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Characterization of estrogens, testosterone, and cortisol in normal bottlenose dolphin (*Tursiops truncatus*) pregnancy

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ABSTRACT

The goal of this study was to describe profiles of serum estrogens, testosterone and cortisol during normal pregnancy in bottlenose dolphins. Predominant estrogens in all categories of dolphin sera pools during estrus and pregnancy (EARLY: Days 0–120; MID: Days 121–240; LATE: Days 241 to parturition; Day 0 = day of conception) were estrone/estrone conjugates (E1-C) and estriol (E3). Serum samples collected throughout 101 normal pregnancies were analyzed for E1-C, E3, testosterone (T) and cortisol (CORT). E1-C was higher ($P < 0.05$) during LATE compared to EARLY and MID, and higher ($P < 0.05$) in nulliparous than multiparous females. E1-C concentrations were also inversely associated with maternal age ($P = 0.05$). E3 was higher ($P < 0.05$) in EARLY than MID and LATE, and higher overall for nulliparous than multiparous females, but concentrations were similar among gestational stages when parity was excluded from analyses. Analysis by indexed month post-conception (IMPC) demonstrated that E1-C increased from IMPC 9 and peaked at IMPC 11. E3 was significantly elevated during IMPC 1, decreased until IMPC 6 and peaked at IMPC 11. T increased ($P < 0.05$) at IMPC 3 and continued to increase throughout gestation ($P < 0.05$). CORT was higher ($P < 0.05$) during LATE compared to EARLY and MID ($P < 0.05$), peaked during IMPC 12, and was not affected by parity. Hormone profiles were not influenced by fetal sex.

1. Introduction

For the purposes of describing reproduction in the bottlenose dolphin (*Tursiops truncatus*), there have been numerous studies on the species' reproductive endocrinology during various physiological states, including estrus and pregnancy. Studies that have been directed toward describing the endocrine changes during bottlenose dolphin pregnancy have usually relied on circulating progesterone analysis as a means to diagnose pregnancy via temporal and quantitative patterns, with data available for the last 11 months (Cornell et al., 1987; Richkind and Ridgway, 1975; Sawyer-Steffan et al., 1983) or for the entire 12 month gestation (O'Brien and Robeck, 2012). Although there exist numerous historical examples of documenting pregnancy based on progesterone monitoring alone, evidence indicates that serum progesterone concentrations of animals with early pregnancy loss or of those housed without males can mimic concentrations of normal pregnancy (O'Brien and Robeck, 2012; Robeck et al., 2001, 2013). Because the earliest ultrasonographic indicators of pregnancy (thickened endometrium and uterine horn fluid) are observed no earlier than 44 days post-conception in the bottlenose dolphin, endocrine markers of a viable conceptus would be helpful to identify, particularly during the preimplantation stages when the majority of pregnancy loss appears to occur (O'Brien and Robeck, 2012; Robeck et al., 2013).

Analysis of serum samples collected at regular intervals across 28 pregnancies showed that bottlenose dolphin relaxin concentrations increase during the different stages of pregnancy, peaking in late gestation (Bergfelt et al., 2011). Based on data from 28 pregnancies, concentrations of thyroid hormones (total and free thyroxine and triiodothyronine) were significantly higher in the first four months of pregnancy (early pregnancy) compared to non-pregnant animals (West et al., 2014). Such findings suggest that thyroid hormones may serve as an early indicator of pregnancy in this species but only when there are non-pregnant samples to use as a comparison.

With the exception of the aforementioned research on gestational progesterone profiles and to a lesser extent, relaxin (Bergfelt et al., 2011) and thyroid hormones (West et al., 2014), the endocrinology of the pregnant dolphin remains poorly understood, especially with regards to estrogens, glucocorticoids, and androgens. Two studies have analyzed estrogens during gestation in the bottlenose dolphin. The first measured estrogens at three, six and nine months of gestation with an increasing trend observed

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during the progression of gestation (Richkind and Ridgway, 1975). A second study examined pregnant, ovulatory and ovariectomized dolphins (Sawyer-Steffan et al., 1983). Estrogens were measurable in all reproductive categories, and samples from pregnant animals had the highest concentrations, but low sample size precluded the ability to clearly characterize estrogen concentrations across pregnancy.

In killer whales, concentrations of urinary estrogen conjugates increase during the third to sixth months of gestation, continuing to rise through the tenth month and then plateau until parturition (Walker et al., 1988). Analyzing estrogen concentrations in bottlenose dolphin serum throughout pregnancy and identifying principal estrogens and metabolites at different gestational stages would be of interest to further characterize the endocrinology of this reproductive state, and would be a useful husbandry tool if an early identifier of pregnancy is revealed.

To our knowledge, no past research has evaluated androgen concentrations in female bottlenose dolphins. Longitudinal measurements of androgens in humans demonstrate a slow increase throughout gestation until birth (Kerlan et al., 1994). Maternal testosterone concentrations during pregnancy are inversely related with birth weight in humans (Carlsen et al., 2005), suggesting that maternal androgens influence the intrauterine environment and the developing fetus, and might therefore serve as an indicator of fetal health. For further characterization of gestation in the bottlenose dolphin, it would be of use to examine the relationship of maternal androgen concentrations with fetal sex, as this has not been reported in any marine mammal.

Glucocorticoids (GCs) have been analyzed in bottlenose dolphins during pregnancy and outside of pregnancy (Richkind and Ridgway, 1975; St Aubin et al., 1996; Suzuki et al., 1998, 2003). GC concentrations remain unchanged during pregnancy (Richkind and Ridgway, 1975) but this study had a limited number of animals and samples and additional data derived from serial sampling efforts would be beneficial to our understanding of GC production during different gestational stages. In a pregnant killer whale, serum cortisol concentrations fluctuated during gestation and were inversely related to those of progesterone (Suzuki et al., 2003) but changes in cortisol and other GCs occurring around parturition have not been documented in any cetacean. The ability to quantify GC profiles throughout bottlenose dolphin pregnancies could aid with predicting the timing of parturition and potentially improve methodologies for the management of labor in this species (Robeck et al., 2012).

A better understanding of the endocrine milieu throughout normal pregnancy can add to the reproductive database of the bottlenose dolphin and may assist with identification and clinical management of abnormal pregnancy. The overall goal of this study was to describe profiles of serum estrogens, androgens (testosterone) and GCs (cortisol) during normal pregnancy in the bottlenose dolphin. The specific objectives were to: (1) identify the estrogens and their metabolites present in serum and urine during estrus, early, mid and late gestation utilizing high performance liquid chromatography and determine the optimal estrogen antibodies for sample analysis during pregnancy monitoring; (2) characterize profiles of serum estrogens, testosterone and cortisol during months and stages of pregnancy; and (3) examine the influence of parity and fetal sex on serum estrogen, testosterone and cortisol concentrations during months and stages of pregnancy.

2. Materials and methods

All samples were collected as part of routine husbandry procedures for the bottlenose dolphin. All procedures described within 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 for the care of Marine Mammals.

2.1. Study animals

Serum samples were collected from 45 animals and 101 normal pregnancies (11 of the 101 were the result of artificial insemination and 20 animals were nulliparous) as classified by those that resulted in the birth of a live calf which survived for at least 30 days. Animals were housed at SeaWorld Parks in Orlando, San Antonio and San Diego. Animals were housed in enclosures containing $\geqslant 850\ m^3$ of either natural processed and manufactured salt-water (ambient temperature, approximately 14–28 °C). Diet consisted of frozen-thawed whole fish (herring, *Clupea harengus*; capelin, *Mallotus villosus*, and Columbia River smelt, *Thaleichthys pacificus*) fed at approximately 4–5% of body weight per day.

2.2. Sample collection

Blood samples ($n = 437$ samples) were collected voluntarily from animals during pregnancy from August 1980 through October 2013. Samples were collected from the ventral tail fluke using a 19 gauge winged blood collection set. Blood was collected into BD Vacutainers (Becton Dickinson, ThermoFisher, Waltham, MA, USA) containing activated thrombin. The thrombin-coagulated blood was centrifuged at 1500 rpm for 10 min, and the serum was decanted and frozen at −80 °C for further testing. Although sampling time was not recorded for every sample, routine blood samples were collected in the mornings (before 12:00 h) as per standard husbandry procedure. Urine samples were collected as previously described (Lenzi, 2000) from unrestrained animals as part of routine endocrine monitoring. Urine samples were aliquoted and stored frozen at −80 °C. The effects of long term storage at ultra-low temperatures on hormone concentrations in bottlenose dolphin sera are unknown and were outside the scope of this study. However, there have been numerous studies with human sera that have demonstrated that hormone concentrations are unchanged (or non-statistically different) when stored frozen (estrogens: stable after 10+ years frozen storage (Kley et al., 1985); cortisol and testosterone: unchanged after 40+ years frozen storage (Stroud et al., 2007)).

2.3. Hormone assays

All hormone concentrations were expressed as ng hormone per ml serum.

2.3.1. Estrogen high performance liquid chromatography (HPLC) analysis

HPLC analysis was performed to identify the immunoreactive estrogens and their metabolites present in bottlenose dolphin urine and serum during different stages of reproduction (ESTRUS and 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 C18 HPLC column (4 μm, 3.9 × 155 mm, NovaPak Waters Corporation, Milford, MA, USA). A pool of bottlenose dolphin urine or serum (ranging from 8 to 14 ml, depending on how much serum or urine was available from 14 different animals and 17 different pregnancies) from these different 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 0.15 ml MeOH (HPLC Grade, ThermoFisher), resuspended by vortexing, and then 0.05 ml loaded onto the column. A 20–80% linear gradient of methanol:water (HPLC Grade, Sigma Aldrich,