General and Comparative Endocrinology 236 (2016) 83–97

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Research paper

Characterization and longitudinal monitoring of serum progestagens and estrogens during normal pregnancy in the killer whale (*Orcinus orca*)

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ARTICLE INFO

Article history:
Received 16 December 2015
Revised 18 April 2016
Accepted 7 July 2016
Available online 9 July 2016

Keywords:
Orca
Pregnancy
Progesterone
Estrogen
Killer whale
5α-Pregnane-3,20-dione

ABSTRACT

The secretory patterns of progestagens and estrogens were characterized throughout 28 normal pregnancies until two month post-partum in eleven killer whales. HPLC analysis of serum from different reproductive stages (luteal phase, EARLY, MID, and LATE pregnancy) identified three major immunoreactive progestagen peaks; progesterone (P4), 5α-pregnane-3,20-dione (5α-DHP) and pregnanediol, with 5α-DHP approximately half of that for P4 in the luteal phase, and EARLY, but approximately 2/3 of P4 during MID and LATE pregnancy. At birth, 5α-DHP was the only significant (>10% immunoreactivity) immunoreactive progestagen detected in placental (umbilical cord) serum. Maternal recognition of pregnancy appears to occur between day 21 and 28 post-ovulation when a significant deviation in progestagen concentrations between conceptive and non-conceptive cycles was detected. Progestagen concentrations during pregnancy displayed a bimodal pattern with significant peaks ($P < 0.05$) in EARLY (indexed month post-conception [IMPC] 2, 3, 4) and MID (IMPC 9, 10) before decreasing ($P < 0.05$) over an 11 day interval to luteal phase concentrations on the day of parturition. Among estrogens, estriol was secreted in the highest concentrations but only estrone (free and conjugated) and estradiol increased ($P < 0.001$) during pregnancy, with peaks observed during the final month of gestation, and an influence ($P < 0.05$) of fetal sex on estradiol production was detected. Collective findings indicate that P4 derived from the corpus luteum is the major biologically active progestagen during the luteal phase and pregnancy, and that 5α-DHP production, possibly from both luteal and placental sources, increases during the second half of pregnancy.

1. Introduction

Despite the worldwide prevalence of killer whales with total population numbers believed to surpass 50,000 animals (Taylor et al., 2013), increasing anthropogenic stressors derived from fisheries, shipping traffic and whale watching activities have combined with approximate reproductive isolation to result in several populations being listed as threatened or endangered (National Marine Fisheries Service [NMFS], 2008; Noren et al., 2009; Poncelet et al., 2010). One of the most thoroughly studied of these populations, the southern residents, is currently listed as endangered (NMFS, 2008). Cyclic changes in food availability, primarily Chinook salmon (Beamish and Mahnken, 2001; Beamish et al., 2009) have been linked to a population decline since reaching a documented maximum of 98 animals in 1995 (NMFS, 2008; Taylor, 2004). The primary factors responsible for this population's decline in response to reduced prey availability are believed to be combined effects of increased mortality rates, across all age classes, and decreased calving rates (Ford et al., 2009; Ward et al., 2009).

The detrimental impacts of reduced prey on both reproduction and mortality are postulated to include a synergistic effect created when females experiencing a caloric debt are forced to mobilize fat stores that contain high concentrations of lipophilic polychlorinated biphenyls (PCBs; Ross et al., 2000; Ford et al., 2009). These and other organic pollutants, found in some of the highest concentrations of any marine mammal within killer whale blubber (for review see Krahn et al., 2009), are then released into circulation. Exposure to these compounds is associated with multiple adverse metabolic and reproductive perturbations in other species (DeGuise et al., 1995; Muir et al., 1996; Ross et al., 2000; Faroon and Ruiz, 2015).

Although research on killer whale reproductive physiology including male maturation, seasonality, estrus and ovulation has been published (Robeck et al., 1993, 2004, 2011; Robeck and Monfort, 2006), few studies have examined the detailed reproductive or metabolic hormonal changes that occur during pregnancy.

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http://dx.doi.org/10.1016/j.ygcen.2016.07.010
0016-6480/

Walker et al. (1988) described consistently elevated urinary pregnanediol-3-glucuronide (PdG) after two months of pregnancy, and rising estrone-conjugates that plateaued between the 6th and 16th month of pregnancy. Robeck (1996) used high-performance liquid chromatography (HPLC) analysis to analyze immunoreactive progesterone metabolites in the urine from killer whales during the luteal phase and pregnancy and based on results suggested that the corpus luteum was the major source of progesterone production throughout pregnancy. Duffield et al. (1995) reported a bimodal pattern of progesterone secretion during gestation as well as higher progesterone concentrations in nulliparous killer whales during their first pregnancy than their second or third pregnancy. In contrast, Katsumata et al. (2006) described the circulating progesterone profile as having one peak, which gradually declined toward parturition, with the two study females displaying higher concentrations of progesterone during their subsequent compared to their first monitored pregnancy.

Progesterone has long been recognized as being primarily a uterotropic hormone necessary for the establishment and maintenance of pregnancy in the majority of eutherian mammals (Spencer and Bazer, 2002; Spencer et al., 2004). Insufficient concentrations have been associated with early embryonic loss, retarded fetal growth, abortions or stillborn calves in a multitude of species (Allen, 2001; Deanesly, 1972; Shelton et al., 1990) including the bottlenose dolphin (Bergfelt et al., 2011; O'Brien and Robeck, 2012; Robeck et al., 2012), a species within the same phylogenic family as killer whales (Delphinidae). Therefore, further clarification of circulating patterns of progesterone and other progestagens during the luteal phase and normal pregnancies of killer whales is warranted to expand upon our existing understanding of gestational physiology in this species.

Estrogens (estrone [E1], estradiol [E2] and estriol [E3]) are associated with having multiple functions during pregnancy in different species. The early post-conceptive rise in E1 and E2 produced by the endometrium and then the conceptus is necessary for maternal recognition of pregnancy in pigs (Bazer et al., 1998, 2009; Bazer, 2013) and early elevations of these estrogens are associated with implantation in some primates (Rao et al., 2007). In humans, E3, produced by the fetal placental unit, increases dramatically (~900–2700%) during pregnancy peaking just prior to parturition and is reflective of fetal health (Peter et al., 1994) and, if abnormal, can be a predictor of fetal growth restriction (Settiyanan et al., 2015). In multiple species, the estrogenic increase in association with approaching parturition is generally associated with regulating periparturient myometrial/endometrial receptivity to oxytocin (Soloff, 1975; Challis et al., 2000). Furthermore, E1 and E2 may be responsible for modulating cervical relaxin receptors prior to parturition (Downing and Sherwood, 1984; Sherwood, 2004). To our knowledge, no descriptions of serum estrogen profiles in killer whales during pregnancy have been reported, and thus the potential for using estrogens as biomarkers of fetal health or impending parturition in this species remains unexplored.

Because the need to better characterize normal gestational physiology is essential for understanding and monitoring of the species' health, whether in the wild or in captivity, the goal of this research was accordingly to characterize longitudinal profiles of progestagens and estrogens in females producing a viable, term calf. The specific objectives were to: (1) identify the predominant progestagens and estrogens present in serum during early, mid and late gestation with comparison to the luteal phase of the estrous cycle; (2) examine the influence of dam age, parity and month of conception on gestation length; (3) characterize profiles of serum immunoreactive progestagens and estrogens during months and trimesters of pregnancy; and (4) examine the influence of month of sample collection, dam age, parity and fetal sex on serum progestagens and estrogens during pregnancy.

2. Materials and methods

2.1. Animals and sample collection

Eleven female killer whales were group housed at three SeaWorld habitats (Orlando, FL, USA; San Diego, CA, USA; San Antonio, TX, USA) and Loro Parque (Tenerife, Spain). Pools contained a minimum of 19,000 m^3 of salt water kept at approximately 14 °C year-round. Animals were fed a diet of frozen-thawed whole fish, which contained some or all of the following fish species: Pacific herring (*Clupea harengus*); Columbia river smelt (*Thaleichthys pacificus*); Atka mackerel (*Pleurogrammus azonus*); and pink salmon (*Oncorhynchus gorbuscha*) at approximately 2–3% of their body weight per day. Inclusion criteria for animals were gestations that resulted in a calf that was alive and nursing at six months post-delivery, and for which at least monthly blood data were available throughout gestation.

Blood samples ($n = 994$ samples) were collected voluntarily from animals using pre-conditioned behavior 2 month before, during and after 28 pregnancies (a mean 35 samples/pregnancy) from June 1983 until Dec 2014. Samples were collected from the ventral tail fluke using a 19 gauge winged blood collection set. Paired blood samples were also collected from post-partum females and from the umbilical cord veins and arteries attached to an expelled placenta of her live, term calf ($n = 5$). Blood was collected into BD Vacutainers (Becton Dickenson, Franklin Lakes, NJ, USA) containing activated thrombin, allowed to clot for 20 min, and then centrifuged at 1000g for 15–30 min. Serum was removed and stored at −80 °C until analysis.

2.2. Reproductive status and stages of pregnancy

Conception dates were known via monitoring of urinary estrogen concentrations ($n = 9$), or where females were artificially inseminated ($n = 4$). For the remaining gestations in which the exact conception date was not known ($n = 15$), the conception date was designated as the midpoint between the last sample with baseline serum progesterone concentration (<0.5 ng/ml) and the first sample displaying progesterone concentrations above baseline (if the interval exceeded 14 d, data from that pregnancy were not included in analyses). Season of parturition was defined as Winter (Dec to Feb), Spring (Mar to May), Summer (June to Aug) and Fall (Sept to Nov).

Serum concentrations of immunoreactive progestagens (Pg), estrone (E1), estradiol (E2) and estriol (E3) during each of the 28 pregnancies from 11 animals were categorized into time components based on their relative relationship to conception or parturition. We used day post-conception (DPC) to evaluate overall hormonal trends during gestation. For comparison of reproductive and gestational stages (STAGE), we placed the samples into 5 groups or stages as follows: Stage 1: Pre-conception (Pre) – from two months prior to conception; Stage 2: Early gestation (Early) – samples collected after conception (>1 d post-ovulation) though month 5; Stage 3: Mid gestation (Mid) – start of the 6th month though the 11th month; Stage 4: Late gestation (Late) – start of the 12th month though the 17th month (or until parturition); and Stage 5: Post gestation (Post) −1 day through 2 months post-parturition.

Due to large variation in gestation length (473–559 d), a monthly correction factor, or index, for each individual pregnancy was created to standardize hormone concentrations relative to

month post-conception among all study females. To do this, the proportion of the killer whale mean pregnancy length (17.5 months, Robeck and Nollens, 2013) contributed by each month of gestation was first determined (1 ÷ 17.5 = 0.057), meaning that each month represents 5.7% of the entire gestation. This percentage was then multiplied by each individual animal's gestation length to determine the total days per indexed month for each unique gestation. For example, a gestation length of 566 days would have an indexed month of 32.3 days (566 × 0.057). Then, a sample's day post-conception was divided by the indexed month value to determine the appropriate indexed month post-conception (IMPC). So, for a sample collected on day 60 post-conception with a gestation length of 566 d and an indexed month of 32.3 d, the IMPC value was 1.86 (60 ÷ 32.3). Data for IMPC 0 was from day 1 post conception until IMPC 0.99; data for IMPC 1 included an IMPC range of 1.00–1.99; data for IMPC 2 included an IMPC range of 2.00–2.99, and so on for all IMPC classes prior to conception and post-parturition.

For comparisons of hormone profiles between the estrous cycle and pregnancy stages, data collected from 12 animals during the first 5 weeks post-ovulation, during conceptive and non-conceptive cycles, were combined at weekly intervals as follows: week 0: day of ovulation; week 1: days 1–7; week 2: days 8–14, week 3: days 15–21; week 4: days 22–28; week 5: days 29 to day 35. This length of time should allow for hormone comparisons of the entire luteal phase, which has been estimated at 25 days (Robeck et al., 2004), with early pregnancy.

2.3. Hormone assays

2.3.1. High performance liquid chromatography (HPLC) analysis

HPLC was performed to identify the immunoreactive estrogens, progestagens and their metabolites present in killer whale sera during different stages of reproduction (LUTEAL, EARLY, MID and LATE pregnancy). An HPLC machine (Beckman System Gold Programmable Solvent Module 125 and Model 168 Diode Array Detector, Beckman Instruments, Brea, CA, USA) was used along with a reverse phase HPLC column (C18, 4 μm, 3.9 × 150 mm, Novapak® Waters Corporation, Milford, MA, USA). A 7.5–10 ml pool of serum from six animals during 13 pregnancies and 14 luteal phases from the five reproductive stages was filtered and concentrated using a SPICE C18 cartridge (Analtech, Newark, DE, USA) to remove contaminants and the eluant dried down. Samples were reconstituted in 0.25 ml MeOH (HPLC Grade, Fisher Scientific, PA, USA), suspended by sonication or with vortexing, and then 0.05 ml loaded onto the column. See Table 1 for specific HPLC details for each hormone class. Briefly, a solvent:water mixture was used in a gradient within the HPLC system at a flow rate of 1 ml/min, with fractions collected every minute over a 60–95 min run-time to separate the different hormones and metabolites. Known standards were run prior to each sample to determine where each steroid and metabolite would elute (as detected by the diode array detector). After completion of each HPLC run, fractions were evaporated and reconstituted in 0.2–0.25 ml of standard enzyme immunoassay buffer (phosphate buffered saline with 0.1% bovine serum albumin) then stored at −20 °C until hormone concentration analysis using the hormone enzyme immunoassays (EIAs) described in the following sections. The total immunoreactivity (TI) for each hormone was determined by first removing a baseline immunoreactivity value (determined using an iterative method whereby values exceeding the mean plus 2 standard deviations were excluded; Brown et al., 1999). Next, the percentage of total immunoreactivity for each peak was determined by dividing its immunoreactivity by the TI. For fractions adjacent to immunoreactive peaks, which similarly displayed high immunoreactivity and therefore could represent immunoreactivity of the previous or following fraction, the immunoreactivity was divided and added equally to the adjacent peaks. Sample immunoreactivity, as detected by each EIA, was compared to the retention times of the known standards to identify the presumptive hormones and metabolites.

2.3.2. Progestagen (Pg) assay

Concentrations of Pg were measured using a direct, single antibody EIA as previously described (Robeck et al., 2005). Briefly, aliquots of serum (0.05–0.0005 ml, depending on the reproductive stage) and standard (4–0.016 ng/ml) were added to a 96 well microtiter plate (Maxisorp, ThermoFisher, Waltham, MA, USA) coated with a monoclonal iP antibody (1:10,000, CL425, Quidel, San Diego, CA, USA, final purification by C. Munro, UC Davis, Davis, CA, USA) along with an enzyme conjugate (horse-radish peroxidase [HRP], 1:40,000, C. Munro). The iP antibody cross-reacts with a wide variety of progestagens including progesterone (100.0%) and 5α-pregnane-3,20-dione (55.0%; see Graham et al., 2001 for a comprehensive list). Parallel displacement of serum compared to the standard curve was demonstrated ($r = 0.988$) and the recovery of known concentrations of standard to serum was 89.8 ± 1.8% (linear regression, $y = 0.89x + 0.15$, $r^2 = 0.994$). Assay sensitivity was 0.02 ng/ml and intra-assay variation was <10%. Inter-assay coefficients of variation for two quality controls binding at 20% and 60% were 5.9 and 11.8%, respectively ($n = 58$).

2.3.3. Pregnanediol-3-glucuronide (PdG) assay

PdG concentrations for HPLC fractions were measured using a direct, single antibody EIA (Munro and Stabenfeldt, 1984). Briefly, 0.05 ml of reconstituted HPLC fractions and standard

Table 1
Methods for HPLC separation for different hormone groups.

Hormone group	Solvent:H_2O	Run time (min)	Known standards (in order of elution)[a]	EIA[b]
Estrogens	20–52% methanol:water	95	Estrone-3-glucuronide (E1-G) Estrone-3-sulfate (E1-S) Estriol (E3) Estradiol (E2)	In-house E1/E1-C In-house E2 In-house E3
Progestagens	20–80% acetonitrile:water	60	Pregnanediol-3-glucuronide (PdG) 17α-Hydroxyprogesterone (17α-OHP) Progesterone (P4) Allopregnanolone 5α-Dihydroprogesterone (5α-DHP) 5β-Pregnanediol (Pd)	In-house PdG In-house Pg

[a] E1-G: 1,3,5(10)-Estratrien-17-one 3-glucuronide; E1-S: 1,3,5(10)-Estratrien-17-one 3-sulfate; E3: 1,3,5(10)-estratrien-3-ol-17-one; E2: 1,3,5(10)-estrattrien-3, 17β-diol; PdG: 5β-Pregnane-3α,20α-diol glucuronide; 17α-OHP: 4-pregnen-17α-ol-3,20-dione; P4: 4-pregnen-3-20-dione; Allopregnanolone: 5α-pregnan-3β-ol-20-one; 5α-DHP: 5α-pregnan-3,20-dione; Pd :5β-pregnan-3α,20α-diol.
[b] HPLC fractions from each hormone group were run in all of the designated EIAs.

(100–1.81 ng/ml) were added to a 96 well microtiter plate coated with a polyclonal antibody (1:10,000, P26, C. Munro) along with an enzyme conjugate (horse-radish peroxidase, 1:30,000, C. Munro). The plate was incubated at room temperature for 2 h before addition of 0.1 ml ABTS substrate solution and the plate incubated for another 20–30 min. The plate was read in a plate reader at 405 nm with reference filter of 540 nm. The antibody cross-reacts with: PdG 100.0%; 20-α-hydroxyprogesterone 60.7%; pregnanediol 7.5%; 20-β-hydroxyprogesterone (2.5%); and <0.1% with other tested progestagens including progesterone Assay sensitivity was 1.9 ng/ml and intra-assay variation was <10%. Inter-assay coefficients of variation for two quality controls binding at 30% and 70% were 3.0 and 10.5%, respectively ($n = 6$).

2.3.4. Estrone and estrone conjugate (E1/E1-C) assay

Concentrations of E1 and E1 conjugates (E1-C) were measured in the same assay using a direct, single antibody enzyme immunoassay (EIA) as previously described (Munro et al., 1991). Briefly, serum (0.05–0.01 ml) and standard (4–0.016 ng/ml) were added to a 96 well microtiter plate coated with a polyclonal estrogen conjugate antibody (1:20,000, R522-2, C. Munro) along with an enzyme conjugate (HRP, 1:250,000, C. Munro). The plate was incubated at room temperature for 2 h before addition of 0.1 ml substrate (tetramethylbenzidine [TMB] in phosphate citrate buffer with sodium perborate, Sigma Aldrich). The reaction was stopped after 15–20 min using 0.05 ml of 0.6 M H_2SO_4 and the samples read in a plate reader at 450 nm with a reference filter of 655 nm. The antibody cross-reacts with: E1 > 100%; E1-G, 100%; E1-S 66.6%; E2 7.8% (personal communication, C. Munro). Parallel displacement of serum compared to the standard curve was demonstrated ($r = 0.994$) and the recovery of known concentrations of standard to serum was 82.1 ± 10.8% (linear regression, $y = 0.87x - 0.74$, $r^2 = 0.996$). Assay sensitivity was 0.03 ng/ml and intra-assay variation was <10%. Inter-assay coefficients of variation for two quality controls binding at 30% and 70% were 10.5 and 11.9%, respectively ($n = 54$).

2.3.5. Estradiol (E2) assay

E2 concentrations were measured using an in-house, single antibody, direct EIA. Briefly, 0.05 ml of serum and standard (5–0.02 ng/ml) were added to a 96 well microtiter plate coated with a poly-clonal antibody (1:12,000, R0008, C. Munro) along with an estradiol-HRP conjugate (1:40,000, C. Munro). Following a 2 h room temperature incubation, 0.1 ml ABTS substrate solution was added and the plate incubated for another 60–90 min. The plate was read at 405 nm with a 540 nm reference filter. Reported cross-reactivity for antibody R0008 was specific for estradiol (100%) and less than 1% for other estrogens (C. Munro, personal communication, 2006). Assay sensitivity was 0.04 ng/ml and intra-assay variation was <10%. Parallel displacement of serum compared to the standard curve was demonstrated ($r = 0.967$) and the recovery of known concentrations of standard to serum was 92.1 ± 9.7% (linear regression, $y = 0.91x + 0.19$, $r^2 = 0.996$). Inter-assay coefficients of variation for two quality controls binding at 30% and 70% were 9.0 and 11.7%, respectively ($n = 45$).

2.3.6. Estriol (E3) assay

E3 concentrations were measured using a single antibody, direct EIA as previously described (Hedriana et al., 2001). Serum (0.05 ml) and standard (80–0.31 ng/ml) were added to a 96 well microtiter plate coated with a polyclonal antibody (1:10,000, R4835, C. Munro) along with an E3-HRP (1:40,000, C. Munro). Following a 2 h room temperature incubation, 0.1 ml ABTS substrate solution was added and the plate incubated for another 20–30 min. The plate was read at 405 nm with a 540 nm reference filter. Reported cross-reactivity for antibody R4835 was specific for E3 with other steroids and metabolites structurally similar to E3 showing <0.1% cross-reaction (Hedriana et al., 2001). A retrospective evaluation of the cross-reactivity of E2 with the E3 antibody was conducted as follows: samples containing a range of E2 concentration (2000–0.976 ng/ml) obtained by serial dilutions of E2 standard in EIA buffer were created and analyzed using the E3 EIA as described in this section. The hormone concentration observed (ng/ml) for the E2 standard at ~50% binding (5.20 ng/ml) was then divided by the known concentration of standard added (also at ~50% binding, 7.81 ng/ml) to determine the percent cross-reactivity of the E2 standard. The results indicated that a 66.5% cross-reactivity of the R4835 (E3) antibody with E2 exists, and therefore E3 values were adjusted to account for this cross reactivity prior to analysis. This one done by removing 65% of the total E2 concentration as determined by the E2 EIA from Total E3 concentrations as determined by the E3 EIA in paired samples. For samples with inadequate volume for paired analysis of both hormones, the E3 sample result was not included in final analyses. Assay sensitivity was 0.31 ng/ml and intra-assay variation was <10%. Parallel displacement of serum compared to the standard curve was demonstrated ($r = 0.983$) and the recovery of known concentrations of standard to serum was 115.5 ± 15.6% (linear regression, $y = 1.16x - 3.68$, $r^2 = 0.991$). Inter-assay coefficients of variation for two quality controls binding at 30% and 70% were 7.6 and 8.4% respectively ($n = 68$).

2.4. Statistical analysis

2.4.1. Gestation length comparisons

Gestation length (d) was evaluated against the fixed variables: age of dam at conception (age), season of birth (season), parity of dam (parity), and sex of calf (sex) using a mixed effects maximum likelihood (ML) regression to determine if any significant effects of these fixed variables on gestational length could be detected. A single level mixed model regression procedure ("Mixed" function, STATA version 13, StataCorp LP, College Station, TX, USA) was used to control for the variance contributed from unbalanced repeated measures of differing numbers of pregnancies (random intercept variable) from each animal (Leckie, 2013; West et al., 2015).

2.4.2. Hormone analysis

A mixed effects ML regression model (Leckie, 2013; West et al., 2015) using the random intercept variables, animal ID (level 2) and pregnancy number (level 3) was used to control for the variance associated with an unequal number of repeated measures during each gestation and an unequal number of pregnancies per animal. For each dependent variable, the use of a two or three level model was determined by comparing the ML estimates of the 2 and 3 level models using the likelihood-ratio (LR) test at $P < 0.05$. Once the effects of random variables were determined (e.g., one or two stage multilevel), all fixed effects were added and then removed iteratively in a backward direction. The two models were then compared (with and without the individual fixed effect variable that had been removed) using the LR test at $P < 0.05$. The variable was then retained or omitted depending on whether or not it contributed significantly toward model explanation of the dependent variable variance. The fixed effect variables included three unique time periods during pregnancy: days post-conception (DPC), STAGE (PRE, EARLY, MID, LATE, POST; as defined in Section 2.2) and IMPC. Covariates included fetal sex (0 = female, 1 = male), parity (0 = nulliparous, 1 = primiparous, 2 = multiparous), age of dam at conception, and for controlling for seasonal effects, month of sample collection (month) and interactions between the final fixed variables in the model (e.g., stage X parity). Comparisons of hormone patterns between week post-ovulation for conceptive and non-conceptive cycles were made using a mixed model regression

with the fixed variables being week post-conception, cycle outcome (conceptive = 0, non-conceptive cycle = 1), and the random intercept variables as animal and pregnancy ID. For evaluation of hormonal changes during the 60 days prior to parturition, data were aligned as day prior to parturition, and for days where less than 3 data points were available, data were combined with the day immediately prior to or after that date until at least 3 points per day were obtained. Data were then analyzed using a mixed effects ML regression using hormone concentration as the dependent variable, time (day prior to parturition) as the fixed variable and animal ID and pregnancy ID as the random intercept variables as appropriate. Comparisons of hormone concentrations between samples of maternal (combined days 1 or 0 prior to parturition) and umbilical cord blood were made using an unpaired *t*-test with unequal variances using the Welch (1947) approximation for degrees of freedom.

All final mixed effects models were checked for normality using quantile plots of the standard residuals. If quantile-quantile (qnorm) plots of standardized residuals exhibited non-normal distribution then data was transformed as predicted by the Shapiro-Wilk test (Ladder command, STATA) until residuals were normalized. Finally, pairwise comparisons of the marginal means between and within fixed variables were made while applying the Sidak correction factor. Marginal means and SEMs of non-transformed IMPC data were calculated for all main effects in the individual hormone models and used to plot hormonal concentrations during pregnancy. For text and tables, transformed data was first back-transformed and then presented as marginal means with 95% confidence intervals (*CI*).

3. Results

3.1. Gestation length

The mean gestation length for the animals (n = 11) used in the study was 532 ± 3.1 d (±SEM, range: 473–559 d, n = 28). The mean coefficient of variation (*cv*) of gestation lengths within all animals that had repeat gestations ($n = 6$) was 2.3% (95% *CI*: 1.3–3.3%). No differences were detected between the gestation lengths of these animals. Overall, gestation length was not influenced by age of dam ($P = 0.97$), fetal sex ($P = 0.23$), or season of parturition ($P = 0.14$). A significant interaction was detected between season and parity ($P < 0.0001$), and when controlling for this interaction, parity had a significant influence ($P = 0.001$) on gestation length. However, inadequate sample distribution across all parity and season periods (e.g., no primiparous females gave birth in the spring) precluded further intergroup comparative analysis.

3.2. Hormone analysis

3.2.1. HPLC results

For Pg, the major immunoreactive peaks (>10% total immunoreactivity [TI]) for each antibody-specific EIA were: (1) Pg EIA: progesterone (P4, all stages); 5α-pregnane-3,20-dione (5α-DHP, all stages); pregnanediol LUTEAL, EARLY, MID); (2) PdG EIA: PdG (LUTEAL, EARLY, LATE); P4 (LUTEAL, MID, LATE; Fig. 1A–D). Total PdG immunoreactivity, as measured using the PdG EIA, compared to total immunoreactivity for the Pg EIA was two to 20-fold lower for the former. Thus, PdG was not used for further sample analysis. P4 had the highest percentage of TI during the luteal phase (22%) and in all stages of pregnancy, peaking in early pregnancy with 42% of TI, decreasing to 16% and 24% during MID and LATE, respectively. Both 5α-DHP and pregnanediol remained between 10 and 12% of the total immunoreactivity during the luteal, EARLY and MID. However, during LATE, 5α-DHP jumped to 14% while pregnanediol decreased to 4% (Fig. 1D). 5α-DHP was the only significant immunoreactive peak (28%) detected in umbilical cord (placental) blood, with P4 at 6% and pregnanediol remaining at 4%.

For estrogens, the major immunoreactive peaks (>10% TI) for each antibody-specific EIA were: (1) E1/E1-C EIA: E1-G (LUTEAL, EARLY, LATE); E1-S (LATE); E1 (EARLY, MID, LATE); (2) E2 EIA: E2 (all stages); (3) E3 EIA: E3 (all stages, Fig. 2A–D); E2 (LUTEAL, EARLY). E1/E1-C had the highest TI at 41.08% during LATE, decreasing to 38%, 27% and final 12% during LUTEAL, EARLY and MID, respectively. E2 had the highest TI during LATE, followed by 47%, 30% and 25% during MID, EARLY and LUTEAL, respectively. E3 TI peaked at 33% during MID, then it fell to 30% during LATE, then to 14% and 9% in EARLY and LUTEAL, respectively (Fig. 2A–D).

3.2.2. Progestagen analysis (Pg)

For Pg analysis a three-level model (animal ID and pregnancy) provided significant ($\chi^2 = 168.8$, $P < 0.0001$) explanation of the variance and was used for the final analysis. Data were transformed (square root) for the final analysis.

3.2.2.1. Pg concentrations relative to days post-conception (DPC). Pg concentrations were significantly influenced by DPC ($P < 0.007$) with a mean concentration throughout pregnancy of 30.04 ng/ml (*CI*: 28.9–31.13 ng/ml).

3.2.2.2. Pg concentrations relative to reproductive STAGE. Pg concentrations within each gestational stage (EARLY, MID, LATE) were significantly different ($P < 0.001$) to PRE and POST pregnancy stages, and EARLY was less than ($P < 0.05$) MID and (Table 2).

3.2.2.3. Pg concentrations relative to indexed month post-conception (IMPC). IMPC ($P < 0.0001$) was the only fixed variable which had a significant effect on the model. During pregnancy, Pg concentrations increased rapidly to significant peaks at IMPC 2 (43.7, *CI*: 38.5–49.3 ng/ml), 3 (37.9, *CI*: 33.0–43.2 ng/ml) and 4 (36.1, CI: 30.13–42.5 ng/ml), declined during IMPC 5 (27.9, *CI*: 24.4–31.4 ng/ml) and 6 (28.1, *CI*: 24.5–31.7 ng/ml), increased again during IMPC 9 (34.9, *CI*: 31.5–38.4 ng/ml) and 10 (34.8, *CI*: 31.3–38.3 ng/ml), then remained unchanged until a significant decline in the final gestational month (22.4, *CI*: 18.3–26.9 ng/ml) (Fig. 3A). All IMPC classes had significantly ($P < 0.001$) greater mean Pg concentrations than the 2 months pre- or post-pregnancy (Fig. 3A).

In an attempt to illustrate the contribution of each Pg identified by HPLC toward total Pg immunoreactivity during each IMPC, the following assumptions were made: 1) Since the proportions of each Pg present in serum relative to total Pg immunoreactivity was calculated during the luteal phase and only three time-points during gestation (early [IMPC 3], mid [IMPC 9], and late [IMPC 15]), a regression analysis using values from luteal (represented as IMPC 0) and the aforementioned IMPC time-points as the independent variable was used to predict percent changes between each time point (as determined by regression coefficients) during the entire gestation. The predictive total immunoreactive contribution of each Pg was then determined for each IMPC. These values (ng/ml) were then graphed to provide a proposed model of Pg production and during gestation (Fig. 3B).

3.2.3. Estrone/estrone conjugate (E1/E1-C) analysis

For *E1/E1-C* analysis a three-level model (animal ID and pregnancy) provided significant ($\chi^2 = 215.0$, $P < 0.0001$) explanation of the variance and was used for the final analysis. Data were inverse square root transformed for the final analysis.

3.2.3.1. E1/E1-C concentrations relative to day post-conception (DPC). Across pregnancy, E1/E1-C concentrations increased significantly ($P < 0.001$) at a rate of 31.5 pg/100 days, and tended to be higher

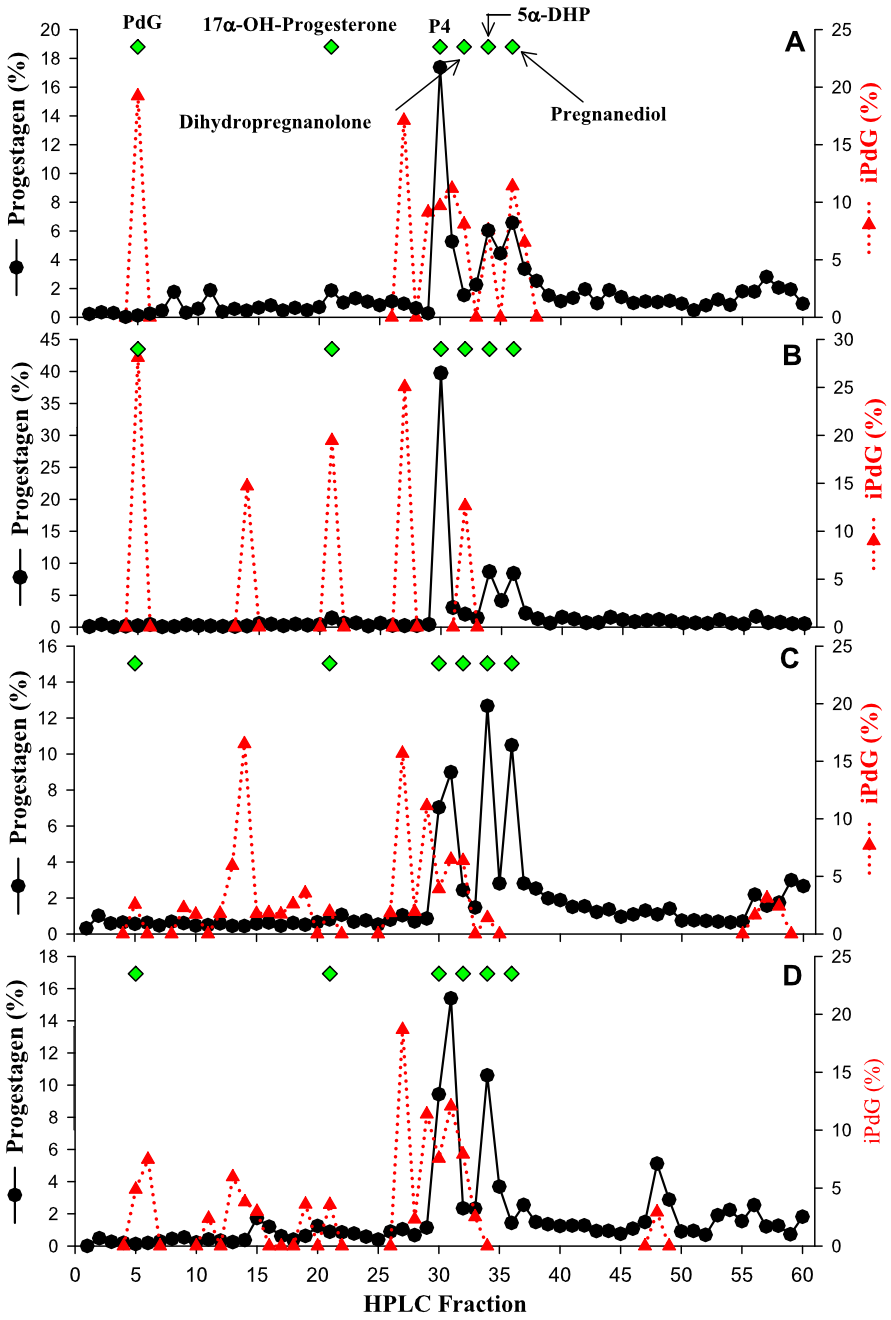

Fig. 1. HPLC immunogram of a pool (n = 13 pregnancies, 14 luteal phases) of killer whale (6 animals) serum collected during the luteal phase (Panel A), early pregnancy (Panel B, Days 0–180; Day 0 = day of conception), mid pregnancy (Panel C, Days 181–360) and late pregnancy (Panel D, Days 361 until term). Data-points for each fraction are presented as the percentage of total immunoreactivity measured in each EIA (the total immunoreactivity excluded values considered to represent baseline immunoreactivity as described in the methods section; for additional clarity only non-zero data points are presented). Immunoreactivity was determined by either a progestagen EIA using a highly cross-reactive progesterone antibody (●) or a specific pregnanediol-3-glucuronide (PdG) monoclonal antibody (▲). Elution positions of reference standards are designated by green diamonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(P = 0.046) for females carrying a male (0.27 ng/ml, CI: 0.25–0.29 ng/ml) than a female fetus (0.24 ng/ml, CI: 0.23–0.26 ng/ml).

3.2.3.2. E1/E1-C concentrations relative to reproductive STAGE. MID and LATE pregnancy had significantly (P < 0.04) elevated concentrations of E1/E1-C when compared to PRE, and EARLY stages of pregnancy and MID were reduced when compared to LATE (Table 2).

3.2.3.3. E1/E1-C concentrations relative to indexed month post-conception (IMPC). E1/E1-C concentrations increased throughout pregnancy with significant elevations during the last half (IMPC 8

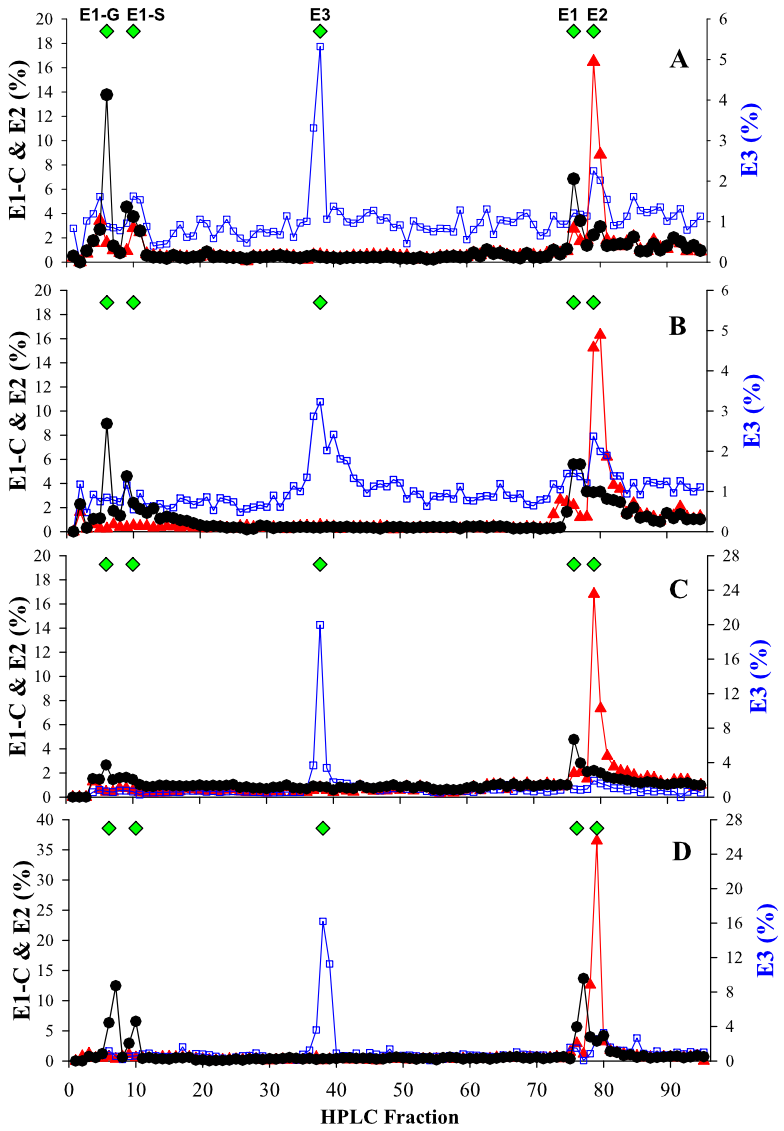

Fig. 2. HPLC immunogram of a pool (n = 13 pregnancies, 14 luteal phases) of killer whale (6 animals) serum collected during the luteal phase (Panel A), early pregnancy (Panel B, Days 0–180; Day 0 = day of conception), mid pregnancy (Panel C, Days 181–360) and late pregnancy (Panel D, Days 361 until term). Data-points for each fraction are presented as the percentage of total immunoreactivity measured in each EIA (the total immunoreactivity excluded values considered to represent baseline immunoreactivity as described in the methods section; for additional clarity only non-zero data points are presented). Immunoreactivity was determined by using one of three estrogen EIAs with different antibodies: estrone/estrone-conjugates (E1/E1-C, ■); estradiol (E2, ▲); estriol (E3, ●). Elution positions of reference standards are designated by green diamonds. Standards eluted in order as follows: estrone-3-gluconuride (E1-G); estrone-3-sulfate (E1-S); estriol (E3); estrone (E1); estradiol (E2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Marginal mean (95% *CI*) hormone concentration during each stage of killer whale pregnancy.

Hormone (ng/ml)[a]	Gestational stage					P value	Sidak groups[b]
	Pre (1)	Early (2)	Mid (3)	Late (4)	Post (5)		
Progestagens	1.19 (0.58–2.02)	25.1 (22.0–28.4)	30.4 (27.5–33.4)	30.1 (27.3–33.0)	0.3 (0.03–0.92)	P > 0.05	1,5 < 2 < 3,4
Estrone/estrone-conjugates	0.23 (0.21–0.25)	0.24 (0.23–0.26)	0.26 (0.24–0.28)	0.28 (0.26–0.30)	0.24 (0.21–0.25)	P < 0.001	1,2 < 3,5 < 4
Estradiol	0.17 (0.15–0.20)	0.18 (0.17–0.20)	0.19 (0.18–0.21)	0.21 (0.19–0.23)	0.17 (0.14–0.22)	P < 0.05	1,2,3 < 4
Estriol	2.18 (1.90–2.51)	2.01 (1.84–2.21)	1.85 (1.68–2.02)	2.17 (1.97–2.38)	2.02 (1.74–2.34)	P < 0.001	3 < 4

Pre: 0 to 2 months prior to ovulation; Early: Day 2 through Month 5 post-ovulation; Mid: Month 6 through Month 11; Late: Month 12 until parturition; and Post: Day 1 to 2 months post-parturition.

[a] Hormones concentrations were back-transformed after the analysis.

[b] Sidak correction factor used for comparisons of marginal means. Only stages that are significantly different from other stages are listed.

onwards; $P < 0.05$) and significant ($P < 0.05$) peaks occurring at IMPC 16 (0.33, CI: 0.29–0.38 ng/ml) and at IMPC 17 (0.36, CI: 0.27–0.45 ng/ml) with a rapid decrease to pre-pregnancy concentrations post-partum (Fig. 4A).

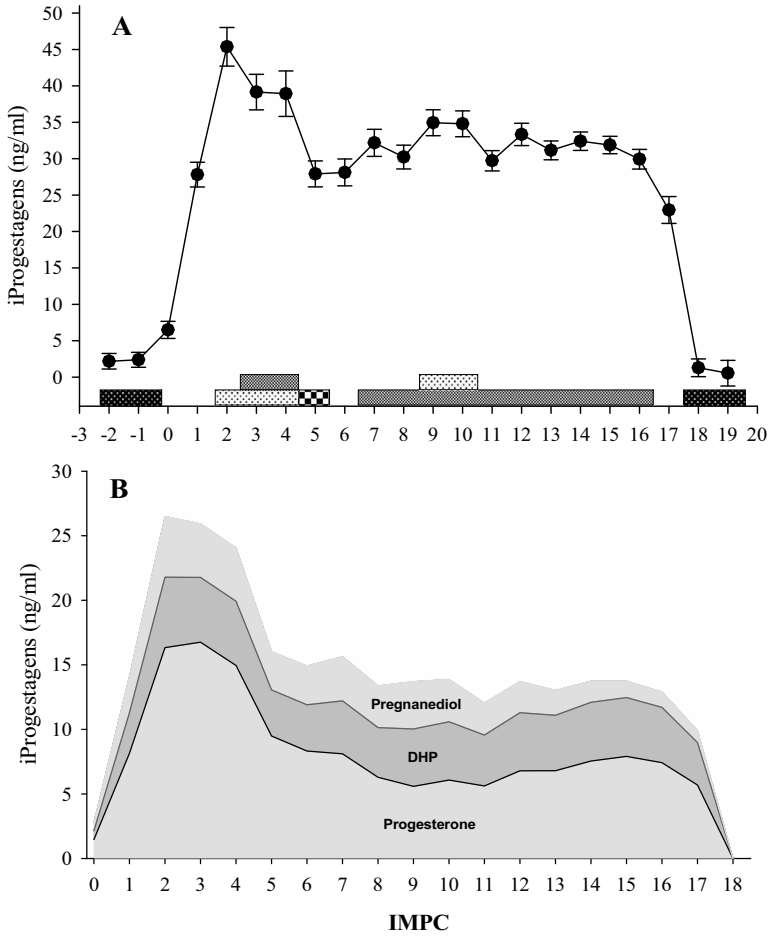

Fig. 3. Panel A. Serum concentrations of immunoreactive progestagens during killer whale pregnancy based on indexed month post-conception (IMPC). IMPC was determined by calculating the percentage that each month contributes to the total gestation. Then, this percentage was multiplied by each individual pregnancy length to determine the total number of days per index month for each gestation. Horizontal bars represent IMPC months where differences were detected. Bars with different shading patterns are different from each other ($P < 0.05$). Values are marginal means (±SEM) for all animals. Panel B. Represents the estimated contribution of the three major sources of immunoreactive progestagens, progesterone (P4), 5α-pregnane-3,20-dione (5α-DHP) and pregnanediol, during pregnancy. Estimated contributions were based on regression curves of the percentage contributions during early, mid and late pregnancy. Percentage of luteal contributions of P4, 5α-DHP, and pregnanediol toward total immunoreactivity were assumed to be unchanged after early pregnancy. The remaining P4 was assumed to represent a placental source.

3.2.4. Estradiol (E2) analysis

For E2 analysis, a two-level model (animal ID) provided significant ($\chi^2 = 255.4.5$, $P < 0.0001$) explanation of the variance and was used for the final analysis. Data were square root transformed or the final analysis.

3.2.4.1. E2 concentrations relative to days post-conception (DPC). DPC ($P < 0.001$) and parity ($P = 0.02$) were the only fixed variables that significantly described E2 concentration variance. Across pregnancy, E2 concentrations increased significantly ($P < 0.001$) at a rate of 8.4 pg/100 days. E2 concentrations of multiparous females (0.17, *CI*: 0.15–0.19 ng/ml) were significantly lower ($P = 0.025$) than those of primiparous females (0.22, *CI*: 0.19–0.26 ng/ml), but were similar to those for nulliparous females (0.21, *CI*: 0.17–0.24 ng/ml).

3.2.4.2. E2 concentrations relative to reproductive STAGE. As with DPC, parity significantly influenced E2 concentrations ($\chi^2 = 8.7$, $P = 0.01$) with concentrations for multiparous females being significantly lower than primiparous females. LATE pregnancy had significantly elevated concentrations of E2 when compared to PRE ($P = 0.005$), EARLY ($P < 0.001$) and MID pregnancy ($P < 0.05$, Table 2).

3.2.4.3. E2 concentrations relative to indexed month post-conception (IMPC). E2 concentrations varied significantly with parity ($P = 0.01$) and across IMPC ($P < 0.0001$), whereby concentrations slowly increased throughout pregnancy with a significant elevation occurring in month 17 (0.28, *CI*: 0.22–0.32 ng/ml) followed by a decrease ($P < 0.05$) to pre-pregnancy values by 1 month (0.16, *CI*: 0.13–0.20 ng/ml) post-partum (Fig. 4B).

3.2.5. Estriol (E3) analysis

For E3 analysis a two-level model (pregnancy ID) provided significant ($\chi^2 = 249.5$, $P < 0.0001$) explanation of the variance and was used for the final analysis. Data were inverse square root transformed for the final analysis.

3.2.5.1. E3 concentrations relative to days post-conception (DPC). No significant ($P > 0.05$) fixed effects of E3 concentrations during gestation were detected.

3.2.5.2. E3 concentrations relative to reproductive STAGE. E3 concentrations were only significantly different between MID (2.01, CI: 1.84–2.21 ng/ml) and LATE (2.17, *CI*: 1.97–2.38 ng/ml) pregnancy (Table 2).

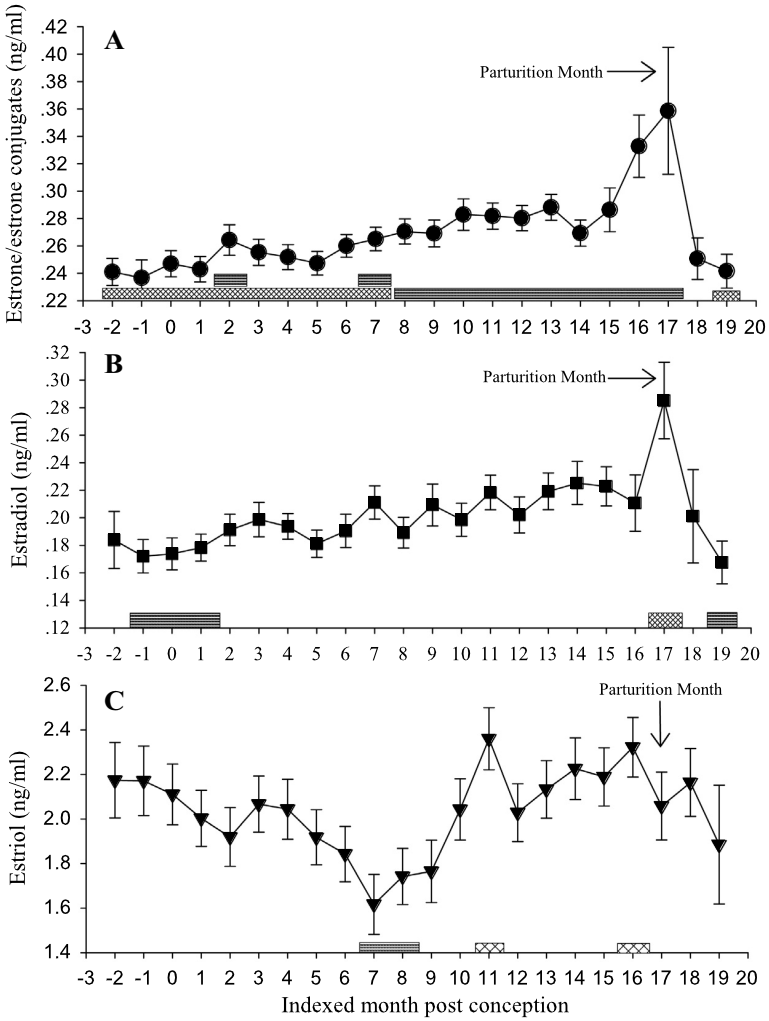

Fig. 4. Serum concentrations of estrone/estrone-conjugates (E1/E1-C; Panel A), estradiol (E2; Panel B) and estriol (E3; Panel C) during killer whale pregnancy based on indexed month post-conception (IMPC). IMPC was determined by calculating the percentage that each month contributes to the total gestation. Then, this percentage was multiplied by each individual pregnancy length to determine the total number of days per index month for each gestation. Horizontal bars represent IMPC months where differences were detected. Bars with different shading patterns are different from each other ($P < 0.05$). Values are marginal means (±SEM) all animals. Horizontal bars represent IMPC months where differences were detected. Bars with different shading are different from each other ($P < 0.05$).

3.2.5.3. E3 concentrations relative to indexed month post-conception (IMPC). Significant ($P < 0.05$) nadir E3 concentrations were observed during IMPC 7–8, compared to peaks during IMPC 11 and 16 (Fig. 4C).

3.2.6. Hormone profiles during the first 5 weeks of pregnancy

Serum Pg concentrations of samples collected from non-conceptive (luteal phase, 218 samples, during 95 cycles in eight whales) and conceptive ovulations (147 samples, from 28 pregnancies) indicated a significant influence of week post-ovulation ($P < 0.0001$), and an interaction ($P < 0.0001$) of week post-ovulation with cycle type (conceptive or non-conceptive). Although no overall differences in Pg concentrations were detected between conceptive and non-conceptive cycles ($P = 0.47$), significant differences between the two groups were detected beginning in week 4 post-conception ($P < 0.05$, Fig. 5A). For non-conceptive cycles, significantly elevated (compared to day 0 and week 1, $P < 0.05$) Pg concentrations occurred during week 2 (3.58, *CI*: 2.09–5.06 ng/ml) and 3 (7.51, *CI:* 6.06–8.96 ng/ml) post-ovulation and returned to baseline (<1 ng/ml) between day 28 (2.9, *CI:* 1.42–4.44 ng/ml) and 35 (0.61, *CI:* 0–2.53 ng/ml) post-ovulation (Fig. 5A).

Among estrogens, only E1/E1-C concentrations were determined during the 5 weeks post-ovulation, since HPLC analysis indicated that E1/E1-C was the major estrogen produced during the luteal phase and pregnancy (Section 3.2.1). E1/E1-C concentrations were not influenced by week post-ovulation ($P = 0.28$) or cycle type (conceptive or non-conceptive, $P = 0.48$), and no significant interactions between these variables were detected (Fig. 5B).

3.2.7. Hormone profiles during the 60 days prior to parturition (DPP)

Overall, Pg concentrations decreased significantly ($P < 0.0001$) from 60 days prior to parturition until 14 days post-partum at a rate of 0.33 ng/day. However, a scatterplot of Pg by time (day prior to parturition) indicated a rapid decrease in Pg concentrations

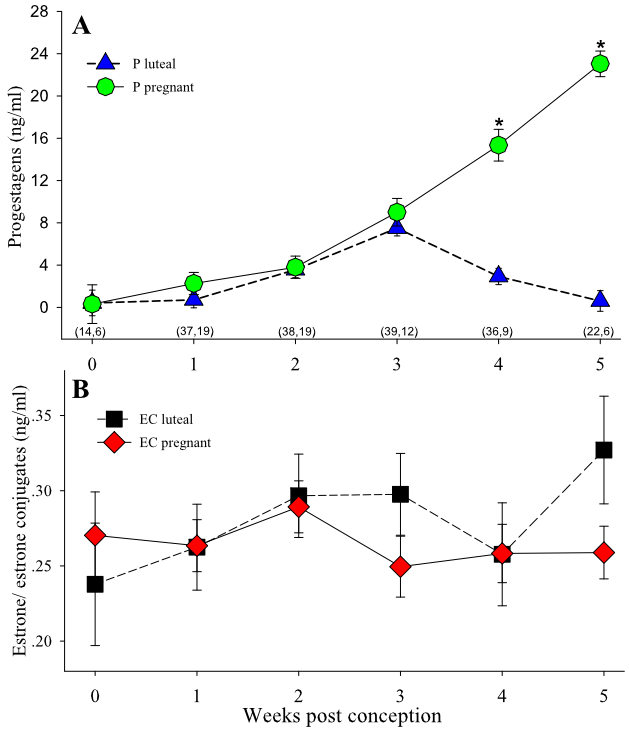

Fig. 5. Weekly serum immunoreactive progestagens (Panel A) and estrone/estrogen conjugates (Panel B) during conceptive and non-conceptive cycles in the killer whale. Day 0 = day of ovulation (confirmed by urinary hormone monitoring and ovarian ultrasound for females undergoing AI or estimated from serum progestagen data). Note the decline ($^{*}P < 0.05$) in progestagen concentrations for non-conceptive cycles compared to conceptive cycles after Week 3. No differences between cycle types were detected in serum concentrations of estrone/estrone conjugates. Values are the mean ± SEM. Values in parentheses (nonconceptive, conceptive cycles) above the x-axis represent the number of data-points used to calculate each mean.

within the last two weeks of gestation (Fig. 6A). From less than 15 d prior to parturition a simple regression model was no different from a mixed model to describe the data ($P = 1.0$), therefore further analysis was performed using linear regression. Based on r^2 values of regression curves starting from Day 15 until birth (3 days post-partum) and repeated iteratively from day 14 until birth, day 13 until birth, etc. though 3 d until birth, day 10 had the "best fit" (Day 11: $r^2 = 0.60$, $P < 0.0001$, Day 10: $r^2 = 0.75$, $P < 0.0001$, Day 9: $r^2 = 0.74$, $P < 0.001$, Day 8: $r^2 = 0.68$, $P < 0.001$). From day 10 prior to parturition until 3 days post-partum, Pg concentrations dropped at a rate of 2.5 ng/day. At parturition, the mean Pg concentration was 9.6 ng/ml (range: 6.8–13.8 ng/ml, $n = 3$) and decreased to 1.7 ng/ml (range 0.33–16.8 ng/ml, $n = 3$) based on data pooled from days 1 to 3 post-partum. A mixed regression of Pg concentrations by DPP (from day 60 to 11 prior to parturition) was significant ($\chi^2 = 4.51$, $P = 0.03$) with progestagens decreasing at a rate of 0.09 ng/day (Fig. 6A). For E2 analysis, no significant effect ($P = 0.46$) of time on hormone concentrations was observed within the 60 days prior to parturition. However, E1/E1-C concentrations rose slowly ($r^2 = 0.07$, $P = 0.02$) until parturition at a rate of 2.1 pg/-day (Fig. 6B).

3.2.8. Hormone concentrations in maternal versus fetal-placental circulation

Serum concentrations of maternal Pg on the day of ($n = 3$) or day prior ($n = 5$) to parturition (13.9 ± 1.95 ng/ml, $n = 8$) were significantly less ($t_{df=4.1} = 4.82$, $P < 0.008$) than those found for serum collected from the umbilical cord (86.1 ± 15.0 ng/ml, $n = 5$). Similarly, maternal concentrations E1/E1-C (0.50 ± 0.12 ng/ml, $n = 4$) and E2 (0.22 ± 0.11 ng/ml, $n = 2$) were significantly less than those of the umbilical cord (E1/E1-C: 13.3 ± 1.6 ng/ml, $n = 5$, $t_{df=4.1} = 8.12$, $P < 0.001$; E2: 13.4 ± 0.94 ng/ml, $n = 5$, $t_{df=4.0} = 13.9$, $P < 0.0001$).

4. Discussion

The results of this research provide the first comprehensive evaluation of circulating concentrations of progestagens and estrogens during normal pregnancy in the killer whale, and yield new information on the relationships of maternal and fetal-derived factors with hormone secretory patterns. Our collective findings based on HPLC analysis of serum pools from the luteal phase and pregnancy stages provide new insight of pregnancy maintenance in the killer whale, which suggests that 5α-DHP may be a biologically important progestagen, particularly during mid to late pregnancy. As shown in previous work (Robeck and Nollens, 2013; Robeck et al., 2015), the gestation length in the current study varied widely, with viable calves born after a gestation of 15.5–18.3 months (mean 17.4 months). Gestation length was shown not to be influenced by factors associated with season (month of conception, or parturition), dam (age, parity), nor fetus (sex) which differs to that for the horse, a seasonally reproductive species, where fetal sex and month of foaling significantly affected gestation length (Davies Morel et al., 2002).

Longitudinal monitoring demonstrated that circulating immunoreactive progestagens were influenced by all the examined time variables (days post-conception, IMPC, gestational stage). Progestagen concentrations increased rapidly to significant peaks early in gestation (starting at IMPC 2), declined at the end of the first trimester (IMPC 5 and 6), increased to a second peak spanning the 9th and 10th months of pregnancy, after which progestagen concentrations remained unchanged until a rapid decline during the last 10 days of pregnancy. This bimodal progestagen pattern confirms an earlier report in killer whales (Duffield et al., 1995). However, contrary to what has been observed in humans (Maccoby et al., 1979; Järvelä et al., 2012), and to the earlier killer whale report using fewer animals and pregnancies (Duffield et al., 1995), no effect of parity on progestagen concentrations was observed in the extensive dataset described herein. Similarly, our results showed that fetal sex did not influence maternal progestagen concentrations, which is in line with most reports in humans (Maccoby et al., 1979; Järvelä et al., 2012), but for swine, umbilical progesterone concentrations were higher for female than male fetuses (Hagen et al., 1983). Because we did not have enough umbilical cord samples from different sexed calves, we cannot determine if a fetal sex effect on progesterone concentrations in cord blood is also present in killer whales.

HPLC results indicate that serum 5α-DHP and pregnanediol were the major progesterone metabolites within maternal circulation during the luteal phase and pregnancy. Pregnanediol is a -biologically inert progestagen, and a major metabolite of P4 in humans where it is produced in the liver or placenta (Atherden, 1959; Tulchinsky and Okada, 1975). Progesterone metabolites are excreted in either the urine or feces with the proportion of excretion being species-dependent. In humans, progesterone is conjugated in the liver to PdG and greater than 50% of this metabolite is then excreted in the urine and around 30% in the bile (Kirkpatrick et al., 1990; Sandberg and Slaunwhite, 1958). In view of the detection of considerable concentrations of pregnanediol in killer whale serum herein and contrary to prior conflicting findings of PdG immunoreactivity in killer whale urine, where some workers found significant PdG excretion during pregnancy (Walker

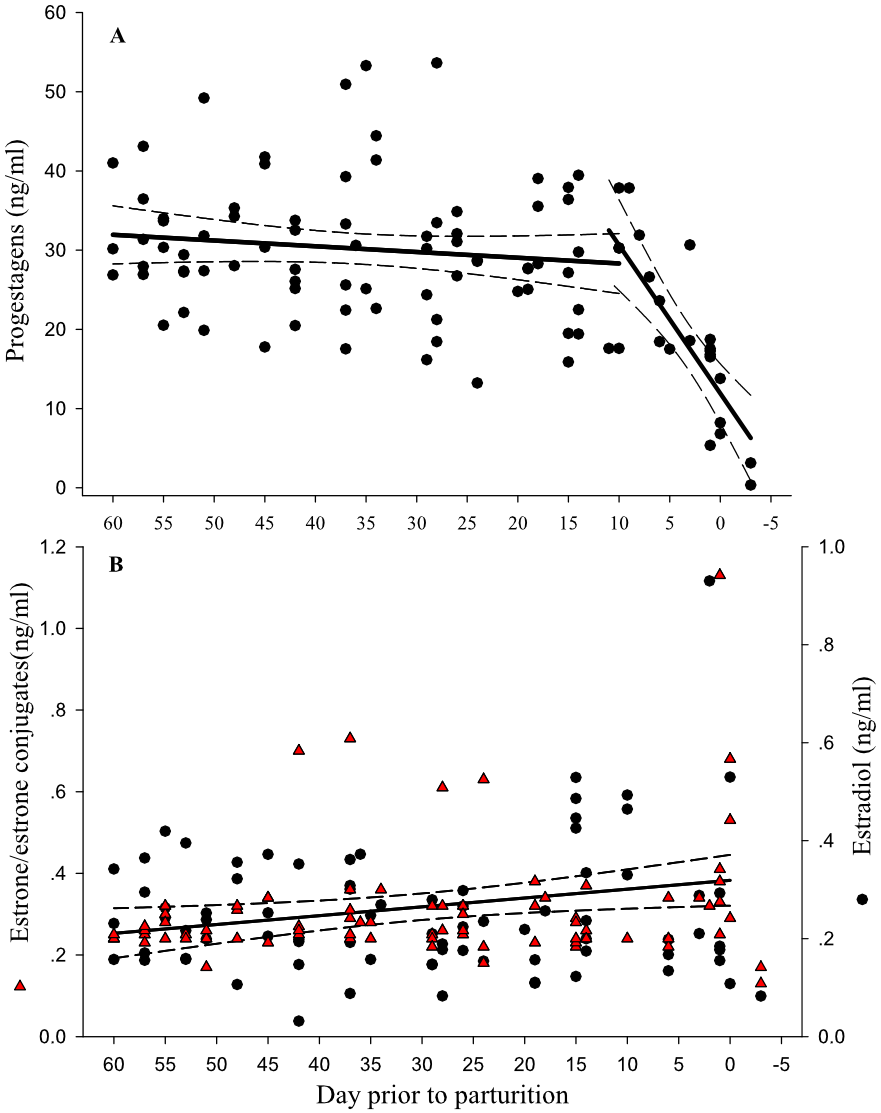

Fig. 6. Scatter plot of serum immunoreactive progestagens (●, Panel A), estrone/estrone-conjugates (■) and estradiol (▲, Panel B) during the last 60 days of pregnancy (DPP) in killer whales. The solid black lines are linear regressions (95% *CI* represented by dotted lines) of progestagen concentrations from −60 to 11 days (Line 1) prior to conception and −10 days to 3 days post-conception (Line 2, Panel A). Predicted mean progestagen concentrations represented by Line 1 significantly decreased at a rate of −0.09 ng/day ($\chi^2 = 4.51$, $P = 0.03$, mixed effects ML regression), this rate accelerated to −2.5 ng/d during the final 10 days of gestation as represented by Line 2 ($r^2 = 0.75$, $P < 0.001$). Line 2 was chosen as the regression line which best (greatest r^2) described the rapid drop in P4 prior to birth based on repeated regression analysis using a range of days starting at day 15 to birth (3 days post partum), then repeating the analysis from 14 to birth, 13 to birth, etc. through 3 d until birth. No significant linear trend was detected between E2 and DPP (Panel B). However, a slight linear (Black line, $r^2 = 0.08$, $P < 0.02$) increasing trend was detected for estrone/estrone conjugates from Days −60 to parturition with concentrations increasing at a rate of 2.1 pg/day (Panel B).

et al., 1988) while others did not (Robeck, 1996), definitive pregnanediol excretory patterns are yet to be determined. Some insight is provided by a recent fecal hormone study during the luteal phase in a single killer whale where virtually no pregnanediol or PdG was detected and the fraction comprising the majority of immunoreactivity co-eluted with P4 (Kusuda et al., 2011), which was similar to our finding whereby serum P4 consistently accounted for the largest immunoreactivity during all phases in our study. However, a complete understanding of the excretory mechanisms of P4 metabolites in killer whales will require further research.

During the luteal phase of killer whales in the current study, the progesterone metabolite 5α-DHP, produced by the enzymatic activity of 5α-reductase, was found in concentrations that were half than those of P4. In humans, high circulating concentrations of 5α-DHP during the luteal phase were initially believed to be the result of target tissue metabolism of progesterone into a bioactive form (Karavolas and Herf, 1971), and support for this theory was demonstrated by a direct negative feedback effect of 5α-DHP on gonadotropin secretion from the pituitary (Zanisi and Martini, 1975). Subsequent efforts determined that, in addition to target tissue metabolism of P4, 5α-DHP was also secreted directly by the ovary in the rat (Ichikowa et al., 1974) and human (Bäckstrom et al., 1986), and that it is the primary progestagen during the luteal phase and early pregnancy in elephants (Hodges et al., 1994, 1997). Those findings in the elephant, combined with evidence that 5α-DHP demonstrated equal affinity as P4 for elephant endometrial P4 receptors, led to speculation that ovarian 5α-DHP was the major bioactive progestagen in the elephant (Meyer et al., 1997). The potent biological activity of 5α-DHP has recently been confirmed in the mare, a species where

pregnanes have long been identified as the primary progestagen during mid to late pregnancy, thus establishing support for the premise that 5α-DHP may be an important bioactive progestagen in other species (Scholtz et al., 2014). In contrast, in the rock hyrax (*Procavia capensis*), a species in which circulating 5α-DHP is found in high concentrations during the luteal phase, and that has high affinity for P4 receptors, it appears that peripheral red blood cells, and not the luteal tissue, are responsible for metabolic breakdown of P4 into 5α-DHP (Kirkman et al., 2001). While the significant contribution of 5α-DHP toward total Pg immunoreactivity lends support for the idea that it is biologically significant, only further research will allow us to determine if its production is due to peripheral metabolism or direct ovarian production.

In addition to the production of 5α-DHP during the luteal phase, we also detected high concentrations of 5α-DHP throughout gestation, with the relative representation of 5α-DHP in total Pg concentrations increasing by 50% in late pregnancy (Fig. 3B). In combination with this trend, the finding that the only major immunoreactive peak in the cord blood was from 5α-DHP suggests that the placenta, at least during the last half of pregnancy, is the major source of 5α-DHP production. In humans, 5α-DHP increases throughout pregnancy from around 4 ng/ml in early pregnancy to well over 27 ng/ml by term, an almost sevenfold increase, and is believed to represent a placental metabolism of P4 (Milewich et al., 1975). Although it appears that luteal P4 production remains the major Pg source during pregnancy, 5α-DHP increases from the last two thirds of gestation toward parturition. This increase in 5α-DHP has also been observed in others species most notably the elephant (Hodges et al., 1994, 1997), manatee (Tripp et al., 2009), and horse (Scholtz et al., 2014), whose major P4 metabolites includes 5α-DHP, and whose secretion pattern tends to mimic placental development (i.e., the ratio of P4: 5α-DHP is high in early pregnancy and low in late pregnancy).

The pattern of progestagen production during the normal killer whale pregnancy reported herein, together with ultrasonographic and histological evidence in this species and other delphinids that the CL is active throughout the entire pregnancy (Harrison and Ridgway, 1971; Robeck et al., 2012; Robeck unpublished observations) and that the CL plays an important role in the maintenance of pregnancy in the killer whale. Prior to the current research, it was unknown if the placenta, or the fetal-placental unit, contributed to circulating progestagen concentrations in this delphinid. In species that do not depend solely on CL-derived progestagens for pregnancy maintenance, the typical pattern of progestagen production includes an initial peak followed by a decline then a secondary peak, the latter of which is attributed to an increase in placental progestagen production (reviewed by Davies and Ryan, 1972). In the killer whale, our findings as well as those by Duffield et al. (1995), also demonstrate a biphasic pattern of progestagen production, with the second hormone rise occurring early in the 2nd trimester during months 7–9. Based on comparative endocrinology, Duffield et al. (1995) postulated that this second peak was indicative of shift toward placental P4 production. Luteal-placental shifts in the production of progestagens occur to varying degrees in many species of mammals and are believed to take place in most species with gestational periods longer than 151 days (Davies and Ryan, 1972). Although placental and fetal contributions toward circulating concentrations of progestagens (specifically 5α-DHP) prior to the 2nd trimester in the killer whale cannot be ruled out, based on the cord blood analysis, our findings indicate that the second progestagen gestational peak may indicate a relative increase in placental production (or more specifically the fetal-placental unit) of 5α-DHP, but that only significant source of immunoreactive P4 appears to be from the CL.

Evidence provided in the current study which supports the hypothesis that, similar to the domestic cow and pig, progestagens are of ovarian and placental origin in the parturient killer whale are twofold. First, and as previously discussed, beyond the early luteal phase (in conceptive cycles) and the early pregnancy stage, we observed a proportional decrease in the secretion of P4 (presumed to be of ovarian origin) as gestation progressed, and an associated increase in 5α-DHP secretion, which, based on HPLC immunograms of umbilical cord blood, we infer reflects 5α-DHP production by the developing placenta. Secondly, progestagen concentrations on the day of parturition of umbilical cord samples were almost eightfold higher than those in the maternal circulation (86.1 and 11.4 ng/ml, respectively), thereby suggesting significant contribution of the fetal-placental unit to circulating progestagen concentrations relative to the CL. Although it was not determined if the cord blood was sourced from the umbilical vein or artery, or if that distinction is important in the diffuse epitheliochorial placentation found in cetaceans (Zhemkova, 1967) versus the hemochorial type found in humans (Harbert et al., 1964; Zander, 1964), the high 5α-DHP concentrations and lack of P4 in cord samples as compared to maternal serum samples suggest that it is placental in origin.

Combined research in bottlenose dolphins indicate that a reduced (compared to luteal production) but biologically important extra-ovarian progestagen source exists in this species (Bergfelt et al., 2011; Robeck et al., 2012). The results from the current research with the killer whale indicate that the majority of immunoreactive placental progestagens, at least at birth, was represented by 5α-DHP and may simply be a result of luteal P4 being metabolized by placental reductase. Further work with placental tissue may allow for enhanced clarity concerning if the fetal placental unit is a significant regulator of progestagen concentrations in the fetal and maternal circulation.

In the present study, progestagen concentrations at week 4 post-ovulation were significantly higher in pregnant than non-pregnant animals, and continued to increase, while analogous values in non-conceptive animals significantly dropped from week 3 to week 4 and returned to baseline (<1 ng/ml) by week 5 (day 35). This divergence in serum progestagens presumptively represents the effects of luteolysis in non-pregnancy and suggests that the maternal recognition of pregnancy (MRP) occurs between day 21 and 28 post-conception in the killer whale. This period is delayed when compared to the bottlenose dolphin where the period of pregnancy recognition is proposed to occur at 12–14 days following conception (O'Brien and Robeck, 2012), which correlates with an approximately 10 day longer estrous cycle for killer whales (42 d, Robeck et al., 2004) as compared to bottlenose dolphins (33 d, O'Brien and Robeck, 2012; 36 d, Robeck et al., 2005).

Results of our circulating estrogens HPLC analysis indicate that E1 with its metabolites and E2 are produced in comparable concentrations during the luteal phase and all periods of pregnancy, whereas E3 concentrations were consistently two to fivefold higher than E1/E1-C or E2 at all examined stages. In humans, E3 is the most abundant estrogen during the luteal phase and pregnancy and it rises continuously toward the end of pregnancy (Peter et al., 1994). However, in killer whales, despite the relative higher production of E3 in comparison with E1/E1-C and E2, no defined pattern of production was documented, except that it had a significant nadir in mid gestation (IMPC 6–8), after which it rebounded to concentrations which were similar to the rest of gestation with a non-significant decline after parturition. In humans, E3 is produced by the placenta through the metabolism of fetal adrenal production of dehydroepiandrosterone sulfate (DHEAS), and abnormally low levels of urinary E3 concentrations during the second trimester are associated with an increased risk of fetal loss (Peter et al., 1994; Santolaya-Forgas et al., 1996; Schleifer et al., 1995). Furthermore, recent evidence suggests that consequences of abnormally low E3 concentrations during the second trimester may be related to fetal growth restriction and low

birth weight (Settiyanan et al., 2015). Because all of the pregnancies evaluated within this research resulted in live births and viable calves, future research will need to determine if even lower concentrations during this period are associated with any of these fetal placental abnormalities found in humans or if some other association with fetal well-being and E3 can be determined. It should be noted that serum concentrations of E3 in human pregnancies undergo a ~33-fold increase by the end of pregnancy when peak values are ~198 ng/ml (Peter et al., 1994), which is markedly higher than the E3 concentrations observed in killer whales herein, which peaked at 2.4 ng/ml, essentially unchanged from pre-pregnancy concentrations. Therefore, despite the observation that E3 was found in the highest concentrations compared to E1/E1-C and E2, it would be surprising to find that circulating E3 concentrations in the killer whale represent useful biomarkers of fetal and placental health as has been observed in humans.

The major circulating E1 metabolite during the luteal phase and pregnancy was E1-G, which was approximately 50% greater than concentrations of either E1 or E1-S during analogous stages. In view of E1-G being the major E1 metabolite in serum, it would be reasonable to expect proportionally high concentrations of this metabolite in killer whale urine. However, using an antibody with similar cross-reactivity as the one used in this research, E1-S was the only immunoreactive urinary estrogen metabolite detected in a previous killer whale study (Walker et al., 1988), so it remains to be determined if the circulating E1-G concentrations detected in the current study are routed for fecal rather than urinary excretion. Conjugation of E2 in the liver and subsequent urinary excretion as E1-S may also occur, and would therefore explain the findings by Walker et al. (1988).

Of the estrogens found in killer whale serum only E1 (and its metabolites) and E2 were significantly elevated in the 3rd trimester of pregnancy compared with all earlier time-points, with both hormones rising slowly throughout gestation and peaking the month prior to parturition. This increase, which may be more pronounced than indicated if blood volume expansions during late gestation were to be considered (Robeck and Nollens, 2013), may reflect fetal-placental production. E1 is the predominant circulating estrogen during pregnancy in the sow (Ash and Heap, 1975; Grzesiak et al., 2014), while E1 and its conjugates predominate in cattle (Hoffmann et al., 1976). Similar to what we observed for E1 and E2, in the sow E1 rises steadily throughout pregnancy toward parturition (Ash and Heap, 1975; Grzesiak et al., 2014). For cattle, sheep and goats, E1 concentrations remain relatively unchanged until 10–20 days prior to term, during which they rise slowly and then peak rapidly 4 days prior to delivery for cattle and on the day of delivery for sheep (Hunter et al., 1977; Challis, 1971) and goats (Challis and Linzell, 1971). For the killer whale, the 28-fold and 33-fold increase in E1/E1-C and E2, respectively, in sera from the umbilical cord compared with the maternal circulation provided some evidence that these estrogens may be placental in source. In cattle, E1/E1-C concentrations in the fetal circulation were almost twice those of maternal values, while E2 concentrations were 15-fold higher in the fetal circulation (Hoffmann et al., 1976). Furthermore, umbilical vein concentrations of total estrogens were double that of maternal serum in the pig, and this was viewed as evidence that estrogen secretion is placental in origin (Ash and Heap, 1975). Additional evidence for fetal-placental production of estrogen in the killer whale is the rapid decline of estrogen concentrations to baseline by day 3 post-partum. This rapid decrease in concentration of estrogens in association with parturition was also observed in the goat (Challis and Linzell, 1971), sow (Ash and Heap, 1975; Baldwin and Stabenfeldt, 1975) and cow (Kindhal et al., 2002). Although both E1/E1-C and E2 were significantly elevated during the last trimester, E2 had a significant increase only during the last month and this may reflect the enhanced biological potency of E2 and increased need for this hormone to help regulate endometrial receptors (e.g., increase in relaxin, oxytocin or prostaglandin receptors) prior to parturition (Challis et al., 2000; Fuchs et al., 1992; Sherwood, 2004; Soloff, 1975). The rapid decline of progesterone combined with the increase in both E1/E1-C and E2 is consistent with the hypothesis for other mammalian species whereby these changes in relative hormone concentrations enhance the mobility and responsiveness of the myometrium before parturition (for review see López Bernal, 2001).

Unlike what has been observed in humans (Troisi et al., 2008), we found no effect of maternal age on concentrations of E1/E1-C, E2 or E3 during pregnancy. However, we did find that multiparous females had significantly lower concentration of E2 than primiparous females across all time component analyses. This approximate relationship where E2 decreases with subsequent gestations is similar to observations in humans and has been postulated to be a consequence of the initial pregnancy(ies) causing a prolonged induction of 16α-hydroxylase activity in the maternal liver, which in turn, increases maternal E2 metabolism (Troisi et al., 2008; Wuu et al., 2002). Because there was no relationship with maternal age and E2 concentrations, our results provide support that this phenomenon is indeed related to pregnancy exposure causing persistent enzyme induction and not maternal age.

Maternal concentrations of E1/E1-C across the entire pregnancy (i.e., as assessed using the time variable DPC) were affected by fetal sex, with killer whales carrying a male fetus having higher concentrations than those carrying a female fetus, but no significant effect was detected when samples were evaluated across trimesters or months. To our knowledge, maternal differences in E1 or E2 concentrations based on fetal sex have not been reported in other animal models (Robertson and King, 1979; Maccoby et al., 1979). However, in human amniotic fluid, E2 was higher in the presence of a female fetus, while E1 was non-significantly higher for females carrying a male fetus (Robinson et al., 1977). This difference is attributed to the selective increased aromatization of androgens to E1 versus E2 by the human placenta (Robinson et al., 1977). In killer whales, it is unknown what biological significance can be attributed to this phenomena, considering the high cross-reactivity of the antibody utilized herein with E1 and its conjugates, and because the more biologically potent estrogen, E2, showed no tendency for this relationship. However, as suggested for human amnionic fluid, elevated maternal serum concentrations of E1 may reflect higher fetal androgen production by the male fetus that in turn results in increased placental E1 production and sequestration into the maternal compartment. Future evaluation of maternal androgens during pregnancy may provide more insight into the mechanisms associated with our findings.

Although the list of threats to wild killer whale populations vary according to their ecotype, reduced prey availability and environmental contaminants represent key threats to the species on a global level (Ross et al., 2000; Ford et al., 2009; Krahn et al., 2009). Defining endocrine changes during normal gestations as described in the research will establish baseline information from which adverse biological responses to the aforementioned threats may be detected. Our dataset of normative steroid concentrations and profiles during the pregnancy of the killer whale can be integrated into ongoing reproductive health monitoring of captive killer whales, and collective findings highlight noteworthy areas of future research regarding the species' gestational biology and that of other Delphinids.

Acknowledgments

We thank the animal care laboratory, animal training and veterinary staff at SeaWorld Orlando, San Antonio, and San Diego,

and Loro Parque, Spain for their assistance with sample collection. We thank the technical staff at the SeaWorld and Busch Gardens Reproductive Research Center for their assistance with sample analysis. We thank Alan Fetter of San Diego Zoo Global for assistance with HPLC analysis. Brad Andrews is also thanked for his continued support of this project. This research was conducted under the NMFS permit number 16193, was funded by SeaWorld Parks and Entertainment, Inc. and is a SeaWorld technical contribution # 2015-23C.

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