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Research paper

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

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ABSTRACT

The secretory patterns of testosterone (T), androstenedione (A4), dehydroepiandrosterone (DHEA), cortisol (C), and corticosterone (Co) were characterized throughout 28 normal pregnancies until two-months post-partum in eleven killer whales. Effects of fetal sex, dam parity or age, and season were evaluated across either day post-conception (DPC), stage of pregnancy (PRE, EARLY, MID, LATE, POST) or indexed month post-conception (IMPC) using a mixed model linear regression with animal ID and pregnancy number as the random variables. Across DPC, DHEA, A4 and T concentrations were affected ($P < 0.05$) by season, with highest concentrations during spring (DHEA, A4, & T) and summer (A4) as compared to the fall. A significant effect of parity on androgen production was observed only for DHEA, with multiparous females having higher ($P = 0.01$) concentrations than nulliparous females. All three androgens significantly increased with each successive pregnancy stage and IMPC with peak concentrations occurring during IMPC 10 (DHEA), 13 (A4) and 14 (T), respectively. Cortisol was affected by season ($P = 0.03$) with highest concentrations being detected during the months of fall, while Co was only affected by parity ($P = 0.003$) with significant increases observed for primiparous females as compared to nulliparous females. Cortisol and Co concentrations peaked ($P < 0.05$) during IMPC 17 (i.e., the month prior to parturition). The C to Co ratio during pregnancy was 7.4 to 1, indicating that cortisol is the major circulating glucocorticoid studied to date in pregnant killer whales. The significant increase in concentrations of maternal androgens throughout pregnancy, which were unrelated to fetal sex, indicates that they play an important role during killer whale fetal development.

1. Introduction

Despite healthy global populations of killer whales, evidence suggests that increasing numbers of regionally sympatric populations or ecotypes are being adversely affected by anthropogenic stressors (Poncelet et al., 2010; NMFS, 2008). These stressors include direct competition for resources (food or space) and indirect pressures such as environmental contaminants (Ford et al., 2009; Krahn et al., 2009; Noren et al., 2009; Ward et al., 2009). Understanding how these factors can affect the reproductive biology of killer whales is extremely important for long-term survival and health of these and any wild animal population. This drive to define the reproductive state of free-ranging animals has led to increased field efforts toward collecting and analyzing biologic samples across numerous taxa (Krey et al., 2015; Norman et al., 2012; Wells et al., 2014). However, in view of the difficulties in collecting longitudinal samples from individual free-ranging animals, normal hormone profiles that occur during different reproductive states (eg., pregnancy, estrus, anestrus) or in response to individual animal variations (parity and/or age), and in regard to potential seasonal and/or environmental effects, must be defined before meaningful interpretations of wild animal data can be accomplished.

Recent efforts to define hormone profiles during pregnancy in captive killer whales has led to an increased understanding of the secretion of progestagens and estrogens during normal pregnancy, and the effects of female parity and age on changes that may occur in the production of these hormones (Robeck et al., 2016). These results established the presence of a bimodal pattern of progestagen secretion during pregnancy, and provide support for placental production of progestagens beginning during the 6th and 7th gestational month. Despite such placental contribu-

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tions, results provided additional evidence that progesterone is the dominant progestagen and the CL is the primary source of circulating progesterone throughout pregnancy. The study also demonstrated that estrone and its conjugates and estradiol increase in late pregnancy, with estradiol concentrations being significantly affected by female parity. While this recent research is the most comprehensive examination of hormone concentrations during pregnancy in the killer whale to date, no information concerning androgens or glucocorticoids was provided and very little has been published for the species in general.

Androgen concentrations during pregnancy vary widely between species. In the rat they appear to increase at midpregnancy, are placental in origin and serve as precursors for luteotropic estrogens (Keyes et al., 1980). In the mare, testosterone concentrations peak at 7 months and decrease to baseline prior to parturition (Silberzahn et al., 1984). This peak in maternal testosterone is believed to represent an intermediate metabolic step in the placental conversion of fetal gonad DHEA toward 17β-estradiol. In the bitch, testosterone concentrations are elevated during the late follicular phase and peak in association with ovulation (Concannon and Castracane, 1985). Androstenedione production in the bitch remains elevated after a preoulatory peak until 2 to 8 days prior to parturition or until the end of the luteal phase (Concannon and Castracane, 1985). Androstenedione appears to parallel progesterone production in the bitch and is believed to be a major precursor for estrogen production during the luteal phase and pregnancy. Abnormally elevated concentrations of testosterone during pregnancy have been negatively associated with fetal size at birth in primates (Carlsen et al., 2006) and sheep (Manikkam et al., 2004), suggesting they are an indicator of fetal health. In addition, maternal concentrations of testosterone have been demonstrated to vary depending on when carrying a male or female fetus(es) in rats (Weisz and Ward, 1980), cattle (Mongkonpunya et al., 1975) and elephants (Duer et al., 2002). In the bottlenose dolphin (*Tursiops truncatus*), a species in the same phylogenetic family as killer whales (Delphinidae), serum testosterone rose significantly by month 4 post-conception, peaking in month 9 with no effect of fetal sex being detected (Steinman et al., 2016). Characterization of testosterone secretion during normal pregnancy in killer whales would be an important first step towards understanding its role in fetal development and health.

Cortisol concentrations during pregnancy have been described as important for fetal organ development (Ballard and Ballard, 1995; Bolt et al., 2001; Liggins, 1994), fetal initiation of labor (Challis et al., 2000; Davies and Ryan, 1972; Hoffman et al., 1976), and are related to the degree of maternal stress during labor (Okada et al., 1974). The only description of cortisol concentrations during pregnancy in a killer whale described 4-month cyclic changes in concentrations which were negatively correlated with serum progesterone (Suzuki et al., 2003). However, in this study, hormone concentrations were not indexed relative to conception or parturition. In the bottlenose dolphin, cortisol concentrations rose throughout pregnancy peaking significantly in the final month of gestation (Steinman et al., 2016). However, no other information was presented concerning changes associated with conception or parturition.

To our knowledge, no publications exist that describe androgens or their precursors during pregnancy in the killer whale. This, together with the paucity of data concerning glucocorticoid production during pregnancy formed the basis of our research goal which was to characterize these hormones from conception to birth in normal killer whale pregnancy. Specific objectives were to: 1) characterize profiles of serum immunoreactive dehydroepiandrosterone, androstenedione, testosterone, cortisol and corticosterone during months and stages of pregnancy (2) examine the influence of season of sample collection, dam age, parity and fetal sex on serum hormone concentrations and; (3) compare periparturient concentrations of circulating maternal hormones with those from the placenta after a normal birth.

2. Materials and methods

All procedures described herein were reviewed and approved by the SeaWorld Parks and Entertainment Incorporated Institutional Animal Care and Use Committee, and were performed in accordance with the U. S. Animal Welfare Act.

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 yearround. 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 to 3% of their body weight per day. All food fish was graded for human consumption. Animals were supplemented with Vita-Zu Marine Mammal tablets (Mazuri, St. Louis, MO, USA), which contain multi-vitamins and folic acid. Inclusion criteria were gestations from nulliparous, uniparous and multiparous dams, with a gestation that resulted in a calf that was alive and nursing at 6 months post-delivery, and for which at least monthly blood data were available throughout gestation.

Blood samples (n = 994 samples) were collected as part of routine health assessments during 28 pregnancies (a mean 35 samples/pregnancy) from June 1983 until Dec 2014. In addition, 85 samples were collected during 80 non-conceptive (luteal phase) estrous cycles in eleven whales. Samples were retrospectively determined to be luteal phase, if they were from a whale that was experiencing estrus cyclicity (based on behavioral and urinary hormone monitoring) and had serum progesterone concentrations greater than 2 ng/ml. Samples were collected voluntarily by staff veterinarians using conditioned behavior from the ventral tail fluke using a 19 gauge winged blood collection set. 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 to 30 min. Serum was collected and stored at −80 °C until analysis.

2.2. Reproductive status and stages of pregnancy

Precise conception dates were known from the monitoring of urinary estrone/estrone conjugate concentrations combined with breeding observations (n = 8) or from dates of artificial insemination (n = 5). For gestations in which the conception date was not known (n = 15), it was designated as the midpoint between the last serum sample with baseline progesterone concentration (<0.5 ng/ml) and the first sample displaying progesterone concentrations above baseline (if the interval exceeded 14 d, data from that female were not included in analyses).

Serum concentrations of each hormone analyzed during each of the 28 pregnancies from 11 animals were categorized into time components based on their relative relationship to conception or parturition (further details are provided in Section 2.4). For gestational stages, samples were designated as one of the five following stages: (1) Pre-conception (PRE) – two months prior to conception; (2) Early gestation (EARLY) – samples collected after conception (>day 2 post-ovulation) through month five; (3) Mid-gestation (MID) – month six through month 11; (4) Late gestation (LATE) –

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