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Relaxin as a hormonal aid to evaluate pregnancy and pregnancy loss in bottlenose dolphins (*Tursiops truncatus*)

Don R. Bergfelt [a,*], Jason L. Blum [b], Bernard G. Steinetz [b], Karen J. Steinman [c], Justin K. O'Brien [c], Todd R. Robeck [c]

[a] Department of Biomedical Sciences, Ross University School of Veterinary Medicine, Basseterre KN 00265, Saint Kitts and Nevis, West Indies
[b] Department of Environmental Medicine, New York University School of Medicine, Tuxedo, NY 10987, United States
[c] SeaWorld and Busch Gardens Reproductive Research Center, San Diego, CA 92109, United States

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ABSTRACT

This study was conducted to critically evaluate weekly and monthly circulating concentrations of immunoreactive relaxin throughout pregnancies that resulted in live births, stillbirths, and abortions in aquarium-based bottlenose dolphins. A relaxin RIA was used to analyze serum collected during 74 pregnancies involving 41 dolphins and 8 estrous cycles as well as 8 non-pregnant dolphins. Pregnancies resulted in live births ($n = 60$), stillbirths ($n = 7$), or abortions ($n = 7$). Relative to parturition (Month 0), monthly changes ($P < 0.0001$) in relaxin was indicated by relatively low concentrations during early pregnancy (Months −12 to −9) which subsequently increased ($P < 0.05$) during mid- (Months −8 to −5) to late (Months −4 to −1) pregnancy; relaxin was highest ($P < 0.05$) at the time of parturition. Post-parturition (Month 1), concentrations decreased ($P < 0.05$). During the first 4 weeks post-ovulation, relaxin concentrations were not different between pregnant and non-pregnant dolphins (status-by-week interaction, $P = 0.59$). Status-by-month interaction ($P < 0.0002$) involving different pregnancy outcomes was due, impart, to an increase in relaxin during early pregnancy ($P < 0.05$) that was comparable among dolphins with live births, stillbirths, and abortions except concentrations were lower ($P < 0.05$; 52%) at mid-pregnancy in association with pregnancy loss. Thereafter, concentrations increased ($P < 0.05$) during late pregnancy in dolphins with stillbirths but not in dolphins with abortions. In conclusion, this study provided new information on the pregnancy-specific nature of relaxin, critical evaluation of the fundamental characteristics of relaxin during pregnancy and pregnancy loss, and clarification on the strengths and limitations of relaxin as a diagnostic aid to determine pregnancy status and assess maternal–fetal health in bottlenose dolphins.

1. Introduction

In bottlenose dolphins, relaxin has only recently has been detected and evaluated for basic and applied purposes (review Bergfelt et al., 2014). A heterologous relaxin radioimmunoassay (RIA) was developed and validated using human relaxin, rabbit anti-porcine relaxin, and synthetic canine relaxin to initially characterize immunoreactivity in samples collected periodically during an approximate 12-month gestation period in aquarium-based dolphins (Bergfelt et al., 2011). In reference to parturition (Month 0), serum relaxin concentrations progressively increased from relatively low concentrations during early pregnancy (Months −11 to −8) to intermediate concentrations during mid-pregnancy (Months −7 to −4), to high concentrations during late pregnancy (Months −3 to 0). However, due to the limited number of samples the results were combined and analyzed over three, 4-month periods or trimesters (early, middle, late) in the previous study (Bergfelt et al., 2011), a more frequent or critical evaluation of circulating concentrations of relaxin is needed to better characterize the temporal changes throughout pregnancy for research and clinical application in bottlenose dolphins and, perhaps, other cetaceans.

While the role of relaxin in animals has historically been associated with reproduction, especially during pregnancy to facilitate parturition (e.g., increased cervical dilatation and pelvic area expansion), it is also recognized as a multifunctional endocrine and paracrine hormone or factor with many roles in female and

* Corresponding author.
E-mail addresses: dbergfelt@rossvet.edu.kn (D.R. Bergfelt), Jason.Blum@nyumc.org (J.L. Blum), berniesteinetz@gmail.com (B.G. Steinetz), Karen.Steinman@SeaWorld.com (K.J. Steinman), Justine.OBrien@SeaWorld.com (J.K. O'Brien), Todd.Robeck@SeaWorld.com (T.R. Robeck).

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male reproduction, the central nervous system, and cardiovascular system (Bathgate et al., 2013; Sherwood, 2004). The structure and heterogeneity of relaxin among species has been reviewed (Bathgate et al., 2013; Sherwood, 2004). In general, relaxin is a 6-kDa heterodimeric polypeptide with A and B chains joined by 3 disulfide bonds. In humans, relaxin is encoded by 3 genes forming H1-, H2-, and H3-relaxin with H2 as the most prominent (Bathgate et al., 2006). Although the size and 2-chain composition of relaxin are comparable within and among species, the degree of homology of the amino acid sequence of relaxin varies within and among species (Sherwood, 2004). In cetaceans, relaxin immunoreactivity was first reported in extracts of luteal tissue from pregnant Antarctic minke (*Balaenoptera acutorostrata*) and Bryde's (*Balaenoptera edeni*) whales using anti-porcine relaxin (Schwabe et al., 1989). Based on a mouse pubic symphysis assay, relaxin bioactivity from the two whales appeared similar to porcine relaxin. Structurally, pig relaxin differed from whale relaxin in number, absence, or presence of several amino acids. Between the minke and Bryde's whales, the amino acid sequence differed by 2–3 residues.

There is extensive diversity regarding the characteristics of relaxin throughout pregnancy among laboratory (e.g., primates, rats, rabbits; Hayes, 2004; Sherwood et al., 1980; Sherwood, 1994), companion (e.g., dogs, cats; Steinetz et al., 1987; Stewart and Stabenfeldt, 1985), domestic (e.g., horses, pigs, sheep; Stewart and Stabenfeldt, 1981; Gordon, 2004; Porter, 1993), and wild (e.g., elephants, rhinoceros, leopards; Steinetz et al., 2005, 2009) animal species. In bottlenose dolphins, the increase in relaxin concentrations that occurs during late pregnancy (Bergfelt et al., 2011) has provided preliminary evidence suggesting that relatively high concentrations prior to parturition may play a role during the birthing process comparable to that in other mammalian species (Porter, 1993; Sherwood, 1994). In addition, elevated concentrations of relaxin may also serve as a diagnostic aid to hormonally detect pregnancy status as proposed in other wildlife species (Bergfelt et al., 2014; Steinetz et al., 2005, 2009).

The implication that relaxin may serve as a hormonal indicator of pregnancy in bottlenose dolphins as proposed in other wildlife species (Bergfelt et al., 2014; Steinetz et al., 2005, 2009) is based, in part, on the pregnancy-specific nature of relaxin in dolphins. Relatively high concentrations of relaxin pre-partum subsequently decreased during the early post-partum period (Bergfelt et al., 2011), concentrations above baseline indicative of pregnancy were detected in pregnant but not in non-pregnant dolphins (Bergfelt et al., 2013), and high concentrations of relaxin (1–3 mg/kg) were detected in tissue extracts from multiple full-term placentas of dolphins (Bergfelt et al., 2011). While the placenta as a source of relaxin in bottlenose dolphins requires clarification, relaxin has also been detected in extracts of luteal tissue from pregnant whales (Schwabe et al., 1989). It is not known if the corpus luteum (CL) of the estrous cycle is a source of relaxin in cetaceans as it is in other mammalian species (Bathgate et al., 2006; Park et al., 2005; Sherwood, 2004). Nonetheless, evaluation of circulating concentrations of relaxin during the estrous cycle and corresponding time during early pregnancy will further clarify the pregnancy-specific nature of elevated concentrations of relaxin during pregnancy for diagnostic purposes in dolphins.

In a preliminary study (Bergfelt et al., 2013) to evaluate the potential of relaxin to diagnose pregnancy in free-ranging bottlenose dolphins, relaxin and progesterone were analyzed in the same samples collected during capture-release health assessments (Fair et al., 2006). Based on photo-identification of cow-calf pairs, progesterone was diagnostic of pregnancy in 9 of 9 dolphins and relaxin in 7 of 9 dolphins. The basis for a false negative in two of the dolphins was speculated to be a result of relatively low concentrations during early pregnancy as previously reported (Bergfelt et al., 2011). However, because the previous study combined relaxin concentrations for the first four months of gestation as representative of early pregnancy, a more critical evaluation of relaxin is needed to characterize month-to-month concentrations during early pregnancy and clarify the strengths and limitations of relaxin as a hormonal aid to diagnose pregnancy status.

Relative changes in circulating concentrations of relaxin during pregnancies that have resulted in stillbirths in bottlenose dolphins were not significantly different to those pregnancies that resulted in live births except that mean concentrations were 42%, 29%, and 34% lower at early, mid-, and late pregnancy, respectively (Bergfelt et al., 2011). The physiological basis for lower concentrations of relaxin in association with stillbirths is not known. In the previous study (Bergfelt et al., 2011), stillbirth was defined to include females in which calves were born dead or died shortly after birth (24–48 h) as well as late-term, spontaneous abortions. Considering the timing and etiology between stillbirths and abortions are not necessarily identical, relaxin may be differentially affected under these varied conditions. Characteristics of circulating concentrations of relaxin between stillbirths and spontaneous abortions are not known in bottlenose dolphins.

The present study was conducted independently from a previous study (Bergfelt et al., 2011) to provide a more comprehensive (weekly and monthly) evaluation of circulating concentrations of immunoreactive relaxin throughout pregnancies that resulted in live and viable births, stillbirths, and spontaneous abortions in aquarium-based bottlenose dolphins. In addition, the study was designed to further evaluate the pregnancy-specific nature of relaxin and clarify the strengths and limitations of relaxin as a potential hormonal aid to diagnose pregnancy status in dolphins and, perhaps, other cetaceans.

2. Materials and methods

2.1. Dolphins and dolphin management

The present study involved a retrospective analysis of archived serum samples from bottlenose dolphins that were collected from 1988 to 2012 during routine animal husbandry practices as part of a proactive health and breeding program at SeaWorld Parks and Entertainment (San Diego, CA; San Antonio, TX; Orlando, FL, USA). Animals were fed and managed in compliance with the US Animal Welfare Act and by the Standards and Guidelines of the Alliance of Marine Mammal Parks and Aquariums. Practices and procedures associated with the study were reviewed and approved by SeaWorld's Animal Care and Use Committee.

A total of 41 adult female bottlenose dolphins (>6 yr and $\geqslant$180 kg) involving 74 pregnant animals or pregnancies and 8 bred, non-pregnant animals or estrous cycles are represented in the present study. Of the 74 pregnancies, 60 resulted in live and viable births, 7 stillbirths and 7 spontaneous abortions. Stillbirth is defined as birth of a dead calf at term (>355 d post-estrus/ovulation; O'Brien and Robeck, 2012).

Transabdominal ultrasonography was used daily to detect ovulation after artificial insemination (AI; Robeck et al., 2005) or weekly to confirm pregnancy by detection of an embryonic vesicle or fetus (Williamson et al., 1990), and diagnose abortion, which was based on observation of a conceptus ultrasonographically at one examination and failure to detect it at the next examination (O'Brien and Robeck, 2012; Robeck et al., 2013). Ultrasonographic examinations were done with a GE Logiqbook (GE LogiqGE Medical Systems, Milwaukee, WI, USA) or an Aloka 900 (Aloka, Wallingford, CT, USA) using a 3.5 MHz wide footprint convex linear-array transducer.

Dolphins were generally housed with other male and female adult and juvenile animals in pools containing $\geqslant$850 m^3 of natural processed salt water or were housed in numerous connected ocean

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