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Evaluation of Single-Sample Analysis of Progesterone in Combination with Relaxin for Diagnosis of Pregnancy in Wild Bottlenose Dolphins (*Tursiops truncatus*)

Don R. Bergfelt,[1] Bernard G. Steinetz,[2] John S. Reif,[3] Adam M. Schaefer,[4] Gregory D. Bossart,[4, 5] Marilyn S. Mazzoil,[4] Eric Zolman,[6] and Patricia A. Fair[6]

[1]*U.S. Environmental Protection Agency, Washington, DC 20460, USA*
E-mail: Bergfelt.don@epa.gov
[2]*New York University School of Medicine, Tuxedo, NY 10987, USA*
[3]*College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA*
[4]*Harbor Branch Oceanographic Institute at Florida Atlantic University, Fort Pierce, FL 39446, USA*
[5]*Georgia Aquarium, Atlanta, GA 30313, USA*
[6]*NOAA's Ocean Service, Center for Coastal Environmental Health and Biomolecular Research, Charleston, SC 29412, USA*

Abstract

The present study is a preliminary evaluation of the potential for an elevation of relaxin to clarify an elevation of progesterone in wild bottlenose dolphins as pregnancy-related since relatively high progesterone in a single-sample analysis is not necessarily pregnancy-specific. Blood samples were collected from adult female bottlenose dolphins in estuaries of Charleston, South Carolina, and the Indian River Lagoon, Florida. Serum progesterone and relaxin concentrations were analyzed in the same samples (n = 74) with respective radioimmunoassays (RIAs). Diagnosis of pregnancy was based on concentrations of progesterone and relaxin > 6.0 and > 11.2 ng/mL, respectively, whereas nonpregnancy was based on respective hormone concentrations less than or equal to baseline concentrations. Progesterone was observed at elevated concentrations considered diagnostic of pregnancy in 20 and relaxin in 12 bottlenose dolphins. In a contingency table analysis with progesterone as the conventional standard for pregnancy diagnosis, there was a 60% positive correspondence with relaxin (12 relaxin-out of 20 progesterone-diagnosed pregnancies) and 40% negative correspondence (8 relaxin-diagnosed nonpregnancies out of 20 progesterone-diagnosed pregnancies). Kappa analysis indicated good agreement (κ = 74%) between relaxin- and progesterone-diagnosed pregnancies. Contingency table results were verified based on known pregnancies of nine cow-calf pairs observed through photo-identification subsequent to health assessments. Concordance between hormone diagnosed and observed pregnancies was 9 of 9 (100%) for progesterone and 7 of 9 (78%) for relaxin. Hence, preliminary results indicated that relaxin may have limited value for pregnancy diagnosis when used alone. However, in combination with an elevation of progesterone in a single-sample analysis, a pregnancy-specific elevation of relaxin can clarify that progesterone is pregnancy-related and, thus, confirm the diagnosis of pregnancy in wild bottlenose dolphins.

Key Words: progesterone, relaxin, pregnancy, wild bottlenose dolphins, *Tursiops truncatus*

Introduction

While changes in concentrations of progesterone or progesterone metabolites in different matrices (e.g., serum/plasma, urine, feces, milk, blubber) have been shown to be indicative of pregnancy status in a variety of managed-care and wild cetaceans (Amaral, 2010), changes in serum hormone concentrations indicative of pregnancy status in wild bottlenose dolphins (*Tursiops truncatus*) have not been documented. In managed-care bottlenose dolphins, immunoanalysis of serum progesterone is well-established as the most practical and conventional approach for pregnancy diagnosis (Sawyer-Steffan & Kirby, 1980; Sawyer-Steffan et al., 1983; Kirby & Ridgway, 1984; Cornell et al., 1987). In a series of fundamental studies (Sawyer-Steffan & Kirby, 1980; Sawyer-Steffan et al., 1983; Kirby & Ridgway, 1984), serum progesterone above a baseline concentration (> 3 ng/mL) was considered ovulatory and conditionally indicative of pregnancy until confirmed as related to pregnancy and not to diestrus (Robeck et al., 2005) or prolonged luteal maintenance (Sawyer-Steffan et al., 1983; Yoshioka et al., 1986; Robeck et al., 2001) associated with nonpregnancy. Typically, repeat blood samples

are collected 6 to 8 wks later during the suspected pregnancy to confirm whether or not the elevation of progesterone in the initial sample is pregnancy-related. In one study (Sawyer-Steffan et al., 1983), analysis of progesterone in repeat serum samples was diagnostic of pregnancy in 16 managed-care dolphins of which 15 calved. A similar hormonal approach has been successfully applied in a managed-care dolphin breeding program (Cornell et al., 1987), except that a slightly higher baseline concentration of serum progesterone (> 6 ng/mL) was chosen as indicative of pregnancy based on the minimum range in concentration as previously reported (Sawyer-Steffan et al., 1983).

Application of progesterone analysis for pregnancy diagnosis in wild bottlenose dolphins has not been fully explored, which may be due to limited opportunities for capture-release, collection of repeat blood samples, and uncertainty of whether or not elevated serum progesterone in a single sample is pregnancy-related (Sawyer-Steffan et al., 1983; Yoshioka et al., 1986; Robeck et al., 2001, 2005). Although it is rare to have more than one opportunity for the collection of blood samples within the same season from wild bottlenose dolphins, measurements of progesterone and other pregnancy-related hormones can be analyzed in the same serum sample and, therefore, potentially provide more certainty to support the diagnosis of pregnancy.

Relaxin is a hormone produced in relatively large quantities during pregnancy by the *corpus luteum* and placenta as reported in humans and laboratory and domestic animals (Gordon, 2004; Park et al., 2005; Bathgate et al., 2006). In dogs, the pregnancy-specific nature of relaxin was exploited to develop and validate an immunological technique to diagnose pregnancy (Steinetz et al., 1989) based on an elevation in serum concentration of relaxin since an elevation in progesterone is not distinctly different between pregnant and nonpregnant or pseudopregnant bitches (Johnston et al., 2001). Considering the clinical success in domestic dogs, homologous and heterologous relaxin radioimmunoassays (RIAs) have been developed and validated for use as potential hormonal methods to diagnose pregnancy in numerous wild animal species (Steinetz et al., 2005, 2009), including marine mammals (Northern fur seals [*Callorhinus ursinus*], Bergfelt et al., 2010, and bottlenose dolphins, Bergfelt et al., 2011). Considering the close relationship between relaxin from baleen whales and pigs (Schwabe et al., 1989), an RIA with antiporcine relaxin was developed and validated as a novel approach to identify the placenta as a source of relaxin and characterize serum concentrations of relaxin in relation to progesterone for each 4-mo period of pregnancy and early *postpartum* in managed-care bottlenose dolphins (Bergfelt et al., 2011). The pregnancy-specific nature of relaxin was indicated by immunoreactive detection in placental tissue and relatively high serum concentrations mid to late pregnancy followed by low or undetectable concentrations after parturition (i.e., nonpregnancy).

The Bottlenose Dolphin Health and Risk Assessment (HERA) project is an ongoing collaborative effort between the National Oceanic and Atmospheric Administration's (NOAA) Ocean Service's Center for Coastal Environmental Health and Biomolecular Research and Harbor Branch Oceanographic Institution at Florida Atlantic University. The project embodies a large collection of data to investigate associations between health status and environmental stressors in various populations of wild Atlantic bottlenose dolphins (Fair et al., 2006). In regard to reproductive health and potential impact that environmental stressors may have on pregnancy, a reliable, accurate, and minimally intrusive method is needed to diagnose pregnancy in wild bottlenose dolphins that may be broadly applicable to the needs of various groups or institutions studying wild populations of cetaceans. While ultrasonography is an authenticated, noninvasive approach for the diagnosis of pregnancy in managed-care bottlenose dolphins (Robeck et al., 2001) and some wild marine mammals (e.g., Northern fur seals; Adams et al., 2007), it has not been fully validated under field conditions in wild bottlenose dolphins. Alternatively, the collection of blood samples at the time of health assessment is an established procedure in the HERA project (Fair et al., 2006). Thus, archived serum was readily available to conduct a preliminary retrospective evaluation to determine whether or not relaxin in combination with a single-sample analysis can support the diagnosis of pregnancy in wild bottlenose dolphins.

The present study was designed in conjunction with the ongoing HERA project as a preliminary evaluation to explore the pregnancy diagnostic potential of single-sample analysis of serum progesterone in combination with relaxin. The hypothesis was tested that an elevation in relaxin is pregnancy-specific and can be used to clarify an elevation in progesterone in the same serum sample as pregnancy-related and, thus, confirm the diagnosis of pregnancy in wild bottlenose dolphins.

Methods

Dolphins and Dolphin Study Sites

All aspects of this study, including techniques involving dolphin capture-release (Fair et al., 2006) were conducted in accord with the National Marine Fisheries Service Permit No. 998-1678-00 and approved by the Harbor Branch

Oceanographic Institutional Animal Care and Use Committee. Capture-release of wild Atlantic bottlenose dolphins was conducted during early summer for each year in 2003 to 2007, 2010, and 2011 along the eastern coast of the United States involving two estuarine sites (Figure 1). The Charleston, South Carolina, site (32° 46'35" N, 79° 55'51" W) included the Charleston Harbor; portions of the main channels and creeks of the Ashley, Cooper, and Wando Rivers; and the Stono River Estuary. The Indian River Lagoon, Florida, site generally ranges from Titusville (28° 36'43" N, 80° 48'27" W) to Stuart, Florida (27° 11'51" N, 80° 15'10" W) and includes portions of the Mosquito Lagoon, Indian River, Banana River, and north and south forks of the St. Lucie River. The present study involved 92 adult female bottlenose dolphins from the two populations of wild bottlenose dolphins that were studied over the various years.

Photo-Identification of Cow-Calf Pairs

The two bottlenose dolphin study sites have been well-established as high fidelity sites based on long-term photo-identification studies (Zolman, 2002; Mazzoil et al., 2005; Speakman et al., 2006). The techniques for photo-identification, boat-based survey methods, and data collection have been previously described (Mazzoil et al., 2005; Speakman et al., 2010). Although documentation of reproductive success using photo-identification has substantial methodological limitations, the calf of a cow-calf pair is typically characterized by being 50 to 75% the length of the cow and in close proximity (Urian & Wells, 1996).

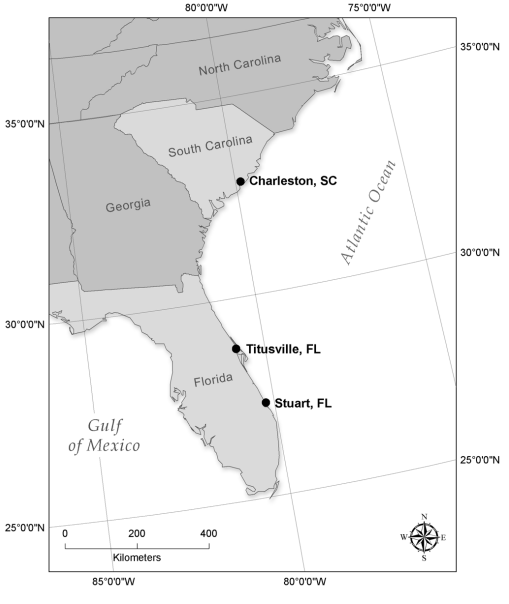

Figure 1. Health and risk assessment and photo-identification studies of two populations of wild Atlantic bottlenose dolphins conducted at two estuarine sites along the eastern coast of the U.S.—(1) Charleston, South Carolina, and (2) the Indian River Lagoon, Florida—which generally ranges from Titusville to Stuart, Florida.

For the present study, photo-identification records were searched for the sighting of cow-calf pairs that corresponded to any of the 92 adult female bottlenose dolphins evaluated herein. Sightings of live cow-calf pairs were used conservatively as an indication of observed or known pregnancies that may be in concordance with hormonal diagnosis of pregnancies based on maternal blood samples collected within 12 mo of a previous health assessment. Cows sighted without calves were not used as nonpregnant because of the periodicity of surveys, unrecognized pre- and post-natal losses, predation of calves, and other events that may occur prior to sightings, all of which precludes a definitive diagnosis of nonpregnancy (Mazzoil et al., 2005; Speakman et al., 2010).

Blood Collection

Techniques for blood collection and processing have been previously described (Fair et al., 2006). Briefly, adult female bottlenose dolphins were physically restrained *in situ*, and blood samples were collected within approximately 10 min after capture. Thereafter, bottlenose dolphins were moved onboard the research vessel for a general health examination and collection of additional biological samples before release.

Blood was collected from the periarterial rete in the flukes using a 19-gauge butterfly catheter attached to 10 mL serum-separator vacutainer tubes. After collection, blood samples were allowed to clot for approximately 30 min at ambient temperature before serum was separated by centrifugation at 3,500 rpm (1,233 × g) for 15 min and transferred to cryovials. Serum samples were labeled with bottlenose dolphin identification and date, and stored at -80° C. For hormone analyses, serum samples were shipped frozen with dry ice to the Animal Health Diagnostic Laboratory (AHDL) at Cornell University (Ithaca, NY, USA) for progesterone analysis and, thereafter, to New York University School of Medicine (Tuxedo, NY, USA) for relaxin analysis. At respective institutions, all serum samples were stored at -20° C until analysis.

Progesterone Analysis

Analysis of serum progesterone was conducted using a commercial solid-phase RIA kit (Coat-A-Count®, TKPG 1423, Siemens Healthcare Diagnostics Inc., Los Angeles, CA, USA) designed

for direct quantification of progesterone in unextracted samples. The assay was adapted for use in bottlenose dolphins as described (Lee et al., 1991; Reimers et al., 1991). Main components of the assay consisted of ^{125}I-labeled progesterone, progesterone antibody-coated polypropylene tubes, and progesterone as reference standard (0.1 to 40 ng/mL). The assay was conducted in accord with instructions from the manufacturer using 100 uL of standards, controls, and appropriate serum sample/tube. Samples were analyzed in duplicate with results reported as the average concentrations (ng/mL) relative to the progesterone standard curve. The lowest detectable hormone concentration or assay sensitivity was 0.02 ng/mL. Intra- and inter-assay CVs were 3.4 and 5.7%, respectively, based on aliquots of serum pooled from individual bottlenose dolphins.

Relaxin Analysis

Analysis of serum immunoreactive relaxin was conducted using a validated RIA developed for use in bottlenose dolphins as described (Bergfelt et al., 2011). Main components of the assay consisted of H2 human relaxin as ^{125}I-labeled ligand (30,000 cpm/tube), rabbit anti-porcine relaxin R6 as the primary antibody (working dilution, 1:40,000), and synthetic canine relaxin 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 hormone concentration or assay sensitivity at 90% specific binding was a mean 11.2 ng/mL (n = 6 assays). Intra- and inter-assay CVs were 16.8 and 10.6%, respectively, based on aliquots of serum pooled from individual samples collected from pregnant bottlenose dolphins under managed care.

Hormonal Criteria for Diagnosis of Pregnancy Status

A single-sample analysis of serum concentration of progesterone > 6 ng/mL was considered diagnostic of pregnancy in accordance with the same baseline concentration considered indicative of pregnancy in bottlenose dolphins under managed care (Cornell et al., 1987). Correspondingly, mean assay sensitivity was used to define a serum concentration of relaxin > 11.2 ng/mL as diagnostic of pregnancy, which is supported by the observation of 15.1 ng/mL as the lowest concentration detected during pregnancy in dolphins under managed care (Bergfelt et al., 2011). Thus, progesterone and relaxin concentrations ≤ 6 and ≤ 11.2 ng/mL, respectively, were considered diagnostic of nonpregnancy.

Statistical Analysis

A contingency table analysis was used to estimate the potential of relaxin to be diagnostic of pregnancy status relative to progesterone. Since progesterone is generally considered the conventional standard for hormonal diagnosis of pregnancy status in bottlenose dolphins under managed care (Sawyer-Steffan & Kirby, 1980; Sawyer-Steffan et al., 1983; Kirby & Ridgway, 1984; Cornell et al., 1987), it was used as the "gold standard" in the contingency table analysis. Various parameters of the contingency table analysis included (1) *sensitivity* – the proportion of bottlenose dolphins diagnosed pregnant with relaxin (> 11.2 ng/mL) when progesterone (> 6 ng/mL) indicated pregnancy; (2) *specificity* – the proportion of bottlenose dolphins diagnosed nonpregnant with relaxin (≤ 11.2 ng/mL) when progesterone (≤ 6 ng/ml) indicated nonpregnancy; (3) *false negative* – the proportion of bottlenose dolphins diagnosed nonpregnant with relaxin when progesterone indicated pregnancy; and (4) *false positive* – the proportion of bottlenose dolphins diagnosed pregnant with relaxin when progesterone indicated nonpregnancy. Kappa (κ) analysis of agreement (*SPSS Statistics*, Version 19; IBM Corporation, 2010) was used to determine the level of concordance between baseline concentrations of progesterone and relaxin that were defined and considered diagnostic of pregnancy. Levels of agreement for κ were considered poor, ≤ 0.20; fair, 0.21 to 0.40; moderate, 0.41 to 0.60; good, 0.61 to 0.80; and excellent, ≥ 0.81 (IBM Corporation, 2010).

Results

There were 92 adult female bottlenose dolphins from which serum samples were available for analysis of progesterone and 91 for relaxin. In 17 bottlenose dolphins, the concentrations of progesterone and relaxin in the same sample were below the sensitivity of respective assays and could not be estimated. Hence, there were 75 observations of progesterone and 74 observations of relaxin available for evaluation (Table 1).

A single-sample analysis of progesterone and relaxin indicated concentrations were above baseline concentrations and, therefore, diagnostic of pregnancy in 20 and 12 bottlenose dolphins, respectively (Table 1). In these potentially pregnant bottlenose dolphins, mean (± SEM) concentrations of progesterone and relaxin were 22.18 ± 2.29 and 461.91 ± 274.0 ng/mL, respectively. For those bottlenose dolphins diagnosed nonpregnant based on progesterone alone (n = 55), the mean (± SEM) concentration was 0.33 ± 0.11 ng/mL. Correspondingly, for those bottlenose dolphins diagnosed nonpregnant based on relaxin alone

Table 1. Single-sample analysis of serum concentrations of progesterone (P4; $n = 75$) and relaxin (Rlx; $n = 74$) in wild adult female bottlenose dolphins; baseline concentrations of P4 (> 6 ng/mL) and Rlx (> 11.2 ng/mL) in the same sample considered diagnostic of pregnancy are shaded light gray. Bottlenose dolphins sighted with a calf through photo-identification are shaded dark grey.

	Bottlenose dolphin	Hormones (ng/mL)			Bottlenose dolphin	Hormones (ng/mL)			Bottlenose dolphin	Hormones (ng/mL)	
No.	ID	P4	Rlx	No.	ID	P4	Rlx	No.	ID	P4	Rlx
1	98106	0.05	ND	26	81703	0.12	ND	51	83703	0.36	ND
2	98306	0.05	ND	27	83103	0.12	ND	52	91703	0.37	ND
3	95507	0.05	ND	28	9C711	0.13	ND	53	9A710	1.37	ND
4	99307	0.05	ND	29	83104	0.13	ND	54	9C311	4.03	ND
5	9A910	0.05	ND	30	83304	0.13	ND	55	95304	5.07	NS
6	98907	0.06	ND	31	9V311	0.14	ND	56	83903	6.65	ND
7	86305	0.06	ND	32	97707	0.14	ND	57	90103	8.18	ND
8	84904	0.06	ND	33	82103	0.18	ND	58	91903	10.83	182.97
9	82303	0.07	ND	34	84303	0.18	ND	59	95904	11.06	3421.0
10	92904	0.07	ND	35	9A310	0.19	ND	60	85905	12.84	ND
11	96705	0.07	ND	36	94704	0.19	ND	61	91904	13.64	100.2
12	91705	0.08	ND	37	83503	0.21	ND	62	82503	16.35	ND
13	91303	0.09	ND	38	9C111	0.22	ND	63	99910	17.82	ND
14	90903	0.09	ND	39	96104	0.22	ND	64	87905	18.41	ND
15	81903	0.09	ND	40	84704	0.23	ND	65	92303	20.25	480.02
16	97706	0.09	ND	41	85104	0.24	ND	66	92103	22.63	92.95
17	9V111	0.09	ND	42	85304	0.25	ND	67	82903	24.01	ND
18	9D911	0.09	ND	43	80103	0.26	ND	68	9A110	26.97	25.76
19	85504	0.09	ND	44	91504	0.27	ND	69	93104	27.50	ND
20	84103	0.09	ND	45	83303	0.27	ND	70	97906	27.95	60.75
21	95104	0.11	ND	46	98707	0.28	ND	71	82703	28.69	105.08
22	9C911	0.11	ND	47	90503	0.29	ND	72	99507	34.03	600.01
23	9D111	0.11	ND	48	98506	0.29	ND	73	94907	38.38	89.15
24	94904	0.11	ND	49	87105	0.33	ND	74	97506	38.63	76.09
25	91503	0.12	ND	50	9D511	0.34	ND	75	91507	38.79	308.91

ND = not detected – below sensitivity of Rlx assay (11.2 ng/mL)
NS = no serum – insufficient volume remaining for analysis

($n = 62$), concentrations were below assay sensitivity (< 11.2 ng/mL; Table 1) and could not be estimated.

From a total of 74 paired progesterone-relaxin observations (Table 1), contingency table analysis (Tables 2 & 3) indicated there were 12 relaxin-diagnosed pregnancies out of 20 progesterone-diagnosed pregnancies that resulted in a positive correspondence (i.e., sensitivity) of 60%. Relaxin was not diagnostic in 8 out of 20 progesterone-diagnosed pregnancies, which resulted in a negative correspondence (i.e., false negative) of 40%. In contrast, there were 54 relaxin-diagnosed nonpregnancies (excludes 8 relaxin false negatives) within the 54 progesterone-diagnosed nonpregnancies that resulted in a specificity of 100% with a false positive rate of 0%. Kappa analysis indicated good agreement ($\kappa = 0.74$) between relaxin- and progesterone-diagnosed pregnancy status (Table 3).

Photo-identification records indicated the sighting of nine cow-calf pairs that corresponded to nine adult female bottlenose dolphins from which blood samples were collected at the previous health assessment and analyzed for serum concentrations of progesterone and relaxin (Table 1). The nine known pregnancies corresponded to nine

Table 2. Contingency table results representing the number of pregnancies and nonpregnancies based on single-sample analyses of serum progesterone and relaxin concentrations in wild bottlenose dolphins

	Progesterone		
Relaxin	Pregnant[1]	Nonpregnant[1]	Total
Pregnant	12	0	12
Nonpregnant	8	54	62
Total	20	54	74

[1] Diagnosis of pregnancy was based on concentrations of progesterone and relaxin > 6 and > 11.2 ng/mL, respectively, whereas nonpregnancy was based on respective hormone concentrations less than or equal to baseline concentrations.

Table 3. Contingency table and Kappa analysis results representing the proportion of relaxin-diagnosed pregnancies (sensitivity) and nonpregnancies (specificity) compared to progesterone as the "gold standard" in wild bottlenose dolphins

Sensitivity[1]	False negative	Specificity[1]	False positive	Kappa (κ)[2]
12/20 (60%)	8/20 (40%)	54/54 (100%)	0/54 (0%)	0.74

[1] Diagnosis of pregnancy was based on concentrations of progesterone and relaxin > 6 and > 11.2 ng/mL, respectively, whereas nonpregnancy was based on respective hormone concentrations less than or equal to baseline concentrations.
[2] Concordance between relaxin- and progesterone-diagnosed pregnancies was considered good (κ = 0.61 to 0.80).

Table 4. Concordance between observed cow-calf pairs sighted through photo-identification and hormonal diagnosis of pregnancy based on collection of blood samples at the time of health assessment in corresponding adult wild bottlenose dolphins

	Progesterone	Relaxin	Sightings
Diagnosed[1]	9 dolphins	7 dolphins	--
Observed	--	--	9 cow-calf pairs
Pregnancies[2]	9/9 (100%)	7/9 (77.8%)	

[1] Diagnosis of pregnancy was based on concentrations of progesterone and relaxin > 6 and > 11.2 ng/mL, respectively, whereas nonpregnancy was based on respective hormone concentrations less than or equal to baseline concentrations.
[2] Refer to Table 1 for results in individual bottlenose dolphins pertaining to each of the sightings and corresponding serum concentrations of progesterone and relaxin.

progesterone-diagnosed (100%) and seven relaxin-diagnosed (77.8%) pregnancies (Table 4). Although relaxin was not detected above assay sensitivity in two of the nine bottlenose dolphins known to be pregnant (22.2% false negative), the lowest detectable concentrations of both hormones in bottlenose dolphins confirmed pregnant by photo-identification was 6.65 ng/mL out of nine samples for progesterone and 60.75 ng/mL out of seven samples for relaxin (Table 1). In 11 bottlenose dolphins with unknown reproductive status, progesterone concentrations were elevated, but relaxin concentrations were either not detected in six (54%) or elevated in five (45%) bottlenose dolphins (Table 1).

Discussion

Pregnancy diagnosis based on repeat blood sampling and analysis of serum concentrations of progesterone is well-established in bottlenose dolphins under managed care (Sawyer-Steffan & Kirby, 1980; Sawyer-Steffan et al., 1983; Kirby & Ridgway, 1984; Cornell et al., 1987). However, in wild bottlenose dolphins, there is typically no opportunity for the collection of repeat blood samples to confirm if an elevation of serum progesterone is pregnancy-related (Sawyer-Steffan et al., 1983; Yoshioka et al., 1986; Robeck et al., 2001, 2005). Recently, the pregnancy-specific nature of elevated serum concentrations of relaxin mid to late gestation has been reported in managed-care dolphins (Bergfelt et al., 2011). The present study in wild bottlenose dolphins indicated that single-sample analyses of serum progesterone and relaxin above respective baseline concentrations were diagnostic of pregnancies as verified through the observation of cow-calf pairs. Although preliminary, the diagnostic value of relaxin alone may be limited. However, in combination with progesterone in a single-sample analysis, an elevation of relaxin has the potential to clarify an elevation of progesterone as pregnancy-related and, thus, confirm the diagnosis of pregnancy in wild bottlenose dolphins.

Progesterone is generally considered the conventional standard for hormonal diagnosis of pregnancy in bottlenose dolphins under managed care (Sawyer-Steffan & Kirby, 1980; Sawyer-Steffan et al., 1983; Kirby & Ridgway, 1984; Cornell et al., 1987); hence, progesterone was evaluated in the present study to determine its diagnostic potential in wild bottlenose dolphins. However, since an elevated serum concentration of progesterone in a single sample is not necessarily related to pregnancy but may be due to progesterone production by the *corpus luteum* (Prelog & Meister, 1949) associated with diestrus (Robeck et al., 2005) or pseudopregnancy (Sawyer-Steffan et al., 1983; Yoshioka et al., 1986; Robeck et al., 2001), the same serum sample was also analyzed for relaxin. In general, the pregnancy-specific nature of relaxin is based on the placenta as a major source of production in some species that leads to increased circulating concentrations dependent on the stage of gestation (Gordon, 2004; Park et al., 2005; Bathgate et al., 2006; Steinetz et al., 2005, 2009). Recently, in managed-care bottlenose dolphins, the pregnancy-specific nature of relaxin was indicated by immunoreactive detection in multiple full-term placentas

(Bergfelt et al., 2011). Moreover, mean circulating concentrations were characterized for each trimester and early *postpartum* as relatively low or undetectable during early pregnancy; high mid to late pregnancy; and low or undetectable following parturition (i.e., nonpregnancy).

In the present study, directional changes in serum concentrations of progesterone relative to baseline concentrations were considered diagnostic of pregnancy (> 6 ng/mL) and nonpregnancy (≤ 6 ng/mL), which is in accord with the successful application in a managed-care dolphin breeding program (Cornell et al., 1987). Unlike progesterone, there is no historical basis for designating a baseline serum concentration of relaxin as diagnostic of pregnancy status; therefore, it seemed reasonable to select the lowest detectable concentration or mean assay sensitivity (11.2 ng/mL) for the purpose of this study. The decision for the latter is supported by the results of a previous study (Bergfelt et al., 2011) in which the lowest relaxin concentration in pregnant bottlenose dolphins under managed care was 15.1 ng/mL. Moreover, in the present study, the lowest relaxin concentration in known pregnant wild bottlenose dolphins was 60.8 ng/mL. Hence, for the present study, directional changes in serum concentrations of relaxin relative to baseline concentrations were considered diagnostic of pregnancy (> 11.2 ng/mL) and nonpregnancy (≤ 11.2 ng/mL).

In two populations of wild bottlenose dolphins associated with estuarine sites along the eastern coast of the U.S. (Charleston, South Carolina, and the Indian River Lagoon, Florida), single-sample analyses of serum progesterone with concentrations above a baseline concentration were diagnostic of pregnancy in 20 bottlenose dolphins. In the same serum samples, analyses of relaxin with concentrations above a baseline concentration were diagnostic of pregnancy in 12 bottlenose dolphins. Considering progesterone as the "gold standard," contingency table analysis indicated a positive correspondence of relaxin with progesterone at 60% (12 relaxin-diagnosed out of 20 progesterone-diagnosed pregnancies) and negative correspondence at 40% (8 relaxin-diagnosed nonpregnancies out of 20 progesterone-diagnosed pregnancies). Kappa analysis indicated relaxin diagnosis of pregnancy status was in good agreement (κ = 74%) with progesterone diagnosis. Notably, the analysis is based on the assumption that all 20 progesterone-diagnosed pregnancies were pregnant. Since an elevation in progesterone based on a single-sample analysis is not necessarily pregnancy-specific in bottlenose dolphins (Sawyer-Steffan et al., 1983; Yoshioka et al., 1986; Robeck et al., 2001, 2005), it is likely that a portion of the 20 progesterone-based pregnancies were nonpregnant. In this regard, the contingency table analysis may have underestimated the number of relaxin-diagnosed pregnancies or positive correspondences at 60% and overestimated the number of false negatives or negative correspondences at 40%.

For verification of the contingency table results, a retrospective evaluation of field data was conducted based on photo-identification of cow-calf pairs that were sighted within 12 mo of previous health assessments. The observation of nine cow-calf pairs corresponded to nine adult female bottlenose dolphins from which blood samples were analyzed for serum concentrations of progesterone and relaxin. The concordance between the hormone-diagnosed pregnancies and observed or known pregnancies was 9 out of 9 (100%) for progesterone and 7 out of 9 (78%) for relaxin. In 2 out of 9 (22%) known pregnancies, relaxin concentrations were not above a baseline concentration (i.e., false negatives) and not diagnostic of pregnancy. Based on photo-identification of these two bottlenose dolphins and the sighting of corresponding newborn calves, the time that health assessments were conducted occurred during their first trimester of pregnancy. Perhaps, therefore, the basis for the apparent false negatives of relaxin is due, in part, to relatively low and undetectable serum concentrations during early pregnancy as reported in managed-care dolphins (Bergfelt et al., 2011). Nonetheless, while additional studies are required to fully characterize the temporal changes in circulating concentrations of relaxin throughout pregnancy in bottlenose dolphins, the novelty of the field evaluation in pregnant wild bottlenose dolphins corroborated a previous evaluation in pregnant managed-care bottlenose dolphins that an elevation of relaxin is pregnancy-specific (Bergfelt et al., 2011).

The present study provided preliminary information indicating a potential limitation for using relaxin alone to diagnose pregnancy that was of various degrees but consistent between the statistical (i.e., predicted) and field (i.e., observed) evaluations. There were nine wild bottlenose dolphins considered pregnant through observation by photo-identification that were used to verify the predicted results from the contingency table analysis. The positive correspondence or sensitivity for pregnancy diagnosis with relaxin compared to progesterone was 60% for predicted and 78% for observed or known pregnancies. In addition, the negative correspondence or false negative rate for relaxin was 40% for predicted and 22% for observed. Unfortunately, the contingency table results for relaxin specificity (100%) and false positives (0%) could not be verified since photo-identification studies cannot be used to definitively diagnose nonpregnancy in cows without a calf due

to the periodicity of surveys, unrecognized pre- and post-natal losses, predation of calves, and other events that may occur prior to sightings (Mazzoil et al., 2005; Speakman et al., 2010). Although more critical studies are required to address the potential limitation (i.e., false negative rate) and application of relaxin to diagnose pregnancy status alone, preliminary results of the combined statistical and field evaluations supported the hypothesis that an elevation in serum relaxin is pregnancy specific and can be used in a single-sample analysis to clarify that an elevation in serum progesterone is pregnancy related and, thus, confirm pregnancy in wild bottlenose dolphins.

In summary, the present study provided preliminary results for hormonal diagnosis of pregnancy in wild bottlenose dolphins based on a single-sample analysis of serum progesterone in combination with relaxin above (pregnant) or below (nonpregnant) respective baseline concentrations. Although the limitations of the present study prohibited a more critical evaluation, relatively high concentrations of both serum progesterone and relaxin in the same sample can potentially confirm the diagnosis of pregnancy in bottlenose dolphins and, perhaps, other cetaceans.

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