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

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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 (Month -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 wk 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, in part, 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.

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Keywords

Bottlenose dolphins; Relaxin; Pregnancy; Stillbirth; Abortion

1. Introduction

In bottlenose dolphins, relaxin has only recently 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 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 to 3 residues.

There is extensive diversity regarding the characteristics of relaxin throughout pregnancy among laboratory (e.g., rats, rabbits; Sherwood et al., 1980; Sherwood, 1994), companion (e.g., dog, cat; Steinetz et al., 1987; Stewart & Stabenfeldt, 1985), domestic (e.g., pigs, sheep; Gordon, 2004; Porter, 1993), and wild (e.g., elephants, rhinoceros, leopards; Steinetz et al., 2005 and 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, 2005 and 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, 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 to 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 to 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 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 850 m³ of natural processed salt water or were housed in numerous connected ocean pools holding 300 to 600 m³ of salt water (ambient temperatures 14 to 28°C). Dolphins were fed at 4 to 5% of their body weight per day with a diet of frozen/thawed whole fish: herring, *Clupea harengus*; capelin, *Mallotus villosus*; and Columbia River smelt, *Thaleichthys pacificus*.

2.2 Collection of blood samples

Blood samples were collected voluntarily from dolphins using preconditioned behavior. In some instances (<10%), samples were collected at the time of AI, which required mild sedation (diazepam, 0.2 mg/kg, po) to stabilize the animals during the insemination

procedure. Preconditioned behavior consisted of signaling the dolphins to move into a dorsal or ventral-up position. Blood was collected via venipuncture of vessels in the ventral aspect of the tail fluke using a 19 to 21-ga winged butterfly needle attached to a Vacutainer tube containing activated thrombin and centrifuged at approximately 1000 x g for 10 min. Serum was decanted into vials which were labeled with dolphin ID and date, and stored at -80°C.

Blood samples were collected once a week for approximately 28 d beginning from day of estimated natural breeding during estrus or ultrasound detected ovulation after AI. Thereafter, samples were collected opportunistically in accord with management practices. Generally, blood samples were collected episodically, at least once a month during early and mid-pregnancy and less frequently during late pregnancy until early post-partum or early post-abortion.

Serum samples for analysis of relaxin (n = 376) were shipped frozen on dry ice from SeaWorld to NYU Langone School of Medicine, Department of Environmental Medicine (Tuxedo, NY, USA) and stored at -20°C until analysis of relaxin.

2.3 Relaxin Analysis

Analysis of serum immunoreactive relaxin was conducted using an RIA previously adapted and validated for use in bottlenose dolphins (Bergfelt et al., 2011). Main components of the assay consisted of H2 human relaxin (Phoenix Pharmaceuticals, Burlingame, CA, USA) as ¹²⁵I-labeled ligand (30,000 cpm/tube), rabbit anti-porcine relaxin R6 as the primary antibody (working dilution, 1:40,000), and synthetic canine relaxin (Steinetz et al., 1996) as reference standard (0.2 to 50 ng/tube). Samples were analyzed in duplicate with results reported as the average concentrations (ng/mL) relative to the relaxin standard curve. The lowest detectable relaxin concentration or assay sensitivity at 95% specific binding was a mean ($\pm$ STD) 5.6 $\pm$ 2.2 ng/mL (n = 6 assays). Variability of the within- and among-assay performances was assessed using a serum pool from several individual samples collected from pregnant bottlenose dolphins with specific binding ranging from 61 to 70% relative to the relaxin standard curve. Intra- and inter-assay CVs were 19 and 10%, respectively.

2.4 Data management and statistical analysis

Comparison of relaxin results between pregnancy and non-pregnancy was done on a weekly basis for 4 wk by adjusting the data for each animal to estrus or ovulation (Week 0). Thereafter evaluation of the relaxin results throughout pregnancy or a 12-month gestation period (approximate 376 d; O'Brien and Robeck, 2012) was done on a monthly basis by adjusting the data for each animal to the known month of parturition or estimated month of parturition (Month 0) in animals with spontaneous abortions. For pregnancies in which samples were collected twice weekly or monthly, the relaxin results were averaged so that there was a single weekly or monthly value to facilitate the week-to-week or month-to-month statistical analysis of relaxin for repeated measures. Prior to statistical analyses, the data were checked for extreme values using Dixon's outlier test (Kanji, 1993), tested for homogeneity using Shapiro-Wilk's test, and transformed by natural log or ranks if necessary. Data were examined by analysis of variance for repeated measures using PROC MIXED procedure in SAS (version 9.2; Statistical Analysis System Institute, Inc., Cary, NC,

USA) to determine the main effects of group (pregnant vs non-pregnant or live birth vs stillbirth vs abortion) and time (weekly or monthly) and interaction (group-by-time). If a significant ($P \leq 0.05$) main effect or interaction was detected, mean differences within weeks or months between groups or among weeks or months within groups were determined using least significant difference (LSD) or t-tests.

3. Results

Month-to-month changes in serum concentrations of relaxin throughout pregnancy and early post-partum period in dolphins with live births adjusted to the time of parturition (Month 0) with results of statistical analyses are shown in Fig. 1. The significant effect of month on relaxin is indicated by relatively low concentrations during early pregnancy (Months -12 to -9) after which relaxin progressively 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 (Month 0). By early post-partum (Month 1), relaxin concentrations decreased ($P < 0.05$).

Week-to-week changes in serum concentrations of relaxin between pregnancy and non-pregnancy adjusted to the time of estrus or ovulation (Week 0) with results of statistical analyses are shown in Fig. 2. Although there was a significant effect of pregnancy status (pregnant vs non-pregnant), there was no significant effect of week or interaction.

Fig. 2 was also prepared to further emphasize the significant increases in relaxin concentrations without high concentrations associated with late pregnancy and parturition obscuring the results. A separate statistical analysis indicated a significant effect of month such that relaxin concentrations were relatively low during the first 4 mo (Months 0 to 3) of pregnancy; thereafter, concentrations progressively increased ($P < 0.05$).

Month-to-month changes in serum concentrations of relaxin throughout pregnancies in dolphins with live births, stillbirths, and abortions adjusted to known or estimated time of parturition (Month 0) are shown in Table 1. In the abortion group, loss of pregnancy was estimated to occur during early (5 of 7, 71%), mid- (1 of 7, 14%), and late (1 of 7, 14%) pregnancy. Hence, there were a limited or nil number of serum samples from month-to-month in the abortion group to accommodate statistical analysis among all groups. Thus, monthly relaxin results were combined to represent three, 4-month periods as shown in Fig. 3. Regardless of pregnancy status, the significant effect of month was attributed to an increase ($P < 0.05$) in relaxin concentrations from early to mid-pregnancy for all groups. A subsequent increase ($P < 0.05$) in concentrations occurred by late pregnancy in the live birth and stillbirth groups but not the abortion group. The significant effects of pregnancy status and status-by-month interaction was primarily due to lower ($P < 0.05$) relaxin concentrations in the stillbirth and abortion groups by mid-pregnancy and abortion group by late pregnancy compared to the live birth group.

4. Discussion

Temporal month-to-month changes in circulating concentrations of immunoreactive relaxin in the present study corroborated and clarified the results of a seminal study in aquarium-based bottlenose dolphins during pregnancy and pregnancy loss (Bergfelt et al., 2011). In

relation to an approximate 12-month gestation period, relatively low concentrations during early pregnancy were followed by a progressive increase in concentrations by mid-pregnancy and high concentrations by late pregnancy in dolphins with live births. Subsequent to parturition, there was an abrupt decrease in relaxin during the early post-partum period. In dolphins with stillbirths and spontaneous abortions, there was an increase in relaxin from early to mid-pregnancy that was comparable to the increase in relaxin in dolphins with live births but the magnitude was significantly lower. Although lower, relaxin continued to increase until parturition in dolphins with stillbirths but remained relatively unchanged in dolphins with abortions.

Aquatic institutions have played a historic role in the conservation of cetaceans through collaboration and development of sustainable populations of animals and by conducting and supporting research and education programs for the benefit of whales, dolphins, and porpoises *in situ* and *ex situ* (O'Brien and Robeck, 2010). The periodic collection and archiving of blood samples is a routine part of management practices to monitor general health and reproductive status of aquatic animals. Although the effect of long-term cryopreservation on serum concentrations of relaxin is unknown, it is known that relaxin is a stable and soluble protein hormone in acid-acetone that can withstand exposure to extreme cold, heat, and long periods at ambient temperature (Doczi, 1963; Steinetz et al., 1962). In a preliminary study to characterize circulating concentrations of relaxin in bottlenose dolphins during pregnancy and pregnancy loss (Bergfelt et al., 2011), the number of monthly samples was limited and, therefore, it was necessary to combine the results into three, 4-month periods or trimesters of pregnancy (early, middle, late) for analysis. Independent from the initial study, the present study involved the collection of serial samples from numerous animals and pregnancies over 24 y provided a comprehensive distribution of samples to critically evaluate the month-to-month changes in relaxin throughout pregnancy. Overall, circulating concentrations of relaxin remained relatively low during the first 4 mo or first trimester of pregnancy (Months –12 to –9); thereafter, concentrations progressively increased during the second (Months –8 to –5) and third (Months –4 to 0) trimesters with a surge-like increase in concentrations pre-partum.

Comparatively, there is extensive diversity regarding the profile of relaxin among laboratory (e.g., rats, rabbits; Sherwood et al., 1980; Sherwood, 1994), companion (e.g., dog, cat; Steinetz et al., 1987; Stewart and Stabenfeldt, 1985), domestic (e.g., pigs, sheep; Gordon, 2004; Porter, 1993), and wild (e.g., elephants, rhinoceros, leopards; Steinetz et al., 2005 and 2009) animal species throughout pregnancy. In general, circulating concentrations of relaxin are elevated by mid-pregnancy in most species; thereafter, concentrations appear to decrease and increase prior to parturition (e.g., horse), plateau (e.g., human), or decline (e.g., dog), or exhibit a surge-like increase pre-partum (e.g., rat). The temporal changes in relaxin concentrations throughout pregnancy in dolphins observed herein appeared to correspond to those in rats (Sherwood et al., 1980). In relation to an approximate 22 d gestation period in rats, serum concentrations of relaxin were not detected during the first 10 d of pregnancy; thereafter, concentrations progressively increased until a pre-partum surge by Day 21. The short-term pattern (e.g., minutes, hours, day-to-day) of secretion of relaxin in dolphins is not known but, in dogs (Steinetz et al., 1990) and pigs (Juang et al., 1996), pulsatile or diurnal secretion of relaxin was not detected. Thus, independent results of the present study

corroborate the results of an earlier study (Bergfelt et al., 2011) and provide a more critical evaluation of the timing and magnitude of the increase in circulating concentrations of relaxin throughout pregnancy in aquarium-based bottlenose dolphins.

Relatively low circulating concentrations of relaxin in pregnant dolphins during the first 4 wk post-ovulation were comparable to those concentrations in bred, non-pregnant dolphins during the estrous cycle. In species in which relaxin has been studied most extensively, the CL is the major source of relaxin with circulating concentrations higher during the luteal than the follicular phase of the menstrual and estrous cycles in humans, non-human primates, and pigs, and maximal at estrus in rats (review Hayes, 2004; Bathgate et al., 2006). Notably, in pigs, relaxin concentrations were much lower during the estrous cycle than pregnancy, and, in women, there was no difference in concentrations between the menstrual cycle and corresponding period of pregnancy. While relaxin has been detected in luteal and placental tissue in pregnant whales (Schwabe et al., 1989) and dolphins (Bergfelt et al., 2011), respectively, future studies are required to clarify the sources of relaxin in pregnant and non-pregnant dolphins as well as the extent that the CL or placenta contributes to the increase in circulating concentrations of relaxin during pregnancy in this species.

In the present study, the increase in relaxin was detected by approximately the end of the first or beginning of the second trimester (i.e., 4 to 5 mo post-estrus/ovulation). Considering that circulating concentrations of relaxin are relatively low during the first trimester, the application of relaxin as a hormonal aid to diagnose pregnancy status would be most reliable during the second and third trimesters. In this regard, single-sample analysis of serum relaxin accurately diagnosed pregnancy in 7 out of 9 known pregnant free-ranging bottlenose dolphins (Bergfelt et al., 2013). The basis for the false negative diagnosis in two of the dolphins was speculated to be attributed to blood samples collected during early pregnancy such that relaxin concentrations did not exceed baseline concentrations indicative of pregnancy. The novelty of the present results clarified that weekly and monthly serum concentrations of relaxin are relatively low during early pregnancy such that single-sample analysis can potentially yield a false negative diagnosis of pregnancy. Despite the apparent limitation of relaxin to falsely diagnose pregnancy during the first trimester of pregnancy, contingency table analysis with progesterone as the “gold standard” indicated a false positive rate of 0% or 100% specificity to diagnose non-pregnancy in dolphins (Bergfelt et al., 2013). Theoretically, therefore, a concentration of serum relaxin above that indicative of pregnancy in single-sample analysis is a strong and reliable indicator of pregnancy in bottlenose dolphins.

Unlike the pregnancy-specific nature of elevated circulating concentrations of relaxin in dolphins, an elevated concentration of progesterone in a single sample is not pregnancy specific. Other pregnancy-related factors have been indicated to have some diagnostic value in domestic and wild animal species. These include early conception factor (Grandy et al., 2001), early pregnancy factor (Ohnuma et al., 2000), alpha-fetoprotein (Morita et al., 2013), pregnancy-associated glycoproteins (González et al., 1999; Szenci et al., 1998), fibrinogen, and other acute-phase proteins (Hart, 1997); C-peptide (Kuniyuki and Hughes, 1992); pregnancy specific protein B (Romano and Larson, 2010); and ceruloplasmin (Willis et al., 2011). Future studies are required to clarify the diagnostic potential of these pregnancy-

related factors in cetaceans. Nonetheless, despite the limitations of progesterone and relaxin for diagnosing pregnancy, especially during the first trimester, relatively high concentrations of both hormones in the same sample can be complimentary and confirmatory of mid- to late pregnancy in bottlenose dolphins.

In the present study, circulating concentrations of relaxin increased from early to mid-pregnancy in dolphins with live births, stillbirths, and abortions; however, mean concentrations were lower (approximately 52%) in association with pregnancy loss. Thereafter, relaxin increased in dolphins with stillbirths in a manner comparable to that in dolphins with live births but mean concentrations were still reduced (approximately 13%), although not significantly, during late pregnancy. Compared to the increase in relaxin concentrations during late pregnancy in association with live births and stillbirths, relaxin did not increase during late pregnancy in association with abortions, which was attributed to the loss of most pregnancies (5 of 7, 71%) during early gestation in the abortion group. In the present study, relaxin results in dolphins with live births and stillbirths are comparable to those in an earlier study conducted independently which involved dolphins with stillbirths and late-term abortions (Bergfelt et al., 2011). The novelty of the present study involved early-term abortions such that the results provides further support to indicate that a relatively high concentration of relaxin during late pregnancy is pregnancy-specific.

Like relaxin, progesterone is primarily produced by the CL and placenta in mammals (Bazer et al., 1998). Conversely, while the need for elevated circulating concentrations of progesterone for the development and maintenance of pregnancy is well accepted among mammals (Bazer et al., 1998), the necessity of elevated systemic concentrations of relaxin requires clarification (Hayes, 2004). Nonetheless, in women (Hayes, 2004) and horses (Ryan et al., 2009), low circulating concentrations of relaxin have been associated with pregnancy loss. In dolphins, circulating concentrations of both relaxin and progesterone were low in relation to stillbirths or late-term abortions (Bergfelt et al., 2011). Correspondingly, early-term abortions in dolphins were associated with decreased concentrations of relaxin as reported herein and progesterone as reported previously (O'Brien and Robeck, 2012). While future studies are required to evaluate the nature of pregnancy loss in relation to luteal or placental synthesis and secretion of relaxin, the present results support the concept that circulating concentrations of relaxin are reduced in association with pregnancy loss regardless if embryo/fetal deaths occur *in utero* or at birth in bottlenose dolphins.

In conclusion, the results of the present 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.

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Highlights

- Weekly or monthly circulating concentration of relaxin were critically evaluated throughout pregnancy and pregnancy loss in bottlenose dolphins
- Relaxin concentrations increased by mid- to late pregnancy in dolphins with live births and stillbirths but were lower in animals with pregnancy loss
- Relaxin concentrations increased to mid-pregnancy in dolphins with early-term abortions but not thereafter
- Relaxin concentrations were not different 4 weeks post-ovulation between pregnant and non-pregnant dolphins

Fig 1.

Mean ($\pm$ SEM) monthly serum concentrations of immunoreactive relaxin with results of statistical analysis throughout a 12-month gestation period and early post-partum relative to parturition (Month 0) in bottlenose dolphins with live births (34 dolphins involving 60 pregnancies). Different super-scripts among means (a,b,c,d,e) indicate statistical changes ($P < 0.05$) among months. Values in parentheses on the x-axis represent the number of observations used to calculate the means.

Fig 2.

Mean ($\pm$ SEM) weekly and monthly serum concentrations of immunoreactive relaxin with results of statistical analysis in pregnant bottlenose dolphins with live births (solid line, 34 dolphins and 60 pregnancies) and non-pregnant dolphins (dashed line, 8 dolphins and corresponding number of estrous cycles). Separate analyses were done for Weeks 0 to 3 and Months 1 to 6 relative to estrus or ovulation (Time=0). Different super-scripts (a,b,c) among means indicate statistical changes ($P < 0.05$) among months. Values in parentheses on the x-axis represent number of observations used to calculate the means within respective groups.

Fig 3.

Mean ($\pm$ SEM) serum concentrations of immunoreactive relaxin combined for early (Months -12 to -9), middle (Months -8 to -5), and late (Months -4 to 0) pregnancy with results of statistical analysis in bottlenose dolphins with live births (solid line, 34 dolphins involving 60 pregnancies), stillbirths (long dashed line, 7 dolphins involving 7 pregnancies), and abortions (short dashed line, 7 dolphins involving 7 pregnancies) relative to known or estimated time of parturition (Month 0). Different super-scripts (a,b,c) among means within groups indicate statistical changes ($P < 0.05$) among months. An asterisk between different group means indicates a statistical difference ($P < 0.05$) between groups. Values in parentheses on the x-axis represent number of observations used to calculate the means within respective groups.

Table 1

Mean ($\pm$ SEM) monthly serum concentrations of relaxin relative to known or estimated parturition (Month 0) in bottlenose dolphins with live births, stillbirths and abortions.

Months from parturition	Relaxin (ng/ml)		
	Live Births	Stillbirths	Abortions ^I
-12	^a 9.5 $\pm$ 1.0 (22)	4.2 $\pm$ 1.0 (2)	7.9 $\pm$ 1.1 (7)
-11	13.2 $\pm$ 3.0 (20)	8.8 $\pm$ 4.4 (4)	10.7 $\pm$ 1.5 (5)
-10	13.3 $\pm$ 2.6 (23)	13.3 $\pm$ 7.1 (3)	11.5 $\pm$ 2.1 (6)
-9	^b 17.2 $\pm$ 3.5 (20)	7.2 $\pm$ 4.0 (4)	11.2 $\pm$ 2.4 (3)
-8	29.6 $\pm$ 5.3 (18)	3.1 $\pm$ 0.0 (2)	12.3 $\pm$ 3.5 (3)
-7	54.5 $\pm$ 7.4 (19)	21.4 $\pm$ 8.3 (5)	55.6 $\pm$ 22.2 (2)
-6	^c 55.6 $\pm$ 9.6 (15)	3.1 $\pm$ 0.0 (1)	40.1 $\pm$ 0.0 (1)
-5	123.5 $\pm$ 17.4 (18)	93.0 $\pm$ 50.0 (3)	70.9 $\pm$ 0.0 (1)
-4	141.8 $\pm$ 14.9 (22)	86.1 $\pm$ 1.9 (2)	62.8 $\pm$ 45.4 (2)
-3	162.7 $\pm$ 28.7 (16)	87.8 $\pm$ 16.8 (3)	--
-2	^d 227.0 $\pm$ 27.7 (19)	151.3 $\pm$ 38.8 (4)	20.9 $\pm$ 0.0 (1)
-1	397.7 $\pm$ 111.4 (14)	149.1 $\pm$ 0.0 (1)	--
0	^e 1289.0 $\pm$ 589.3 (4)	1455.8 $\pm$ 0.0 (1)	--
Overall	^x 118.1 $\pm$ 17.0	^y 90.1 $\pm$ 41.5	^y 16.8 $\pm$ 3.4

^I Abortions were detected between Months -11 to -4.

^{a,b,c,d,e} Differences (P<0.05) among months within the live birth group.

^{x,y} Differences (P<0.05) among groups for the overall means.

Parentheses represent the number of observations included in the mean