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Investigation of an Immunoreactive Chorionic Gonadotropin-Like Substance in the Placenta, Serum, and Urine of Pregnant Bottlenose Dolphins (*Tursiops truncatus*)

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Abstract

This study was designed to test the hypothesis that an immunoreactive chorionic gonadotropin (CG)-like substance is present in full-term dolphin placentas and to determine if CG immunoreactivity can be detected in corresponding serum and urine samples for potential application to diagnose pregnancy. Six placentas were collected immediately after parturition from four captive bottlenose dolphins in 2003, 2007 (Experiment 1), and 2011 (Experiment 2). Serum and urine were collected during early, middle, and late pregnancy from the same dolphins. In Experiment 1, an eCG radioimmunoassay (RIA) was used to analyze dilutions of supernatants from the homogenates of each placenta for eCG immunoreactivity, and a commercial hCG Enzyme-Linked Immunosorbent Assay (ELISA) was used to analyze individual serum samples and dilutions of pools of serum and urine for hCG immunoreactivity. Specific CG immunoreactivity was not detected above assay sensitivities in any of the supernatants of respective placental homogenates, including the highest concentrate (100 mg/mL), nor in any of the individual samples and pools of serum and urine. In Experiment 2, the highest placental homogenate was increased five-fold (500 mg/mL), sensitivity of the eCG RIA was increased six-fold, and a different combination of hCG antibodies was used in an alternative commercial "sandwich"-type ELISA. Despite the optimization, specific CG immunoreactivity in placental tissue and individual serum and urine samples was not detected above assay sensitivities. In conclusion, the hypothesis that an immunoreactive CG-like substance is present in full-term dolphin placentas was not supported. In addition, non-immunoreactivity of a CG-like substance in serum and urine samples collected during various stages of pregnancy precluded the development and application of a CG-based immunoassay for diagnosing pregnancy status in dolphins.

Key Words: chorionic gonadotropin, placenta, immunoanalysis, pregnancy, bottlenose dolphin, *Tursiops truncatus*

Introduction

Diagnosis of pregnancy status in dolphins and other cetaceans using ultrasonography and progesterone analysis has been well-described and reviewed (Schroeder, 1995; Robeck et al., 2001). While ultrasonography is considered the most accurate approach for pregnancy diagnosis, aquatic facilities or institutions that house or study captive and wild dolphins may not have the technical expertise, available funds, or infrastructure to routinely apply, purchase, and support the technology. Alternatively, analysis of circulating concentrations of progesterone has been the most conventional hormonal method for pregnancy diagnosis in cetaceans (Schroeder, 1995; Robeck et al., 2001). However, with single-sample analysis and no accompanying history of reproductive or breeding status, there is a degree of uncertainty whether or not an elevation in serum progesterone is pregnancy-related since progesterone is also elevated during diestrus and prolonged luteal

maintenance or pseudopregnancy in dolphins (Schroeder, 1995; Robeck et al., 2001). Recently, detection of a placental and pregnancy-specific increase in circulating concentrations of relaxin has been documented in dolphins (Bergfelt et al., 2011). Although serum relaxin has the potential to serve as a hormonal measure for diagnosing pregnancy status in dolphins, the relaxin radioimmunoassay is currently not readily accessible. Thus, an alternative hormonal method with specificity and broader accessibility and applicability can potentially enhance reproductive management practices and conservation efforts for diagnosing pregnancy status in dolphins and, perhaps, other cetaceans.

Placental production of chorionic gonadotropin (CG) is thought to be unique to humans, some primates, and equids. In humans and most other primates, hCG is necessary as a luteotropic hormone that "rescues" the primary corpus luteum (CL) from regression and supports continued production of progesterone, which is essential for the development and maintenance of early pregnancy prior to a luteal-placental shift in progesterone production (Zeleznik & Pohl, 2006). Correspondingly, in equids, the luteotropic effect of eCG is involved in the "resurgence" of the primary CL (Bergfelt et al., 1989) and development of supplemental CL (Ginther, 1992; de Mestre et al., 2011) to support production of progesterone, which is also essential for embryo/fetal growth and survival during early pregnancy. In humans (Norman et al., 1987) and equids (Roser & Lofstedt, 1989), placental production of hCG and eCG leads to secretion and excretion such that relatively high circulating concentrations of CG during early pregnancy can be detected immunologically in a single-sample analysis of blood or urine. In this regard, hCG/eCG-based immunoassays have been developed, validated, and commercialized for clinical application in human and veterinary medicine as a hormonal means to diagnose pregnancy status in women and mares.

In bottlenose dolphins, preliminary results with captive animals indicated the presence of an apparent placental CG-like substance (Hobson & Wide, 1986; Watanabe et al., 2007). In one study (Hobson & Wide, 1986), the substance in a full-term placenta displayed apparent bioactive, immunoreactive, and structural similarities to hCG as follows: (1) a mouse bioassay detected an increase in uterine weights that was dose-responsive to dolphin and human placental extracts in a linear and parallel manner to an hCG reference standard, (2) an hCG antiserum neutralized a dolphin CG-induced increase in mouse uterine weight, (3) an hCG radioimmunoassay of dolphin and human placental extracts detected immunoreactivity for intact hCG and hCG α- and β-subunits, and (4) gel chromatography and zone electrophoresis of dolphin and human placental extracts detected similarities in molecular size and charge between a dolphin CG-like substance and hCG. In another study (Watanabe et al., 2007), immunohistology with rabbit anti-ovine LH-β detected a positive stain for an apparent dolphin LH/CG-like substance in a full-term placenta. In the same study, cloned cDNA encoding apparent dolphin LH/CG α- and β-subunits were prepared from placental tissue and amplified. The amino acid sequences of the dolphin LH/CG α- and β-subunits were structurally homologous with equid LH α- and β-subunits (83 to 87% and 81 to 85% combined across several species; Watanabe et al., 2007). In addition, dolphin LH/CG is also structurally related to human hCG α- and β-subunits (71 and 72%, respectively; Watanabe et al., 2007). Thus, confirming the presence of a dolphin immunoreactive CG-like substance in placental tissue and the novelty of detecting the substance in corresponding serum or urine samples are prerequisites before exploring the full potential for application of a CG-based immunoassay as a more specific hormonal measure than progesterone to diagnose pregnancy status in captive and wild populations of dolphins.

The objectives of the present study using specific and sensitive CG-based immunoassays with captive bottlenose dolphins were to (1) corroborate the results of previous studies and test the hypothesis that an immunoreactive CG-like substance is present in full-term placentas and (2) determine if CG immunoreactivity can be detected in corresponding serum and urine samples collected during various stages of pregnancy for application of a CG-based immunoassay to diagnose pregnancy status in dolphins.

Materials and Methods

Dolphins and Dolphin Management

The study involved four female bottlenose dolphins housed at Dolphin Quest Hawaii in Waikoloa and included one founder dolphin and three other dolphins born under human care. Pregnant and postpartum females were kept with their respective calves and other adult and juvenile male and female dolphins in an outdoor habitat or lagoon that contained approximately 7.6 million L of natural seawater. Dolphins were housed and fed in compliance with the U.S. Animal Welfare Act and by the *Standards and Guidelines of the Alliance of Marine Mammal Parks and Aquariums*.

Experiment 1

Collection of Placentas, Blood, and Urine—Collection and archiving of placentas, and serum and urine samples were done during routine animal husbandry practices as part of a proactive health and breeding program at the Dolphin Quest facility in compliance with the U.S. Department of Agriculture-approved Program of Veterinary Care. Placentas were collected within 8 h after delivery of live calves in 2003 (Placentas A, B, C & D) and 2007 (Placenta E). After retrieval from the birthing pool, each of the five full-term placentas (A to E) was inspected for abnormalities and frozen intact at -20° C.

Blood samples were collected episodically from the same dolphins for four pregnancies and urine from three pregnancies during early (Months 1 to 4), middle (Months 6 to 9), and late (Months 10 to 12) pregnancy. Months of pregnancy were estimated retrospectively based on the date of parturition and a 12-mo gestation period (Cornell et al., 1987). Blood and urine were collected voluntarily from each dolphin following preconditioned behavior (Sweeney et al., 2003). Blood was collected from the arterio-venous plexus of the tail with a 21-gauge needle, transferred from a syringe to a 10-mL serum separator tube, and centrifuged within 45 min. After the dolphin was signaled to urinate, urine was collected mid-stream in a sterile container. Serum and raw urine were transferred to one or more cryovials, labeled with dolphin identification and date, and stored frozen at -20° C.

Placentas, and serum and urine samples were temporarily stored on-site at the Dolphin Quest facility and later transferred and stored frozen (-20° C) at Hawaii Pacific University in Kaneohe. Placentas were shipped frozen with dry ice to Louisiana State University Agricultural Center in Baton Rouge for eCG analysis, and serum and urine samples were shipped under similar conditions to the Smithsonian Conservation Biology Institute (Front Royal, VA, USA) for hCG analysis. At respective institutes, all samples were stored frozen (-20° C) until processing and hormonal analysis.

Processing and Analysis of Placental Tissue—Frozen placentas were thawed overnight in a refrigerator at 5° C. To maximize the potential to detect small amounts of placental protein in the least amount of tissue, the greatest amount of tissue to buffer ratio was used in the homogenization process. The amount of tissue sampled was based, in part, on hCG immunoreactivity detected in a precipitate of placental proteins after solvent extraction of one half of a whole placenta (Hobson & Wide, 1986) and eCG biological and binding activities (13,000 IU/mg protein; Licht et al., 1979). In the present study and with the understanding that the dolphin placenta is of a diffuse nature (Slijper, 1966), portions of tissue were excised from several randomly selected sites of each placenta, combined, and placed in cold phosphate buffered saline (PBS) as follows: Placenta A, 16.4 g in 164.0 mL; Placenta B, 8.2 g in 82.0 mL; Placenta C, 6.4 g in 64.0 mL; Placenta D, 9.6 g in 96.0 mL; and Placenta E, 7.3 g in 73.0 mL. Each sample was homogenized using a Waring blender at maximum speed for 30 s. Homogenates were adjusted with PBS to a final tissue concentration of 100 mg/mL.

Analysis of the supernatants of respective placental tissue homogenates was done using an in-house validated radioimmunoassay (RIA) for eCG (Thompson et al., 1982). Main components of the assay included equine LH (eLH) as the ^{125}I-labeled ligand (40,000 cpm/tube), polyclonal rabbit anti-eCG as the primary antibody (working dilution, 1:4000/tube), goat anti-rabbit immune gamma globulin (IgG, working dilution, 1:20) as the secondary antibody, and eCG as the reference standard (0.1125, 0.225, 0.45, 0.9, 1.8, 3.6, 7.2, and 14.4 IU/mL).

Analysis of placental tissue homogenates for eCG immunoreactivity was done by centrifuging (1,200 × g) a portion of the homogenate concentrates (100 mg/mL) from each placenta at 5° C for 15 min. From the 100 mg/mL supernatant, serial dilutions (10, 1, 0.1, 0.01, and 0.001 mg/mL) were prepared with PBS supplemented with 0.1% gelatin (PBS-GEL). A competitive RIA approach was conducted by incubating 200 μL/tube of supernatants, anti-eCG, and ^{125}I-eLH together at 5° C for approximately 24 h. Thereafter, anti-IgG was added to all tubes and incubated at 5° C for approximately 48 h. The assay was terminated by adding cold PBS to all tubes, which was followed by centrifugation (1,200 × g) at 5° C for 30 min. Concentrations of eCG immunoreactivity were estimated and reported as the average of duplicate tubes or samples. The limit of detection (i.e., sensitivity) of the assay was based on average counts per minute for the maximum specific binding minus 2 SDs. Assay variability was based on eCG concentrations in quality control samples (i.e., pregnant mare serum with a known concentration of eCG) that were used to calculate intra- and inter-assay CVs.

Based on initial inspection of results from the competitive RIA approach, a noncompetitive approach was conducted to increase assay sensitivity. The noncompetitive approach was similar to the competitive approach except that the supernatants of the three highest tissue homogenates (100, 10, and 1 mg/mL) of each placenta were allowed to incubate with anti-eCG for 48 h before adding ^{125}I-eLH.

To determine if placental tissue eCG immunoreactivity could be artificially reduced as a result of the homogenization process, eCG was added to newly collected tissue samples. Placental tissue was sampled from Placentas D and E and added to cold PBS as follows: Placenta D, 4.3 g in 43.0 mL, and Placenta E, 6.0 g in 60.0 mL. A fixed volume of serum from a pregnant mare with a known concentration of eCG (160 IU/mL) was included with respective tissue samples and mixed. An aliquot was collected before homogenization for comparison of eCG immunoreactivity after homogenization. After homogenization and centrifugation, the supernatant of each homogenate was adjusted to a final concentration of 100 mg/mL with PBS-GEL and analyzed using a noncompetitive RIA approach similar to that previously described except the supernatant volume was 50 uL/tube.

Analysis of Serum and Urine—The analysis of dolphin serum and urine was done using a commercial 96-well Enzyme-Linked Immunosorbent Assay (ELISA) kit (No. 07BC-1027; MP Biomedicals, Orangeburg, NY, USA) designed for clinical quantification of serum hCG. Main components of the capture or "sandwich"-type assay consisted of goat anti-hCG-α as the primary capture antibody, mouse monoclonal anti-hCG conjugated to horseradish peroxidase as the secondary detection antibody, and hCG (WHO, 1st IRP/3rd IS, 75/537) as the reference standard (5, 20, 50, 150, and 300 mIU/mL). The assay was conducted in accord with instructions from the manufacturer using 50 uL of standards, controls, and serum or urine sample/well.

Analysis of hCG immunoreactivity was done with individual serum samples collected at various stages of pregnancy, as well as pools of serum and urine. Pools of serum and urine were prepared by combining aliquots (100 uL of serum or 50 uL of urine) from selected samples collected at each stage of pregnancy. To each pool of serum and urine, PBS supplemented with 0.5% bovine serum albumin (PBS-BSA) was added to prepare serial dilutions of respective pools (1:1, 1:2, 1:4, 1:8, 1:16, and 1:32). Correspondingly, similar dilutions of the urine pool were prepared with distilled water or zero standard provided in the assay kit to investigate the affect of different dilution mediums. Concentrations of hCG immunoreactivity were estimated and reported as the average of duplicate wells or samples. The limit of detection (i.e., sensitivity) of the assay was based on average absorbance for the zero standard minus 2 SDs, and assay variability was based on hCG concentrations in quality control samples (i.e., low control, a pool of the three lowest hCG standards; high control, a pool of the two highest hCG standards) that were used to calculate intra- and inter-assay CVs.

Experiment 2

To clarify the apparent non-immunoreactivity of CG in archived tissues in Experiment 1, a placenta, and serum and urine samples that had been frozen for < 1 y were analyzed. From one of the same four dolphins studied in Experiment 1, and under the same conditions, a full-term placenta and corresponding serum and urine samples were collected and shipped frozen to respective laboratories for analysis. Processing and analysis of placental homogenates for eCG were similar to that described in Experiment 1, except the placenta was processed (8.8 g of placental tissue plus 8.8 mL of PBS) to yield a higher homogenate concentrate (500 mg/mL) from which a lower concentrate (100 mg/mL) was also prepared. For each concentrate, supernatant volumes of 200, 100, 50, and 25 µL/tube were analyzed for eCG immunoreactivity using the more sensitive noncompetitive RIA approach.

Correspondingly, analysis of serum and urine samples for hCG was similar to that described in Experiment 1, except the analysis involved a different commercial 96-well ELISA kit (No. 4201-16; Diagnostics Automation, Inc., Calabasas, CA, USA) specifically designed for clinical quantification of total serum hCG-β. Main components of the assay consisted of anti-hCG as the primary capture antibody, mouse monoclonal anti-hCG-β conjugated to horseradish peroxidase as the secondary detection antibody, and hCG reference standard (5, 20, 50, 150, and 300 mIU/mL). The assay was conducted in accord with instructions from the manufacturer using 50 uL of standards, controls, and serum or urine sample/well.

Results

Analysis of Placental Tissue

In Experiments 1 and 2, performance of the eCG RIA combined over multiple assays was indicated by intra- and inter-assay CVs that were 6 and 9%, respectively, and sensitivity that was estimated at 0.006 IU/mL (46 ng eCG/mg of placental tissue) for the competitive RIA approach and 0.001 IU/mL (0.77 ng eCG/mg of placental tissue) for the more sensitive, noncompetitive approach.

In Experiment 1, eCG immunoreactivity was not detected above the sensitivities of the competitive and noncompetitive RIA approaches for any concentration of tissue homogenate from Placentas A to E (Table 1). Effectiveness of the homogenization process was indicated by the amount of eCG added before homogenization, which was not different from the amount detected in the supernatants after homogenization (Table 2).

In Experiment 2, apparent eCG immunoreactivity was initially detected in a 200 uL/tube of

Table 1. Supernatants of respective tissue homogenates of five placentas (A to E) collected from dolphins at the time of parturition were diluted with PBS-GEL and analyzed using an eCG RIA in a competitive and noncompetitive manner (Experiment 1).

Dolphin placenta	Tissue homogenate (mg/mL)	eCG immunoreactivity (IU/mL)	
		Competitive	Noncompetitive
A to E	100	--	< 0.001
A to E	10	< 0.006	< 0.01
A to E	1	< 0.06	< 0.1
A to E	0.1	< 0.6	--
A to E	0.01	< 6	--
A to E	0.001	< 60	--

Table 2. Tissue of two placentas (C and D) collected from dolphins at the time of parturition were homogenized with a fixed amount of eCG. Supernatants of respective tissue homogenates were analyzed using an eCG RIA in a noncompetitive manner (Experiment 1).

Dolphin placenta	Tissue homogenate (mg/mL)	eCG immunoreactivity (IU/mL)		Recovery (%)
		Before homogenization	After homogenization	
C	100	39.5	37.8	96
D	100	41.9	41.8	99

supernatant using 100 mg/mL of placental tissue homogenate. Concentration of apparent eCG immunoreactivity was estimated at 0.041 ng/mL, which was equivalent to about 0.0053 IU eCG/mL and slightly above sensitivity of the noncompetitive RIA approach. Apparent eCG immunoreactivity was also detected in a 100 and 200 µL/tube of supernatant using 500 mg/mL of homogenate. However, estimates of the concentrations were not five-fold higher, and both dilution volumes were near assay sensitivity with no indication of a decrease in specific binding typically seen with an increase in volume of analyte.

Analysis of Serum and Urine

In Experiment 1, performance of the hCG ELISAs over multiple assays was indicated by intra- and inter-assay CVs that were both 3% and, in Experiment 2, by an intra-assay CV that was 4%. Assay sensitivity in both Experiments 1 and 2 was estimated at 2 mIU/mL.

In Experiment 1, hCG immunoreactivity was not detected above assay sensitivity in any of the individual serum samples collected during early, middle, and late pregnancy from the same dolphins that yielded placentas in 2003 and 2007 (Table 3). Similarly, hCG immunoreactivity was not detected above assay sensitivity in corresponding serial dilutions of pools of serum and urine or in dilutions of pools of urine prepared with different media (Table 4).

In Experiment 2, with a different combination of hCG antibodies, immunoreactivity was not detected above assay sensitivity in any of the individual serum and urine samples collected during early (urine, $n = 8$), middle (serum, $n = 2$; urine, $n = 5$), and late (serum, $n = 2$; urine, $n = 3$) pregnancy from the same dolphin that yielded a placenta in 2011 (data not shown).

Discussion

The rationale for the present study was based on previous studies in which an apparent immunereactive CG-like substance was detected in full-term placentas of bottlenose dolphins with anti-hCG (Hobson & Wide, 1986) or anti-ovine LH-β (Watanabe et al., 2007). Unlike the original study (Hobson & Wide, 1986), the present study used anti-eCG in an eCG-based RIA (Thompson et al., 1982). Preference for use of anti-eCG was based on the high degree of immunoreactivity between anti-eCG and eLH (344% cross-reactivity; Thompson et al., 1982), homology of the amino acid sequences of eLH α- and β-subunits (83 to 87% and 81 to 85% combined across several equine species) with dolphin LH/CG α- and β-subunits (Watanabe et al., 2007), and homology of eCG α- and β-subunits (78 and 80%, respectively) with hCG α- and β-subunits (Ward et al., 1982; Sugino et al., 1987). In addition, dolphin LH/CG also appears structurally related to the α- and β-subunits of LH in other species (cattle, 92 and 84%; pigs, 97 and 94%; sheep, 92 and 84%; dogs, 94 and 91%; Watanabe et al., 2007) in which the eCG RIA used herein has been applied (cattle

Table 3. Serum samples collected during various stages of pregnancy from dolphins as represented by respective placentas (A to D) were analyzed using an hCG ELISA (Experiment 1).

Dolphin placenta	Stage and month of pregnancy			hCG immunoreactivity (mIU/mL)
D	Early	1 to 4	(4)	< 2
A, D	Middle	6 to 9	(3)	< 2
B, C	Late	10 to 12	(4)	< 2

Parentheses indicate number of serum samples.
Month of pregnancy was determined retrospectively from month of parturition (Month 0).

Table 4. Serum and urine collected during various stages of pregnancy from dolphins as represented by respective placentas (A to D) were portioned, pooled, diluted with PBS-BSA, and analyzed using an hCG ELISA. The urine pool was also diluted with distilled water or zero standard and analyzed (Experiment 1).

			hCG immunoreactivity (mIU/mL)		
Dolphin placenta	Pool	Serum or urine dilutions (v/v)	PBS-BSA	Distilled water	Zero standard
A, B, C, D	Serum	1:1 to 1:32	< 2	--	--
B, C, D	Urine	1:1 to 1:32	< 2	< 2	< 2

and pigs, Thompson et al., 1984, 1985; sheep and dogs, D. L. Thompson, pers. comm., 30 January 2012). Thus, the eCG RIA used in the present study was considered a comparable, specific, and sensitive approach to that used in earlier studies (Hobson & Wide, 1986; Watanabe et al., 2007) to test the hypothesis that an immunoreactive CG-like substance is present in full-term placentas of dolphins.

In Experiment 1, eCG immunoreactivity was not detected above the limit of RIA sensitivities (competitive, 0.006 IU/mL; noncompetitive, 0.001 IU/mL) in any of the five full-term placentas collected in 2003 and 2007. Even with the noncompetitive RIA approach in which there was a six-fold increase in sensitivity and ten-fold increase in placental tissue (20 mg/tube) compared to the competitive approach, no sample had eCG activity ≥ 0.77 ng eCG/mg of placental tissue (Licht et al., 1979). In the previous study (Hobson & Wide, 1986), apparent hCG immunoreactivity was detected in a precipitate of proteins extracted with acetone and diethyl ether from one half of a whole placenta. In the present study, collection of placental tissue from several different sites of each placenta, homogenization in PBS, and analysis of the supernatants was considered a more efficient, less stringent, and direct approach to detect a dolphin CG-like protein. Furthermore, the effectiveness of the PBS homogenization process used herein was indicated by the addition of a known amount of eCG to tissue samples before homogenization and detection of the same amount (96 to 99% recovery) after homogenization.

Considering that the placentas in Experiment 1 had been archived for 4 to 8 y and that long-term freezer storage may result in tissue degradation and loss of protein integrity and stability (National Academy of Science [NAS], 1991), Experiment 2 was conducted with a full-term placenta collected in 2011. Collection, freezer storage, and processing of the 2011 placenta were similar to the 2003 and 2007 placentas except that it was examined within 2 mo of collection using the more sensitive, noncompetitive eCG RIA approach. Similar to the placental tissue concentration in Experiment 1 (100 mg/mL), analysis of the supernatant resulted in detection of apparent eCG immunoreactivity (0.0053 IU/mL), which was slightly above assay sensitivity (0.001 IU/mL). However, when a five-fold increase of homogenate (500 mg/mL) was analyzed, immunoreactivity was unchanged, and there was no decrease in specific binding between the two highest volumes of supernatant (100 and 200 uL/tube) that would be typical of dose-response inhibition (i.e., no indication of a parallelism with the eCG standard curve). Although the nature of the apparent eCG immunoreactivity in the placenta collected in 2011 could not be clarified, consideration of the combined results from Experiments 1 and 2 indicated nonspecific immunoreactivity.

The reason why an immunoreactive CG-like substance was detected in full-term dolphin placentas in previous studies (Hobson & Wide, 1986; Watanabe et al., 2007) but not in the present study is not known. Although the methodology may have been different among studies, immunoreactivity to eCG in the present study was expected to be specific, especially considering the high degree of structural homology among eCG, hCG, oLH, eLH, and dolphin LH/CG (Ward et al., 1982; Sugino et al., 1987; Watanabe et al., 2007) and

cross-reactivity between eCG and eLH (Thompson et al., 1982). Apart from methodology, the preliminary nature of the previous studies involved one dolphin or placenta per study (Hobson & Wide, 1986; Watanabe et al., 2007) compared to four dolphins and six full-term placentas in the present study. Nonetheless, the present study did not corroborate previous results or support the hypothesis that an immunoreactive CG-like substance is present in full-term placentas of dolphins.

In Experiments 1 and 2, multiple serum and urine samples collected during early, middle, and late pregnancy from the same dolphins that yielded placentas collected postpartum were analyzed for hCG immunoreactivity using commercially available, clinically based ELISAs. Despite the novelty of using different combinations of hCG antibodies in "sandwich"-type ELISAs for capture and detection, there was no immunological indication of a dolphin CG-like substance in serum or urine during pregnancy; immunoreactivity for either hCG α- or β-subunits was consistently below the detectable limits of the assays (2 mIU/mL) in all individual samples and pools of serum and urine.

Reportedly, carboxyl terminal peptides of the hCG β-subunit serve as an effective linker to enhance hormone secretion (Muyan et al., 1996; Nakav et al., 2006). When compared to native β-hCG, the secretion of β-hCG devoid of a carboxyl terminal region was reduced two-fold (Muyan et al., 1996). In dolphin placental tissue, an apparent LH/CG-like substance was detected and cloned (Watanabe et al., 2007). Upon analysis of the cDNA, there was no indication of carboxyl terminal peptides or related sequences associated with the LH/CG β-subunit. In the present study, the non-immunoreactivity of CG in serum and urine collected during various stages of pregnancy does not necessarily preclude the presence of a CG-like substance in the prepartum placenta in dolphins. In consideration of an apparent dolphin CG-like substance that is devoid of a carboxyl terminal region (Watanabe et al., 2007), perhaps secretion and excretion of placental CG is unappreciable for immunological detection in serum and urine during pregnancy in this species.

In the present study, Experiments 1 and 2 were not designed to be comprehensive or definitive for determining whether or not a CG-like substance can be detected in the postpartum placenta of dolphins as previously reported (Hobson & Wide, 1986; Watanabe et al., 2007). Instead, the experiments were designed to corroborate and elaborate on the presence of a dolphin immunoreactive CG-like substance in the placenta, and serum and urine samples as prerequisites before exploring the full potential for application of a CG-based immunoassay as a more specific hormonal measure than progesterone to diagnose pregnancy status in dolphins. Perhaps with the use of contemporary molecular-based technology and increased species specificity, future studies will clarify if the dolphin placenta produces and secretes/excretes a CG-like substance to the extent that it can be exploited for application to enhance reproductive management practices and conservation efforts in captive and wild populations of dolphins and, perhaps, other cetaceans.

In conclusion, the results with a specific and sensitive eCG-based RIA did not corroborate previous results or support the hypothesis that an immunoreactive CG-like substance is present in full-term dolphin placentas. In addition, non-immunoreactivity of a CG-like substance in serum and urine samples collected during various stages of pregnancy using different combinations of hCG antibodies in clinical-based ELISAs precluded the development and application of a CG-based immunoassay for diagnosing pregnancy status in dolphins.

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