarker capable of differentiating pseudopregnancy from pregnant animals and conclude that such a biomarker likely falls outside of the traditional progesterone metabolic pathway.

1. Introduction

The majority of pinniped species have highly seasonal synchronized mating schemes enabled by a reproductive cycle with several defining features including: embryonic diapause, delayed implantation, and pseudopregnancy (Boyd, 1991a; Cassini, 1999). Globally, pinniped species have undergone large-scale declines (>70% since the 1970's) from historical levels, and as a group, have the highest variability in recovery success when compared to all marine mammal species (Magera et al., 2013). As a result, reproductive success among pinnipeds has become a matter of concern and an area of increased research. The largest of the otariids, Steller sea lions (*Eumetopias jubatus*), were listed as threatened under the Endangered Species Act in 1990 (55 FR 29793), following significant population declines (>80%) that began in the 1970 s. While the reproductive biology of female Steller sea lions has been well described (Perlov, 1971; Pitcher and Calkins, 1981; Pitcher et al., 1998; Pitcher et al., 2001; Jenison, 2016; Hastings et al., 2017), very few studies have characterized the associated endocrinology of the

* Corresponding author.
E-mail address: erin.legacki@usda.gov (E. Legacki).

https://doi.org/10.1016/j.ygcen.2022.114069
Received 17 November 2021; Received in revised form 17 May 2022; Accepted 2 June 2022
Available online 6 June 2022
0016-6480/Published by Elsevier Inc.

female reproductive cycle (Sattler and Polasek, 2017; Sattler et al., 2018). In general, Steller sea lions reach reproductive maturity at approximately 4.6 years of age, with pupping and breeding occurring in mid-May to mid-July (Pitcher and Calkins, 1981). Like most Otariids, after breeding, pregnant Steller sea lion females undergo an approximate 3-month delayed implantation (Pitcher and Calkins, 1981), which allows for synchronicity of births ensuring that pups are born at the optimal time of year for weather, prey abundance, and general availability of resources necessary for maximal survival of offspring (Boyd, 1991a). Implantation of the conceptus occurs late September – October and is followed by an approximate 8-month active gestation (Pitcher and Calkins, 1981). Adult females that do not become pregnant have a prolonged increase in circulating progesterone concentrations (pseudopregnancy) which is endocrinologically indistinguishable from pregnant females (Sattler and Polasek, 2017) when using traditional single-steroid immunoassays to measure hormone concentrations.

While systemic progesterone concentration cannot be used to differentiate pregnancy from pseudopregnancy in pinnipeds, it is not the sole physiologically significant pregnane (a C21 steroid hormone) measurable throughout gestation. Traditionally, progesterone was thought to be the only pregnane responsible for preparing and maintaining the uterine environment for implantation and fetal development. While it is the initial pregnane synthesized after ovulation by the corpus luteum, in many mammals after implantation and subsequent placental development, progesterone is metabolized by placental enzymes into many other progestogenic compounds. Significant metabolism of progesterone occurs in the placenta of other marine mammals including three species of toothed whales (Legacki et al., 2020) producing a suite of pregnane metabolites (Fig. 1). Measurements of these metabolites may be used to define developmental transitions in steroid synthesis throughout gestation.

Traditionally, the measurement of steroid hormones is conducted through immunoassays, which use antibodies raised to a compound of interest for detection. Since steroid hormones are extremely similar in structure, it is questionable if antibodies can reliably differentiate between multiple like compounds for accurate measurement (Wynn et al., 2018). In addition, hormones act in concert with each other, and accurate measurement of multiple compounds (ideally in a single assay) is needed to visualize endocrine pathways responsible for various physiologies. In general, immunoassays are designed to measure a single hormone at one time but liquid chromatography tandem mass spectrometry (LC-MS/MS) can accurately and reliably identify and measure, with specificity, multiple compounds in a single assay. The combined specificity and multiplexing capabilities of LC-MS/MS methodology allows the measurement of multiple hormones and their longitudinal patterns throughout a reproductive cycle. These patterns will help endocrinologically characterize the stages of the pinniped reproductive cycle and may identify differences between pseudopregnant and pregnant animals.

Our objectives were to further delineate endocrine profiles

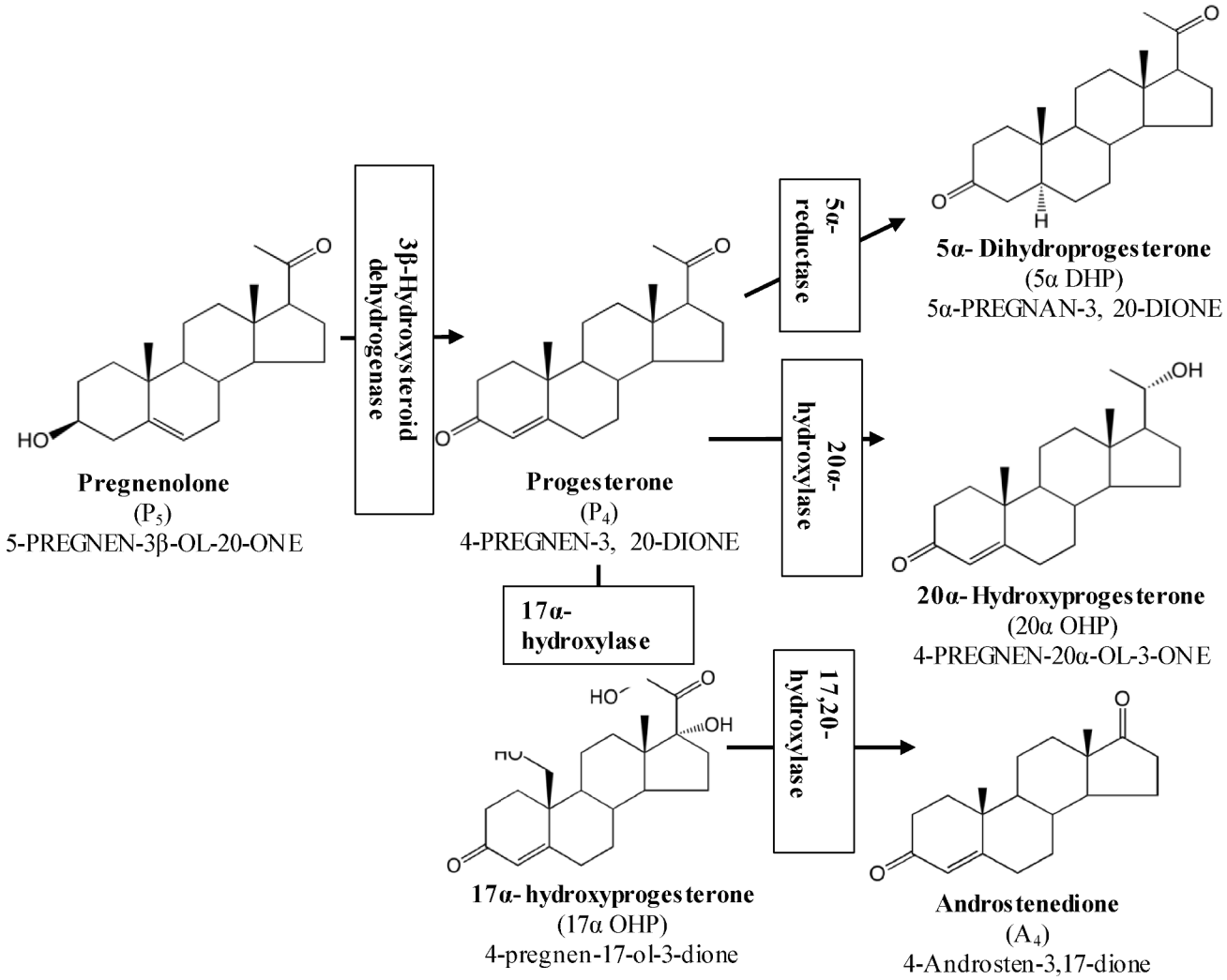


Fig. 1. A schematic representation of progesterone metabolism by three main steroidogenic enzymes 1) 5α-reductase, 2) 20α-hydroxylase and 3) 17α-hydroxylase.

throughout Steller sea lion gestation by 1) quantifying longitudinal profiles of hormone metabolites in pregnant and non-pregnant female sera, and 2) evaluating hormone profiles during early gestation for an early pregnancy biomarker.

2. Methods

2.1. Study animals

Three captive adult female Steller sea lions held at the Alaska SeaLife Center, Seward, AK were sampled from 2011 to 2016 (Table 1). In 2011, sample collection began with two nulliparous females, EJ00004 and EJ0005, who were 11 years old. Female EJ03011, a nulliparous 12-year-old, was brought into the study in 2015. Sea lions were housed in an indoor/outdoor public display enclosure, exposed to seasonal variations in temperature and natural light. Beginning in May, non-pregnant females were housed with the breeding male until copulation was observed. Parturient females had daily exposure to the male seven days after pupping until copulation was observed. Four pregnancies occurred during our study; EJ00004 had two live birth pups, and EJ00005 and EJ03011 each had a stillborn pup carried to full term. The hormone profiles from the females with still-births pregnancies were included in the study because the pups were confirmed alive within 1–5 days before birth through ultrasound, which detected fetal heart rate, pup movement, and found the pups to be fully developed with no obvious abnormalities. Therefore, we did not have any evidence to suggest these pregnancies represented abnormal hormone profile throughout gestation.

2.2. Sample collection

Blood was collected from our three study animals at varying frequency from 2011 to 2016 and in total represented four pregnancies and five pseudopregnancies (Table 1). Females identified as non-pregnant by ultrasound imaging in January were assumed to not have undergone fertilization, implantation, or resorption. In general, blood was collected every other month, August – May, drawn from the caudal gluteal or rear flipper interdigital veins under veterinary supervised general anesthesia (National Marine Fisheries Service Permit No. 18534, ASLC IACUC R12-03-02, (Sattler and Polasek, 2017)). Blood was collected into non-gel clot activator red top tubes (Greiner BioOne Monroe, NC, USA), cooled for a minimum of 30 min, and then spun at 3500 rpm for 5 min, and the serum extracted and stored at −80° C until hormone analysis.

2.3. Hormone measurement

Steller sea lion sera were analyzed for 8 pregnanes (5α-dihydroprogesterone, allopregnanolone, 5α-pregnan-3β, 20α-diol, 20α dihydroprogesterone, pregnenolone, progesterone, 17αOH-progesterone, 20αOH-progesterone), 4 androgens (androstenedione, testosterone, 5α-dihydrotestosterone, dehydroepiandrosterone) and two estrogens (19-norandrostenedione, estrone) using LC-MS/MS. Standards were purchased from Steraloids (Newport, RI): 5α-dihydroprogesterone (5α-pregnan-3, 20-dione, DHP), allopregnanolone (5α-pregnan-3α-ol-20-one or 3αDHP), 5α-pregnan-3β, 20α-diol (3β,20αDHP), 5α- pregnan-20α-ol-3 one (20αDHP), pregnenolone, progesterone, 17αOH-progesterone (17OHP), 20αOH-progesterone (20OHP), androstenedione (A_4), testosterone, 5α-dihydrotestosterone, 19-norandrostenedione, dehydroepiandrosterone (DHEA). Internal standards were purchased from Cerilliant (Round Rock, TX): d_7-androstenedione (A_4- d_7), d_3-testosterone (T-d_3) and d_7-progesterone (P_4- d_7). A mixture of all reference standards was prepared and diluted using HPLC grade water and methanol (Burdick and Jackson, Muskegon, MI) in 10, 1, 0.1 and 0.01 ng/ml concentrations. Formic acid and methyl-tert buytl ether were of ACS grade and obtained from EMD (Gibbstown, NJ).

2.4. Steroid analysis

A previously described LC-MS/MS method was used to quantify hormone profiles in Steller sea lion sera. This method was previously described and validated for equine serum/plasma (Legacki et al., 2016) to detect progesterone, pregnenolone, DHEA, A_4, testosterone, DHT, 17OHP, 20OHP, DHP, 3αDHP, 20αDHP, and 3β,20αDHP. No extra Steller sea lion sera was available to be used for method optimization but there were no matrix effects detected when using equine sera. In brief, 100 μL of internal standard mix (A_4-d_7, T-d_3 and P_4-d_7) was added to all serum samples, stored at 4 ∘C for 10 min to equilibrate, and extracted with methyl-tert buytl ether (1:5). Calibrators were prepared alongside the samples and ranged from 0.1 ng/mL–100 ng/ml with four levels of quality controls (QC; 0.6 ng/mL, 1.5 ng/mL, 20 ng/mL, and 80 ng/mL) which consisted of known standard amounts spiked into charcoal stripped serum. Sample and calibrator supernatant were transferred into 12 × 75 mm glass tubes and dried with a Zymark Turbovap concentrator (Hopkinton, MA) at 45 °C under N_2 and reconstituted with 200 μL of 50:50 mix of water and methanol by volume. The reverse-phase gradient separation was performed on an Agilent UHPLC C18 analytical column (2.1 mm × 50 mm, 1.8 μm pore size) with two mobile phases delivered at 0.4 mL / min, an injection volume of 20 μL and a column temperature of 40 °C. Mobile phase A and B were water (+0.2% formic acid) and methanol (+0.2% formic acid), respectively. An elution gradient was held at 40% B for the first 0.2 min, 40% – 60% B from 0.2 min to 1 min, 60% – 80% B from 1 min to 10 min, 80% – 90% B from 10.0 min to 10.1 min, held at 90% B from 10.1 min to 11.1 min, 90% – 40% from 11.1 min to 11.2 min and held at 40% B until 13.10 min. Ionization utilized an atmospheric-pressure chemical ionization (APCI) source. Tandem mass spectral detection was accomplished using a Bruker EVOQ (Bruker Daltronics Inc., Billerica, MA). Detection and quantitation of all analytes were accomplished using scheduled multiple reaction monitoring with a minimum of two transitions per analyte. All analytes measured were ≤15% deviation from expected concentrations for the three highest QC concentrations (1.5 ng/mL, 20 ng/mL, and 80 ng/mL) with a ≤15% coefficient of variation (% CV). For the lowest QC concentration (0.6 ng/mL) pregnenolone, 20αDHP and 3β,20αDHP had ≤20% deviation from expected concentrations and all other analytes

Table 1
The dates of breeding and pupping for the three captive Steller sea lions participating in this study and totaling 4 pregnancies and 5 non-pregnant cycles.

	2012	2013		2014		2015		2016
Animal ID	Bred	Pupped	Bred	Pupped	Bred	Pupped	Bred	Pupped
EJ00004	5/24	6/20	7/2	7/20	8/1	Not Pregnant	Not Observed	7/1
EJ00005	6/8	5/27 *	6/9	Not Pregnant	Not Observed	Not Pregnant	6/10	Not Pregnant
EJ03011							7/2	6/24 *

* Still Born
Animal was not involved in the reproductive program

had ≤15% deviation. All analytes measured had a percent accuracy (percentage of the nominal concentration) of >90%. Analyte precision, percent relative standard deviation was <15%. The responses for all analytes were linear with correlation coefficients (R^2) of > 0.99.

2.5. Data analysis

To compare steroid concentrations between bred but not pregnant and pregnant Steller sea lions, the nonbreeding season (August–June, Table 1) was divided into EARLY, MID, and LATE luteal/gestational stages, based on the believed period of implantation (prior to October; Pitcher and Calkins, 1981). Therefore, sera samples from across our animals were categorized by reproductive status (pregnant and non-pregnant) and collection date. Serum samples collected in August – November were representative of the luteal phase in a non-pregnant female (n = 11) and the pre-implantation stage of pregnancy (n = 7) and referred to as EARLY. Samples collected in December – February represented a non-reproductive phase in non-pregnant females (n = 6) and the embryonic stage in pregnant females (n = 11) and referred to as MID. Blood collected March – May represented the fetal stage in pregnancy (n = 9), referred to as LATE and we did not have any samples during this time period in non-pregnant females. Differences in steroid concentrations between reproductive status and between luteal/gestational stages (EARLY, MID, and LATE) were assessed using a repeated measures standard least squares fit model and linear regression in JMP (Cary, NC). To visualize longitudinal patterns in steroids throughout gestation, all samples from pregnant females were grouped into 50-day bins and presented as means ± standard error of the mean (SEM). Pearson's correlation co-efficient were determined using multivariate analysis (JMP) and $p \leq 0.05$ was considered significant. Data from the pregnant Steller sea lions during late gestation in this study were recently published in a comparative study of hormone profiles (Conley et al., 2021) across marine and terrestrial mammals during late gestation, but the hormone profiles of EARLY and MID-gestation in pregnant, along with the non-pregnant females were not reported.

3. Results

Of the eight pregnanes, five androgens, and two estrogens we tested for, only five steroids were detected: P_4, DHP, 17OHP, 20OHP, and A_4. A comparison of P_4 concentrations derived from LC-MS/MS with concentrations derived from immunoassays used in Sattler and Polasek (2017), were similar and had a significant correlation (Fig. 2A; $r^2 = 0.813$, $p \leq 0.001$). Across the suite of steroids evaluated, P_4 and DHP were present in highest concentrations. P_4 was the major pregnane at all stages in pregnant animals with a maximum of 24.38 ng/mL ± 1.06 ng/mL at MID P and a minimum of 13.82 ng/mL ± 1.67 during EARLY P (Fig. 2B; $p \leq 0.01$). There was a significant difference in P_4 concentrations between EARLY NP and MID NP stages ($p \leq 0.01$) as well as EARLY and MID P ($p \leq 0.01$). P_4 concentrations between NP and P Steller sea lions were significantly different by MID gestation ($p \leq 0.01$). DHP concentrations were not significantly different between NP and P females during EARLY gestation, but significantly differed by MID gestation (Fig. 3A, $p \leq 0.01$). Pregnant animals had significantly different concentrations of DHP between EARLY (4.46 ng/mL ± 0.46 ng/mL) and MID (9.82 ng/mL ± 0.62 ng/mL) gestation ($p \leq 0.01$). Conversely, NP animals did not have significant differences in DHP concentrations between the EARLY (5.19 ng/mL ± 0.34 ng/mL) and MID (3.56 ng/mL ± 0.56 ng/mL) time period. There was no observed difference in 17OHP during EARLY in NP or P animals (Fig. 3B). However, there were significant differences between MID NP and P ($p \leq 0.01$) along with EARLY P and MID P ($p \leq 0.01$). Within P animals, 17OHP had a maximum of 2.46 ng/mL ± 0.26 ng/mL in MID and a minimum of 1.10 ng/mL ± 0.41 ng/mL in LATE. Concentrations of 20OHP were consistently present throughout pregnancy and never reached more than 2 ng/mL at any time during gestation (Fig. 3C). Patterns of differences between 20OHP and sea lion reproductive status and stage were similar to 17OHP, but with an additional significant difference ($p \leq 0.01$) between EARLY NP (0.69 ng/mL ± 0.03 ng/mL) and MID NP (0.35 ng/mL ± 0.14 ng/mL). The only androgen in a measurable range was A_4 which was detectable in some samples throughout pregnancy in all stages (Fig. 3D), but only in one sample from the Early period. P animals did have A_4 concentrations which were significantly different in EARLY (0.2 ng/mL ± 0.2 ng/mL) than both MID (0.15 ng/mL ± 0.03 ng/mL) and LATE (0.09 ng/mL ± 0.04 ng/mL). Comparatively, NP females didn't have significantly differences in A_4 concentrations between EARLY (0.05 ng/mL ± 0.02 ng/mL) and MID (0.08 ng/mL ± 0.03 ng/mL).

When considering the longitudinal profiles of all hormones, P_4, DHP, 20OHP, and A_4 peaked in concentration between 80 days and 130 days before parturition (Fig. 4). 17OHP was the exception and reached peak concentrations between 180 days – 230 days before parturition. Approximately 80 days before parturition, all hormone concentrations began to decrease.

Patterns of correlation between the measured hormones varied with reproductive status and stage of gestation (Table 2). P_4 and 17OHP were correlated in EARLY NP ($r^2 = 0.77$, $p \leq 0.01$) but not in EARLY P (Fig. 5A, r2 = 0.71, p = 0.07). In the MID P stage 17OHP was again, not correlated with P_4 while the correlation remained in MID NP animals

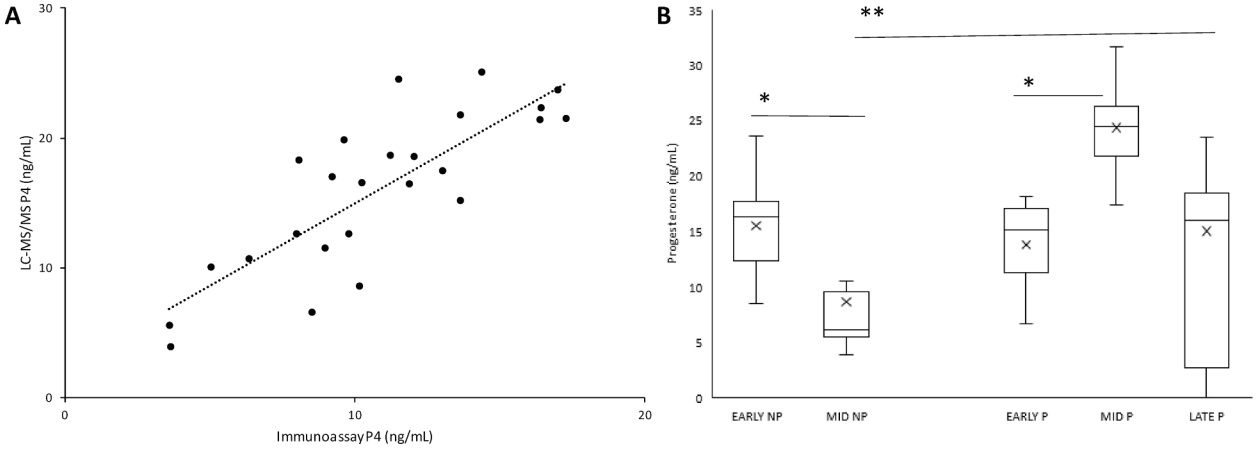


Fig. 2. A) There was a significant correlation ($r^2 = 0.8128$, $p \leq 0.001$) between progesterone concentrations measured by immunoassay by Sattler and Polasek, 2017 and LC-MS/MS generated for this publication. B) Box and whisker plot of progesterone concentrations measured by LC-MS/MS innon-pregnant (NP) and pregnant (P) Steller sea lions. EARLY NP (n = 11), MID NP (n = 6), EARLY P (n = 7), MID P (n = 11), LATE P (n = 9). The X indicates the mean of the samplesand the bar indicates the median of the samples and significant levels were set as ** ≤ 0.01, * ≤ 0.05.

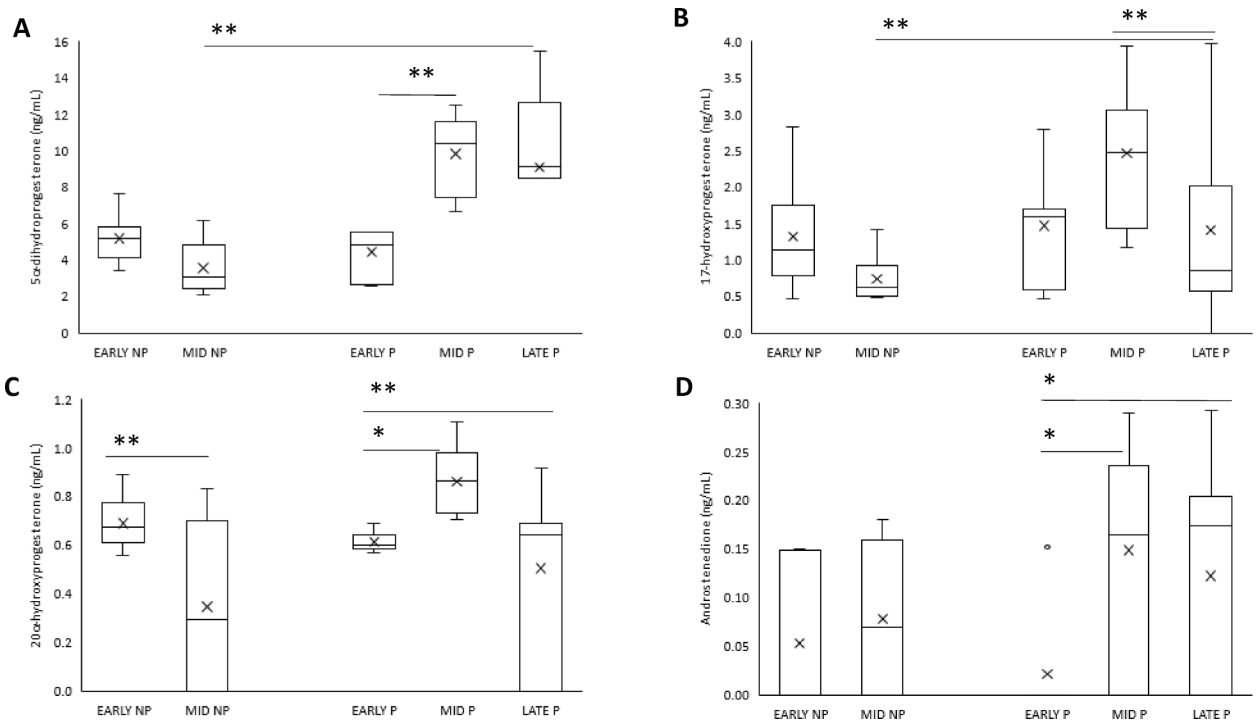


Fig. 3. Box and whisker plots of hormone concentrations during Early, Mid, and Late gestation in pregnant (P) and non-pregnant(NP) Steller sea lions. A) 5α-dihydroprogesterone (DHP), B) 17α-hydroxyprogesterone (17OHP), C) 20α-hydroxyprogesterone (20OHP), and D) androstenedione (A_4), the EARLY P time point had only one sample with detectable A_4. The nondetectable samples were given a concentration of 0.0 ng/mL.Numbers for each plot box are: EARLY NP (n = 11), MID NP (n = 6), EARLY P (n = 7), MID P (n = 11), LATE P (n = 9). The X indicates the mean of the samplesand the bar indicates the median of the samples and significant levels were set as ** ≤ 0.01, * ≤ 0.05.

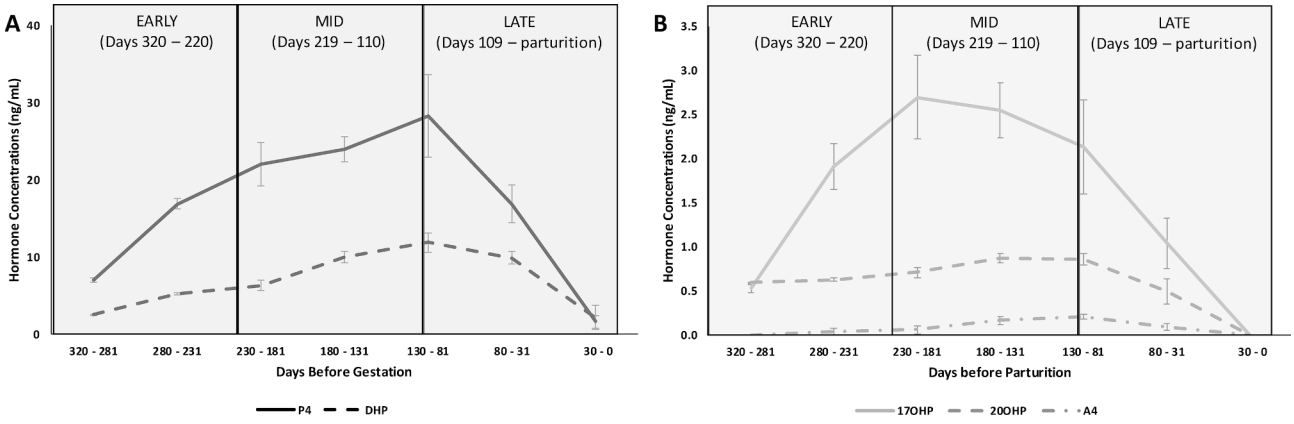


Fig. 4. Steroid hormone values for A) Progesterone (P_4) and 5α dihydroprogesterone (DHP), and B) 17α hydroxyprogesterone (17OHP), 20α hydroxyprogesterone (20OHP), and androstenedione (A_4) from four pregnancies grouping data points into 50-day bins and averaged to show fluctuations throughout the Steller sea lion gestation period. Shaded boxes indicate the EARLY, MID, and LATE stages of the gestation period and day 0 represents parturition.

Table 2
Correlation matrix of Progesterone (P_4), 5α dihydroprogesterone (DHP), 17αhydroxyprogesterone (17OHP), and 20αhydroxyprogesterone (20OHP) in pregnant and nonpregnant Steller sea lions during Early (August – November) and Mid (December- February) gestation. Statistical significance is set at * ≤ 0.05 and ** ≤ 0.01.

Variable	By Variable	EARLY	MID	EARLY	MID
		Pregnant		**Non-Pregnant**	
P4	17OHP	0.77**	0.90**	0.71	0.33
P4	20OHP	0.58	0.81*	0.21*	0.67*
P4	DHP	0.85**	0.90**	0.99**	0.3

(Fig. 5B, r2 = 0.90, p ≤ 0.01). However, a correlation between P_4 and 17OHP began in LATE P animals ($r^2 = 0.88$, p ≤ 0.01, data not shown). Similarly, DHP was highly correlated with P_4 in both EARLY P and EARLY NP animals (Fig. 5C), but this correlation was lost in MID P females yet remained for the MID NP females (Fig. 5D). There were correlations between DHP and 20-OHP in EARLY NP ($r^2 = 0.68$, p ≤ 0.05) and MID NP ($r^2 = 0.90$, p < 0.001), while P animals only exhibited this correlation during MID and LATE periods. Both EARLY P and NP animals had no significant correlation between A_4 and pregnanes measured. However, in MID NP, A_4 was significantly correlated with 17OHP ($r^2 = 0.82$, p ≤ 0.05) but not with the other pregnanes in that stage. MID and LATE term P sea lions had no correlation between pregnanes and A_4.

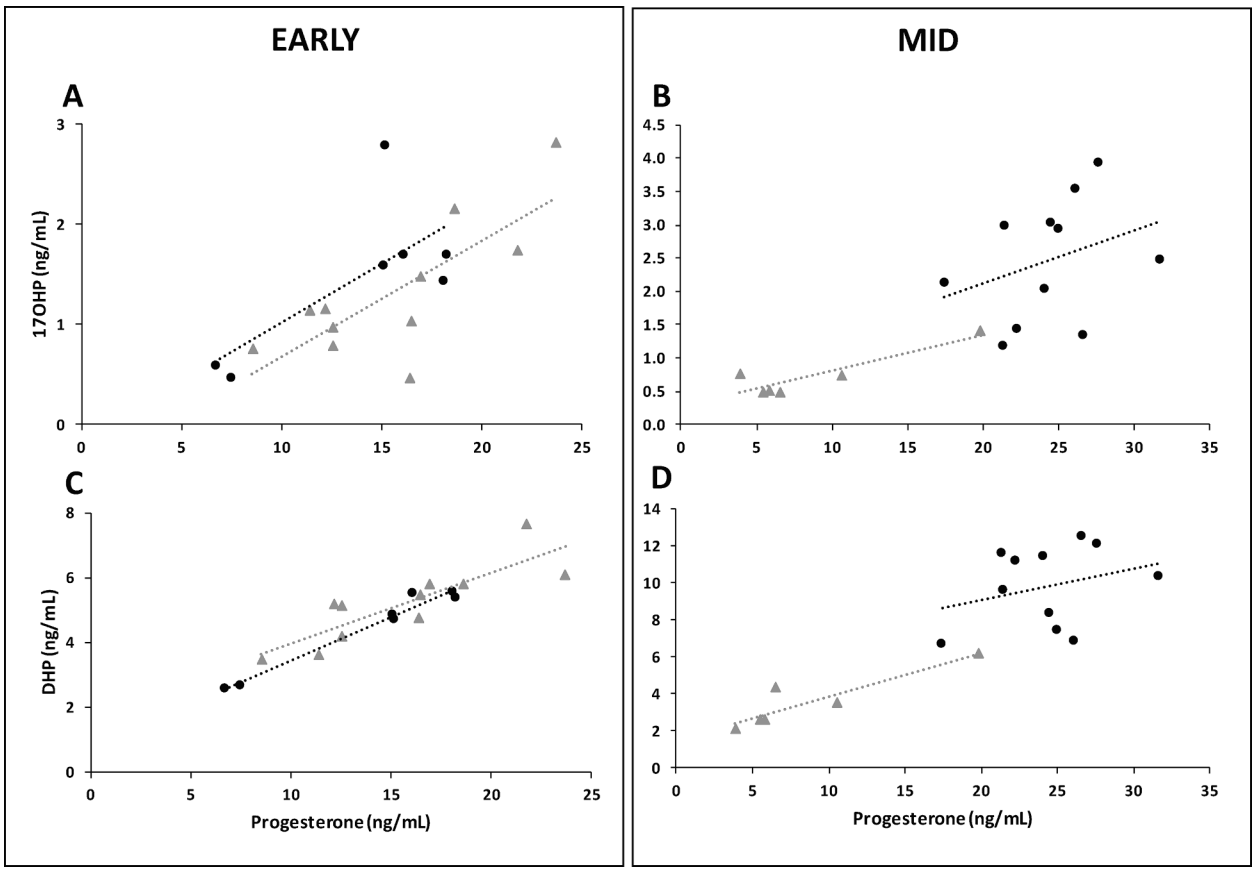


Fig. 5. Linear correlations between EARLY (August – November) and MID (December – February) gestational windows in nonpregnant () and pregnant animals (). A) Correlations between 17OHP and P in EARLY P wasn't significant (NS) and EARLY NP was significant B) Correlations between 17OHP and P_4 in MID P were NS and MID NP were significant. C) Correlations between DHP and P_4 in EARLY P and EARLY NP were both significant, D) Correlations between DHP and P_4 in MID P were NS and MID NP were significant. Significance was ($p \leq 0.05$).

4. Discussion

The primary goal of this study was to quantify pregnane metabolites in pregnant and non-pregnant Steller sea lions with the intent of defining a pattern of hormones unique to varying stages of gestation. While hormone patterns were not significantly different between our temporally defined stages, most notably immediately following implantation, there were key changes which support the need for future investigation. Here we present the most comprehensive longitudinal examination of peripheral sex steroids measured in pregnant and non-pregnant pinnipeds reported to date.

Out of the 8 pregnanes measured using LC-MS/MS, only P_4, DHP, 17OHP, and 20OHP were detected in all samples. Mean P_4, DHP, 17OHP, and 20OHP were all elevated during early pregnancy in both pregnant and ultimately non-pregnant females. The observed elevation in pregnanes, even without an implanted embryo, is a key feature in the reproductive cycle of most pinnipeds (Pomeroy, 2011); Antarctic fur seals (*Arctocephalus gazelle*, Boyd, 1991a); Australian sea lions (*Neophica cinereal*, Tedman, 1991); harbor seals (*Phoca vitulina*, Reijnders, 1990); and harp seals *Pagophilus groenlandicus*, Renouf et al., 1994). Additionally, Shero et al. (2018) found no correlation between estradiol, estrone, progesterone, or relaxin hormone profiles and morphological changes in reproductive structures in Northern fur seals during the re-initiation of embryonic growth (i.e., EARLY P). Like Sattler and Polasek (2017), pregnancy was endocrinologically distinguished at the beginning of the MID gestational period (December) through the measurement of significantly elevated pregnane concentrations. All pregnane concentrations declined close to parturition, a finding corroborating observations made by Sattler and Polasek (2017). This overall decline in pregnane concentrations is similar to that measured in dogs which exhibit peak progesterone concentrations between days 25 and 35 post ovulation and slowly decline until parturition (Papa and Kowalewski, 2020; Hinderer et al., 2021). This decline in progesterone is partially due to a reduction in the luteal steroidogenic acute regulatory (STAR) protein supply of cholesterol, the initial steroidogenic substrate (Kowalewski, 2014). If Luteal STAR protein concentrations were measured throughout gestation in Steller sea lions, it could be determined if the observed pregnane decline nearing parturition is driven by a similar mechanism of action.

While the patterns of P_4 concentrations are similar to those reported in previous pinniped studies, a novel pattern in the changes in 17OHP concentrations throughout gestation emerged. Pregnant animals exhibited an increase in 17OHP during the beginning of MID-gestation (approximately 200 days before birth) and then declined approximately 150 days before parturition. The peak in 17OHP comes prior to the observed peak in P_4, DHP, 20OHP, and A_4 which occurred closer to 130 days before parturition. Horses exhibit a similar early gestational increase in 17OHP which is attributed to the release of chorionic gonadotropin (CG). In horses elevated CG concentrations stimulate secondary ovulation and corpus luteum (CL) formation at approximately 10 weeks of gestation, often referred to as secondary CLs or accessory CLs These structures are correlated with the rise in 17OHP. While CG hasn't been measured in Steller sea lions it has been measured in the placenta of other pinnipeds. In pregnant grey seals (*Halichoerus grypus*) the total amount of placental CG increased significantly from 170 days after conception (approximately 20 days after implantation) until the end of pregnancy (Hobson and Boyd, 1984), which parallels the pattern of 17OHP measured in Steller sea lions. Secondary or accessory CLs have

not been reported in Steller sea lions indicating that CG doesn't induce ovulation post-implantation. Changes in the size and structure of fetal gonads during pinniped gestation suggests these tissues have hormonal production capability *in utero* (Amoroso et al., 1951; Bonner, 1955), potentially due to CG stiumulation. This phenomenon was observed in several seal species including Grey seals, Harbor seals, Northern fur seals (*Callorhinus ursinus*), Weddell seals (*Leptonychotes weddellii*), Crabeater seals (*Lobodon carcinophaga*), Leopard seals (*Hydrurga leptonyx*), and Southern elephant seals (*Mirounga leonine*). Cytochrome P450 17A1 (CYP17), the enzyme responsible for the metabolism of progesterone to 17OHP, was detected in Northern fur seal ovaries (Browne et al., 2006) and suggests that fetal gonads may also have CYP17 and are capable of 17OHP synthesis. Further investigation into the location of 17OHP metabolism could provide more insight into the fetal development of pinnipeds and provide a biomarker for monitoring fetal health.

The only androgen detected in the longitudinal analysis of the Steller sea lion pregnancies was A_4. While A_4, a metabolite of 17OHP, was correlated with 17OHP in MID NP animals it was not correlated with 17OHP in any of the pregnant animal stages. Only one out of seven samples in the EARLY P time period had detectable A_4 compared with EARLY NP animals where four out of ten samples had detectable A_4. All A_4 concentrations measured were close to the limit of detection (0.1 ng/mL) and possibly weren't detected in some samples due to our methods sensitivity. However, the correlation between 17OHP and A_4 in MID NP animals compared with a lack of correlation in MID P animals suggests that the production of these two hormones occurs in different tissues. Previous publications have suggested that DHEA and A_4 are involved in the regulation of delayed implantation in Northern fur seals (Browne et al., 2006), mink (*Mustela-Vison*, Stoufflet et al., 1989), Japanese black bears (*Ursus thibetanus japonicus*, Tsubota et al., 2001), and European badgers (*Meles-Meles*, Mondainmonval et al., 1983). DHEA was not detected in the Steller sea lion samples from this data set and it is possible that any elevated A_4 which may have occurred at implantation was missed in this study due to sampling frequency. To determine if DHEA or A_4 are reliable biomarkers of implantation, pre-implantation samples should be collected from Steller sea lions and analyzed for these and additional androgens. Signals of implantation and the origins of those signal may lead to earlier pregnancy detection.

The assumption that all pregnanes measured during pregnancy are only synthesized in the CL, is held for the majority of carnivores. This assumption is partially true for Steller sea lions, along with other pinnipeds studied, as their CL contains enzymes required for the synthesis of progesterone, a cholesterol side chain cleavage enzyme (P450scc) and 3β-hydroxysteroid dehydrogenase (3 β HSD, Ishinazaka et al., 2001; Ishinazaka et al., 2002) and the placenta does not. However, correlations between hormones, which are used to indicate where hormones are synthesized or metabolized, suggest a different story for Steller sea lions. All non-pregnant sea lions studied had a strong correlation between P_4 and 17OHP concentrations during EARLY NP and MID NP stages. These correlations suggest that 17OHP is metabolized at the same site that P_4 is synthesized, the ovary. However, in pregnant animals there was no correlation between 17OHP and P_4 during EARLY or MID gestation. This lack of correlation suggests that 17OHP production could be from either placenta or fetal tissues but most likely not from the CL. Additionally, in pregnant animals DHP was highly correlated with P_4 during early gestation but this correlation was lost during MID gestation yet persists in non-pregnant animals. At the LATE stage of gestation all the pregnanes measured are correlated with P_4. Collectively, these changes may indicate a shift in the site of P_4 metabolism, a phenomenon which occurs in many mammals but has not been reported in pinnipeds. The Steller sea lion placenta has only been shown to express steroid synthesizing enzyme, aromatase (P450arom, Ishinazaka et al., 2001), but has not been analyzed for CYP17, the enzyme responsible for 17OHP production.

Endocrine similarities between species tends to dominate the field of comparative endocrinology (Wildt et al., 2010). While similarities are important, differences between species could lead to answers for more complicated reproductive questions, such as early pregnancy detection in carnivore species whose reproductive strategy includes delayed implantation and pseudopregnancy. Current literature suggests that carnivore placentas are not able to synthesize progesterone (Ishinazaka et al., 2001; Tsubota et al., 2001; Concannon, 2009), but as the majority of carnivores have not been studied this presumption may be premature. For example, the domestic cat has an increase in placental 3β HSD during late gestation (Siemieniuch et al., 2012) suggesting that the cat placenta begins to produce progesterone for the support of late pregnancy, which is also similar to patterns observed in cattle (Conley et al., 2019). It is also assumed that canids, similar to pinnipeds, all exhibit pseudopregnancy, but New Guinea singing dogs (*Canis dingo hallstromi*) don't have prolonged progesterone as they resume estrus if the previous copulation didn't result in pregnancy (Koler-Matznick et al., 2003; Nagashima and Songsasen, 2021). Advanced technologies, such as LC-MS/MS, can provide a more complete view of species' reproductive endocrinology and give insight into unique gestation pathologies previously undetected.

Measuring pregnane concentrations throughout Steller sea lion pregnancy provided insight into circulating pregnanes responsible for the support of gestation. While we were not able to differentiate early pregnancies from pseudopregnant animals, these data suggest that identifying a hormone biomarker capable of differentiating pregnancy likely falls outside of the traditional P_4 metabolic pathway and that fetal tissue could play a role in progesterone metabolism. Future work should investigate steroidogenic enzymes in fetal gonads and adrenal glands, as well as additional enzymes which may be expressed in the placenta to broaden the scope and source of biomarker investigations in Steller sea lions.

Funding

Funding was provided National Oceanic and Atmospheric Administration, Grant number: NA12NMF4390066.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank staff at the Alaska SeaLife Center for providing animal care, training, and veterinary care: all of which made this work possible. A special thanks goes out to Terril Efird and Brandon Russell for assistance in sample acquisition and processing and Dr. Lori Polasek and Brett Long for their role in building the foundational Steller sea lion research project that this work was built on. The authors would like to express their gratitude to the technical staff at the Equine Analytical Chemistry Laboratory, School of Veterinary Medicine, University of California, Davis, especially the support of Dr. Ben Moeller, Dr. Heather Knych, Daniel McKemie, Teresa Bowers, Dr. Go Sugiarto and Sandy Yim, who provided expertise, technical support and guidance for analysis of samples by liquid chromatography tandem mass spectroscopy.

References

Amoroso, E.C., Harrison, R.J., Matthews, L.H., Rowlands, I.W., 1951. Reproductive organs of near-term and new-born seals. Nature 168 (4279), 771–772.
Bonner, W.N., 1955. Reproductive organs of foetal and juvenile elephant seals. Nature 176 (4490), 982–983.
Boyd, I.L., 1991a. Environmental and physiological factors controlling the reproductive-cycles of pinnipeds. Can. J. Zool. 69 (5), 1135–1148.
Browne, P., Conley, A.J., Spraker, T., Ream, R.R., Lasley, B.L., 2006. Sex steroid concentrations and localization of steroidogenic enzyme expression in free-ranging

female northern fur seals (Callorhinus ursinus). Gen. Comp. Endocr. 147 (2), 175–183.
Cassini, M.H., 1999. The evolution of reproductive systems in pinnipeds. Behav. Ecol. 10 (5), 612–616.
Concannon, P.W., 2009. Endocrinologic control of normal canine ovarian function. Reprod. Domest. Anim. 44 (Suppl 2), 3–15.
Conley, A.J., Legacki, E.L., Corbin, C.J., Stanley, S., Dahlen, C.R., Reynolds, L.P., 2019. Serum and tissue pregnanes and pregnenes after dexamethasone treatment of cows in late gestation. Reproduction 157 (5), 413–422.
Conley, A.J., Loux, S.C., Legacki, E.L., Stoops, M.A., Pukazhenthi, B., Brown, J.L., Sattler, R., French, H.M., Tibary, A., Robeck, T.R., 2021. The steroid metabolome of pregnancy in Perissodacyla, Cetartiodactyla and Carnivora. Reprod. Domest. Anim. 56, 5.
Hastings, K.K., Jemison, L.A., Pendleton, G.W., Raum-Suryan, K.L., Pitcher, K.W., 2017. Natal and breeding philopatry of female Steller sea lions in southeastern Alaska. PLoS ONE 13 (4), 1–17.
Hinderer, J., Lüdeke, J., Riege, L., Haimerl, P., Bartel, A., Kohn, B., Weber, C., Müller, E., Arlt, S.P., 2021. Progesterone concentrations during canine pregnancy. Animals (Basel) 11 (12), 3369.
Hobson, B.M., Boyd, I.L., 1984. Gonadotropin and progesterone concentrations in placentae of grey seals (Halichoerus-Grypus). J. Reprod. Fertility 72 (2), 521–528.
Ishinazaka, T., Suzuki, M., Mizuno, A.W., Harada, N., Mason, J.I., Ohtaishi, N., 2002. Immunohistochemical localization of steroidogenic enzymes and prolactin receptors in the corpus luteum and placenta of spotted seals (Phoca largha) during late pregnancy. J. Vet. Med. Sci. 64 (4), 329–333.
Ishinazaka, T., Suzuki, M., Yamamoto, Y., Isono, T., Harada, N., Mason, J.I., Watabe, M., Tsunokawa, M., Ohtaishi, N., 2001. Immunohistochemical localization of steroidogenic enzymes in the corpus luteum and the placenta of the ribbon seal (Phoca fasciata) and steller sea lion (Eumetopias jubatus). J. Vet. Med. Sci. 63 (9), 955–959.
Koler-Matznick, J., Brisbin, I.L., Feinstein, M., Bulmer, S., 2003. An updated description of the New Guinea singing dog (Canis hallstromi, Troughton 1957). J. Zool. 261 (2), 109–118.
Kowalewski, M.P., 2014. Luteal regression vs. prepartum luteolysis: regulatory mechanisms governing canine corpus luteum function. Reprod. Biol. 14 (2), 89–102.
Legacki, E.L., Robeck, T.R., Steinman, K.J., Conley, A.J., 2020. Comparative analysis of steroids in cyclic and pregnant killer whales, beluga whales and bottlenose dolphins by liquid chromatography tandem mass spectrometry. Gen. Comp. Endocr. 285, 113273.
Legacki, E.L., Scholtz, E.L., Ball, B.A., Stanley, S.D., Berger, T., Conley, A.J., 2016. The dynamic steroid landscape of equine pregnancy mapped by mass spectrometry. Reproduction 151 (4), 421–430.
Magera, A.M., Mills Flemming, J.E., Kaschner, K., Christensen, L.B., Lotze, H.K., Stergiou, K.I., 2013. Recovery trends in marine mammal populations. PLoS ONE 8 (10), e77908.
Mondainmonval, M., Bonnin, M., Scholler, R., Canivenc, R., 1983. Plasma androgen patterns during delayed implantation in the european badger (Meles-Meles L). Gen. Comp. Endocr. 50 (1), 67–74.
Nagashima, J.B., Songsasen, N., 2021. Canid reproductive biology: norm and unique aspects in strategies and mechanisms. Animals (Basel) 11 (3), 653.
Papa, P.C., Kowalewski, M.P., 2020. Factors affecting the fate of the canine corpus luteum: potential contributors to pregnancy and non-pregnancy. Theriogenology 150, 339–346.
Perlov, A.S., 1971. The onset of sexual maturity in sea lions. Proc. All Union Inst Mar. Fish. Oceanogr. 80, 174–187.
Pitcher, K.W., Burkanov, V.N., Calkins, D.G., Le Boeuf, B.J., Mamaev, E.G., Merrick, R.L., Pendleton, G.W., 2001. Spatial and temporal variation in the timing of births of Steller sea lions. J. Mammal. 82 (4), 1047–1053.
Pitcher, K.W., Calkins, D.G., 1981. Reproductive-biology of steller sea lions in the Gulf of Alaska. J. Mammal. 62 (3), 599–605.
Pitcher, K.W., Calkins, D.G., Pendleton, G.W., 1998. Reproductive performance of female Steller sea lions: an energetics-based reproductive strategy? Can. J. Zool. 76 (11), 2075–2083.
Pomeroy, P., 2011. Reproductive cycles of marine mammals. Anim. Reprod. Sci. 124 (3–4), 184–193.
Reijnders, P.J.H., 1990. Progesterone and estradiol-17-beta concentration profiles throughout the reproductive-cycle in Harbor Seals (Phoca-Vitulina). J. Reprod. Fertil. 90 (2), 403–409.
Renouf, D., Taylor, R., Gales, R., 1994. Pseudopregnancy in harp seals (Phoca-Groenlandica). J. Reprod. Fertil. 101 (1), 31–36.
Sattler, R., Bishop, A., Woodie, K., Polasek, L., 2018. Characterizing estrus by trans-abdominal ultrasounds, fecal estrone-3-glucuronide, and vaginal cytology in the Steller sea lion (Eumetopias jubatus). Theriogenology 120, 25–32.
Sattler, R., Polasek, L., 2017. Serum estradiol and progesterone profiles during estrus, pseudopregnancy, and active gestation in Steller sea lions. Zoo Biol 36 (5), 323–331.
Shero, M.R., Bergfelt, D.R., Testa, J.W., Adams, G.P., 2018. Pairing ultrasonography with endocrinology to elucidate underlying mechanisms of successful pregnancy in the northern fur seal (Callorhinus ursinus). Gen. Comp. Endocr. 255, 78–89.
Siemieniuch, M.J., Jursza, E., Szostek, A.Z., Skarzynski, D.J., Boos, A., Kowalewski, M.P., 2012. Steroidogenic capacity of the placenta as a supplemental source of progesterone during pregnancy in domestic cats. Reprod. Biol. Endocrin. 10 (1), 89.
Stoufflet, I., Mondainmonval, M., Simon, P., Martinet, L., 1989. Patterns of plasma progesterone, androgen and estrogen concentrations and invitro ovarian steroidogenesis during embryonic diapause and implantation in the mink (Mustela-Vison). J. Reprod. Fertility 87 (1), 209–221.
Tedman, R.A., 1991. The female reproductive-tract of the Australian Sea Lion, Neophoca-Cinerea (Peron, 1816) (Carnivora, Otariidae). Aust. J. Zool. 39 (3), 351–372.
Tsubota, T., Taki, S., Nakayama, K., Mason, J.I., Kominami, S., Harada, N., Kita, I., 2001. Immunolocalization of steroidogenic enzymes in the corpus luteum and placenta of the Japanese black bear, Ursus thibetanus japonicus, during pregnancy. Reproduction 121 (4), 587–594.
Wildt, D.E., Comizzoli, P., Pukazhenthi, B., Songsasen, N., 2010. Lessons from biodiversity–the value of nontraditional species to advance reproductive science, conservation, and human health. Mol. Reprod. Dev. 77 (5), 397–409.
Wynn, M.A.A., Esteller-Vico, A., Legacki, E.L., Conley, A.J., Loux, S.C., Stanley, S.D., Curry Jr., T.E., Squires, E.L., Troedsson, M.H., Ball, B.A., 2018. A comparison of progesterone assays for determination of peripheral pregnane concentrations in the late pregnant mare. Theriogenology 106, 127–133.

Further reading

Boyd, I.L., 1991b. 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 gazella). J. Reprod. Fertil. 91 (2), 637–647.
Hastings, K.K., Jenison, L.A., 2016. Age-specific variation in timing of parturition in Steller sea lions at Forrester Island Complex, Alaska. PLoS One 32 (2), 777–785.