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Ovarian Follicular Dynamics During the Luteinizing Hormone Surge in the Bottlenose Dolphin (*Tursiops truncatus*)

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Characterizing the relationship between ovarian follicular dynamics and the luteinizing hormone (LH) surge in the bottlenose dolphin (*Tursiops truncatus*) requires detailed daily monitoring due to the transitory nature of LH and ovulation. Utilizing conditioned dolphins and non-invasive sampling techniques, such as urine collection and trans-abdominal ultrasound exams, provides the means to accurately monitor these fleeting processes. Urine samples and ultrasound exams used in this study were originally performed for the purposes of artificial insemination and controlled natural breeding. The LH surge was identified by a rapid immunochromatographic assay (ICG), and real-time B-mode trans-abdominal ultrasound imaging was used to identify pre-ovulatory follicles (POF). Increases in urinary progesterone levels along with the disappearance of the POF verified ovulation. This study found that POF diameters during the LH surge were 1.942 +/- 0.098 cm (n = 9), and time to disappearance of the POF from the last recorded LH peak sample was 37.475 +/- 12.346 h (n = 6). Peak LH surge levels, based on samples collected 2 to 4 times daily, lasted 6.050 +/- 1.332 h (n = 6). Data suggests that bottlenose dolphins, like many other mammals, have brief ovulatory LH surges followed by ovulation within 48 hours.

The study of reproduction often requires identification and assessment of fleeting but key physiological processes. The relationship between the ovulatory luteinizing hormone (LH) surge and ovulation is one such example. Often such processes take place within a few hours. Time from the ovulatory LH surge to ovulation in domestic species, with the exception of the mare, occurs within 24-40

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hrs (Pineda & Dooley, 2003). In the African and Asian elephant (*Elephas maximus*), the ovulatory LH surge is estimated to last 28 hrs (Brown, Schmitt, Bellen, Graham, & Lehnhardt, 1999). The LH surge in the giant panda (*Ailuropoda melanoleuca)* is estimated to last between 12 hrs to 3 days (Durrant, Ravida, Spady, & Cheng, 2006). In the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) and killer whale (*Orcinus orca*), ovulation occurs within 31 and 38 hrs respectively following the ovulatory LH surge (Robeck et al., 2004; Robeck et al., 2009).

To accurately study the ovulatory LH surge, ovarian follicle dynamics, and ovulation in bottlenose dolphins (*Tursiops truncatus*), multiple daily sampling is required. One of the benefits of studying reproduction in zoo and aquarium species is the ability to obtain numerous samples from conditioned animals. In particular, with dolphins, non-invasive techniques such as urine collection and trans-abdominal ultrasound exams can be conducted multiple times daily. In this study, the dolphins were being monitored for artificial insemination and controlled natural breeding; therefore, samples were opportunistically collected based on clinical needs. Of the 10 dolphin ovulatory estrous cycles characterized, 9 were natural cycles and one was altrenogest induced. Altrenogest treatment is a method used to synchronize bottlenose dolphin estrous cycles (Robeck et al., 2005). The LH surge was identified by a commercially available rapid immunochromatographic assay (ICG) designed to detect serum LH in the domestic dog (*Canis familiaris*). This is a previously discussed method for identification of bottlenose dolphin urinary LH (Muraco et al., 2009). Additionally, the same canine ICG LH assay has been used to identify LH in the Giant Panda (Durrant et al., 2006). The structure of LH is well conserved among mammal species allowing for cross-reactivity of various LH antibodies (Liao et al., 2003). Real-time B-mode trans-abdominal ultrasound imaging was used to characterize ovarian follicular dynamics before, during and after the LH surge. Ultrasound has previously been proven to be an accurate method for identification of the pre-ovulatory follicle (POF) and ovulation in dolphins (Brook, 2001). Urinary progesterone assays were performed to ensure ovulation had occurred. Urinary steroid metabolites have been monitored in bottlenose dolphins by electrochemiluminescence immunoassay (ECLIA) and enzyme-linked immunosorbant assay (ELISA) (Muraco et al., 2009; Robeck et al., 2005).

The goal of the study was to better understand the relationship between ovarian follicular dynamics and the LH surge in the bottlenose dolphin. This knowledge will further understanding of dolphin reproduction, assist in the accurate timing for artificial insemination, and maximize controlled natural breeding programs.

Method

Experimental ethics

All animals, materials and methods used in the study were individually evaluated by each facility's Internal Animal Care and Use Committee (IACUC) to ensure safety, health and well-being

of the animals. Samples were taken during routine behaviors in which the animals were conditioned to participate.

Animals

Bottlenose dolphins used in the study consisted of 8 females from 4 different facilities (Table 1). Animals 1 and 2, located at Six Flags Discovery Kingdom (SFDK; Vallejo, CA, USA), were housed in a 2,722,281-L manufactured seawater pool maintained at 21-22^{o}C year round. Animals 1 and 2 were fed a diet of 10.4 and 5.9 kg, respectively, of whole frozen thawed herring (*Clupea harengus*) and capelin (*Mallotus villosus*) daily. Animals 3 and 4, located at Dolphin Research Center (DRC; Grassy Key, FL, USA), were housed in 1,097,209 L and 555,955 L, respectively, of natural seawater with an average temperature of 21.5^{o}C December-March. Animal 3 received a daily diet of 5.9 kg of whole frozen thawed herring (*C. harengus*), capelin (*M. villosus*), and silversides (*Menidia menidia*). Animal 4 was fed a daily diet of 5.4 kg of whole frozen thawed herring (*C. harengus*), capelin (*M. villosus*), and marine smelt (*Hypomesus pretiosus*). Animal 5, located at Theater of the Sea (TOTS; Marathon, FL, USA), was housed in 549,550 L of natural seawater with an average temperature of 31.3 ^{o}C in July and August. Animal 5 was fed a daily diet of 6.22 kg of herring (*C. harengus*), capelin (*M. villosus*), squid (*Loligo opalescens*), and lake smelt (*Osmerus eperlanus mordax*). Animals 6-8, located at The Mirage Hotel (Mirage; Las Vegas, NV, USA), were housed in a 3,141,892-L manufactured seawater pool, maintained at 21-23°C year-round. Animals 6-8 received a diet of frozen thawed herring (*C. harengus*), capelin (*M. villosus*), sardine (*Sardina pilchardus*), and squid (*L. opalescens*) for daily totals of 8.62, 7.26 and 8.16 kg, respectively.

Table 1
Bottlenose dolphins in the study

Animal	Facility[a]	Sex	Birth Date	Weight (kg)	Reproductive History[b]
1	SFDK	F	7/1979[c]	184.09	Parous
2	SFDK	F	7/1986[c]	189.09	Parous
3	DRC	F	11/1984[d]	189.55	Parous
4	DRC	F	2/2001[d]	168.64	Nulliparous
5	TOTS	F	1/1983[c]	172.36	Parous
6	Mirage	F	3/2000[d]	159.66	Nulliparous
7	Mirage	F	5/1997[d]	176.44	Nulliparous
8	Mirage	F	11/1975[c]	191.86	Parous

Note: [a]SFDK, Six Flags Discovery Kingdom; DRC, Dolphin Research Center; TOTS, Theater of the Sea; Mirage, The Mirage Dolphin Habitat. [b]Reproductive history prior to urine collection used in the study. [c]Estimated birth date for wild caught animals. [d]Aquarium born.

Statistics

Arithmetic means and standard deviations (*SD*), presented as mean +/- *SD*, were calculated using SYSTAT software (Systat Software, Inc. 225 W Washington St., Suite 425, Chicago, IL 60606).

Behavioral conditioning

Operant conditioning techniques were used to train the dolphins for ultrasound exams and urine sampling. Behaviors were positively reinforced and slow approximations were taken until each dolphin was fully conditioned for daily sampling and exams.

Ultrasonography

Real-time B-Mode trans-abdominal ultrasound imaging was utilized. The dolphins were trained to float laterally and stationary at the water's surface. The blowhole was submerged, but the

animal could lift her head and take a breath as needed. To visualize the ovaries and POF, the ultrasound transducer was placed between the junction of the rectus abdominus muscle and the hypaxialis lumborum muscle (Brook, 2001). For animals 1-2 and 4, POF's were visualized with a Sonosite Titan portable ultrasound machine with a 5-2 MHz curvilinear transducer (Sonosite Inc. 21919 30th Drive SE, Bothell, WA 98021-3904, USA). Images were digitally captured using the internal digital memory of the machine or by the use of an Archos 605 Audio/Visual Player (Archos Inc. 7951 E. Maplewood Avenue #260 Greenwood Village, CO 80111 USA). For animals 3, 7, and 8, an SSD 900 Aloka ultrasound machine with a 3.5 MHz transducer (Aloka America**,** 10 Fairfield Boulevard Wallingford, CT 06492 USA) was used and images recorded with a Sony digital video cassette recorder (Sony Corporation of America, 550 Madison Avenue, 27th Floor, New York, NY 10022-3211 USA). For animals 5 and 6, a Sonosite 180 Plus ultrasound machine with a 5-2 MHz transducer (Sonosite Inc., 21919 30th Drive SE, Bothell, WA 98021-3904, USA) was used and images recorded using either the internal digital memory of the machine or by the use of an Archos 605 Audio/Visual Player (Archos Inc., 7951 E. Maplewood Avenue #260 Greenwood Village, CO 80111 USA). For all animals, ultrasound exams were conducted 1-3 times daily (Table 2) and exams typically lasted 3-5 minutes for both the left and right lateral positions.

Urine collection and hormone assays

The dolphins were trained to urinate on cue. Initially the trainer placed firm hand pressure to the dolphin's bladder, which often resulted in reflexive urination. This reflexive behavior was paired with primary reinforcement to increase the frequency. Over time, each dolphin was conditioned to urinate by the application of gentle pressure over the area of the bladder. For animals 1-5, urine collection was achieved by placing the floating dolphin in ventral recumbency, allowing the genital slit to be wiped clean with a dry cloth. A sterile plastic 25-ml syringe was used to draw up the urine once it was free-flowing from the urethra. For animals 6-8, the dolphins were partially pulled out of the water so that the genital slit was dry. First, the trainer stood on a dry ledge and grasped the tail flukes of the dolphin that was stationary in a dorsal position floating at the pool surface. The dry ledge was 7 to 10 cm higher than the water surface. Next, the trainer gently stepped backward pulling the dolphin's flukes backward and laterally over the ledge until the dolphin's genital slit was laterally out of the water. The dolphin's head and pectoral flippers remained in the water. The genital slit was wiped clean with gauze. A 50-ml sterile plastic specimen cup was placed under the urethra and gentle pressure was applied to the bladder. The urine was caught in the cup as it freely flowed from the urethra. An average of 5-10 ml of urine was collected from each study animal. Urine volumes of 3-5ml were decanted into plastic storage vials and then immediately frozen at -70^{o}C (animals 1-2), -17^{o}C (animals 3-5), and -33.9^{o}C (animals 6-8).

Urinary progesterone (uP) was measured using an ECLIA tested with the Elecsys 2010 instrument (Roche Diagnostics, Mannheim, Germany) at Clinical Pathology Laboratory in Las Vegas (4275 S. Burnham Ave, Suite 325, Las Vegas, NV 89119, USA). Urine was shipped frozen to the laboratory from each facility.

Urinary luteinizing hormone (uLH) was measured using the ICG Canine Witness® Luteinizing Hormone Assay (Witness Synbiotics Corp., Kansas City, MO, USA). The assay provides a rapid, semi-quantitative visible color band in the presence of dolphin LH (Muraco et al., 2009). Dolphin urine was applied to the test strip either directly following collection, or, if frozen, after thawing. Urine was applied to LH assays either neat (nUr)(Animals 1-6) or after concentrating (cUr) (Animals 7-8). For the nUr samples, a 100-µl volume was placed onto the test strip and results were obtained in less than 1 h after application. For the cUr samples, urine was centrifuged and the supernatant was concentrated on a Microcon-10 filter device (Millipore Corp., Bedford, MA, USA) at 13,900 g for 35 min, before application of 100 µl cUr to the LH assay. Results were obtained in less than 1 h following application.

Table 2
Bottlenose dolphin LH and POF data

Animal	Date	Time[e]	LH Score	Time[f]	POF (cm)	Conceptive
1	5/17/2009	1040	0	930	1.7	Yes – AI+
		1450	0	-	-	
	5/18/2009	900	3	900	2	
		1130	2	1300	2.1	
		1400	2	-	-	
		1500	4	-	-	
	5/19/2009	900	2	840	1.5	
		1420	1	1305	0*	
2	5/25/2009	1315	0	840	1.72	Yes – AI
	5/26/2009	1100	4	1000	2	
	5/27/2009	1100	2	1030	0	
3[c]	11/11/2005	-	-	-	1.8**	Not Bred
	11/12/2005	-	-	-	0**	
3[d]	11/12/2005	930	1	1800	1.8	Yes – AI
	12/12/2005	900	4	-	-	
		1130	3	-	-	
		1340	3	-	-	
		1500	3	1800	1.9	
	12/13/2005	-	-	-	-	
4	2/28/2009	915	1	930	2.08	Yes – AI
		1600	2	-	-	
	3/1/2009	1300	4	1345	2.08	
	3/2/2009	945	1	1345	2.14	
	3/3/2009	-	-	1625	0*	
5[a]	7/6/2008	1400	1	-	-	Not Bred
	7/7/2008	1130	4	-	-	
		1400	3	1430	1.9	
	7/8/2009	1130	1	-	-	
	7/9/2008	-	-	1430	0*	
5[b]	8/11/2008	-	-	1600	1.8	Yes – NB++
	8/12/2008	1130	4	1030	1.9	
	8/13/2008	-	-	-	-	

Table 2 (cont.)
Bottlenose dolphin LH and POF data

Animal	Date	Time[e]	LH Score	Time[f]	POF (cm)	Conceptive
6	6/11/2006	900	1	1030	1.5	No – NB
	6/12/2006	800	4	1000	1.8	
		1200	4	-	-	
		1400	4	1430	1.9	
		1600	3	-	-	
	6/13/2006	900	1	930	2	
	6/14/2006	-	-	930	0*	
7	10/12/2004	800	0	-	-	No – AI
		1500	0	-	-	
	10/13/2004	800	3	-	-	
		1500	4	1515	1.8	
	10/14/2004	800	1	-	-	
8	10/11/2004	800	0	-	-	No – AI
		1500	0	-	-	
	10/12/2004	800	0	1000	1.7	
		1500	4	1530	1.7	
		1930	4	1900	1.9	
	10/13/2004	800	1	900	1.7	
	10/14/2004	-	-	900	0*	

Note: [a]First natural cycle. [b]Second natural cycle. [c]Atrenogest cycle. [d]Natural cycle. [e]Indicates time of urine collection. [f]Indicates time of ultrasound exam. – Indicates no data collected. * Indicates POF had ovulated. **Data not included in calculated means. + Indicates artificial insemination. ++ Indicates natural breeding.

All LH test band results were scored from 0-4 based on color band intensity. Test bands were scored as follows (Figure 1):

0 = no visible test band
1 = faint test band
2 = test band less intense than control band
3 = test band and control band equally intense
4 = test band more intense than control band

Estrous cycle monitoring

Animal 3 was given 110 mg/kg p.o. of altrenogest (Regu-Mate, Intervet Inc., Millsboro, DE, USA) once a day for 113 d for the purposes of estrous synchrony. She was monitored for ovulation following the altrenogest treatment, and the subsequent natural estrous cycle, by ultrasonography of the POF, uLH, and uP. Animals 1-2 and 4-8, experienced natural estrous cycles without any altrenogest pre-treatment. Monitoring for ovulation included ultrasonography of the POF, uLH, and uP.

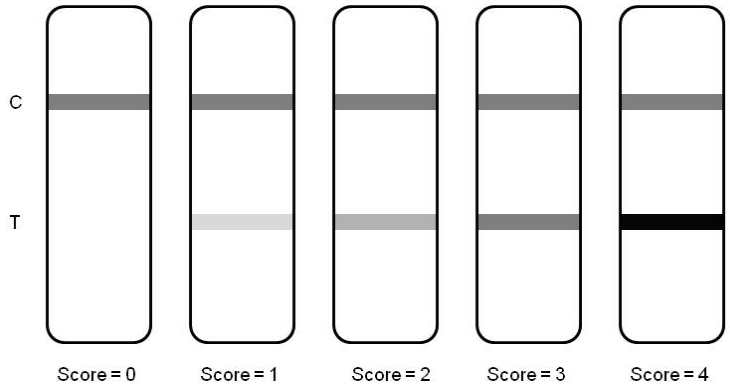

Figure 1. LH test band color intensity scores. C = Control. T = Test.

Results

Ultrasound was used to measure the POF vertical diameter before, during, and after the LH surge as well as to identify the disappearance of the POF indicating that ovulation had occurred (Table 2, Figs. 2, 4). Mean POF diameters 18-29 h prior to the LH surge were 1.767 +/0.189 cm (n = 6). Mean POF diameters during the LH surge were 1.942 +/- 0.098 cm (n = 9). Mean POF diameters 14-24 h following the last recorded elevated LH score were 1.835 +/- 0.289 cm (n = 4). Animals 1 and 2 ovulated 24 and 24 h 30 min, respectively, from the maximum recorded POF diameter during the LH surge. Mean time to disappearance of the POF from the last recorded LH surge was 37.475 +/- 12.346 h (n = 6). In animal 1, fluid evacuation from the follicle during ovulation was visualized and photographed (Figs. 2c,d). Complete evacuation of the follicular fluid from 1.5 cm to 0.0 cm was 4 h 20 min. A 2.0 cm corpus luteum (CL) was visible 6 d post-ovulation and a 3.0 cm CL was visible 22 d post ovulation (Fig. 5a). An embryo was visualized 57 d post ovulation (Fig. 5b). CL's were visualized at 100 and 142 d post ovulation to show the relationship between fetal positioning and the ovary (Fig. 5c,d).

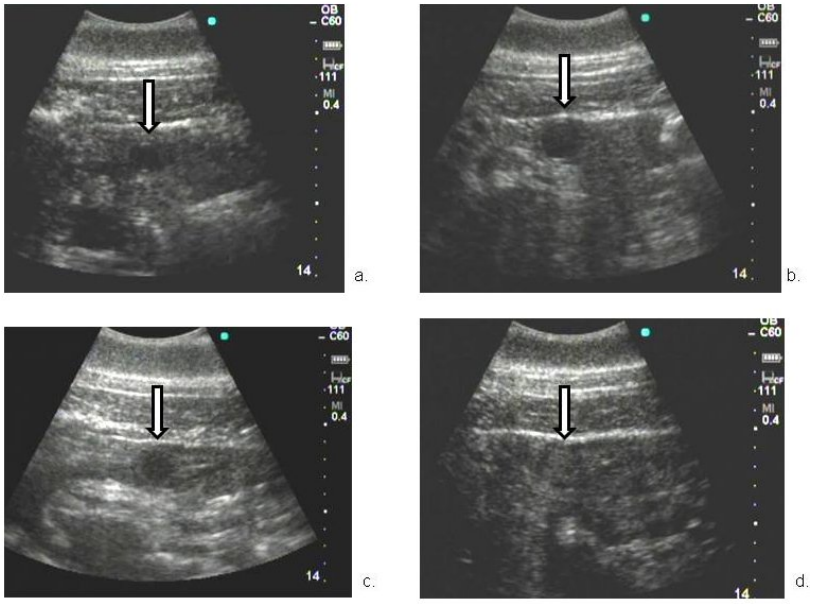

Figure 2. Bottlenose dolphin ovarian follicular dynamics before, during and after ovulation. Arrows indicate follicle. a. 1.9 cm Preovulatory follicle (POF) before the luteinizing hormone (LH) surge. b. 2.0 cm POF during the LH surge. c. 1.5 cm follicle during ovulation. d. Fully evacuated follicle following ovulation.

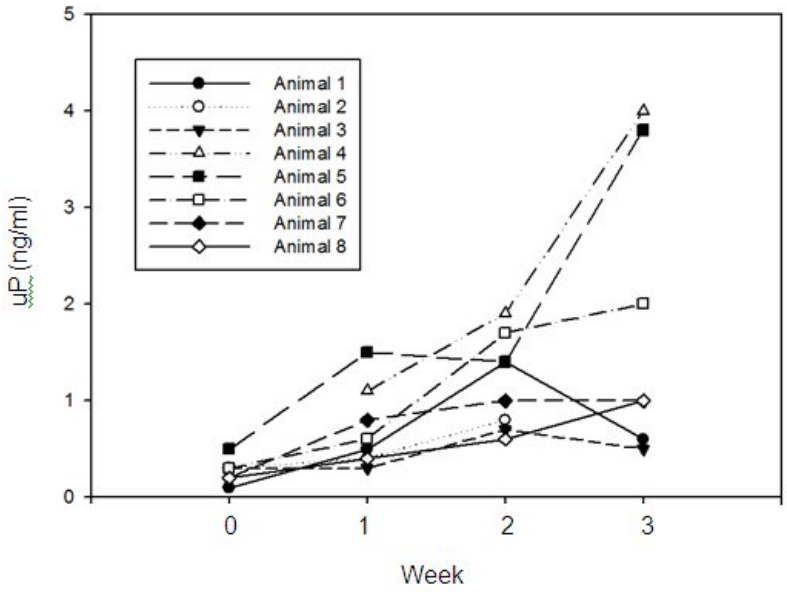

Figure 3. Bottlenose dolphin urinary progesterone levels showing increases in basal levels following ovulation. Week 0 indicates day of LH surge with ovulation occurring 24-48 hours following the last recorded elevated LH surge score.

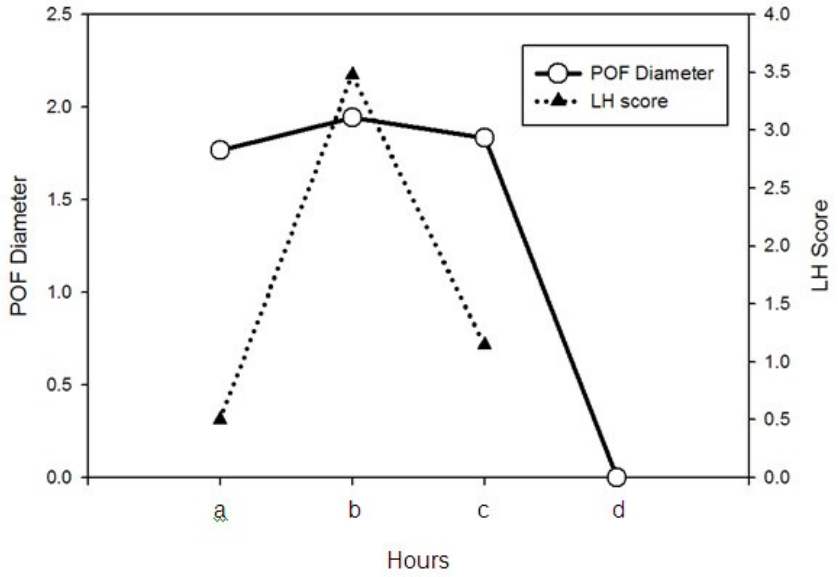

Figure 4. Preovulatory follicle (POF) diameter before, during and after the luteinizing hormone (LH) surge. a) 7-29 hours prior to the LH surge, b) LH Surge, c) 14-24 hours post LH surge, d) Ovulation.

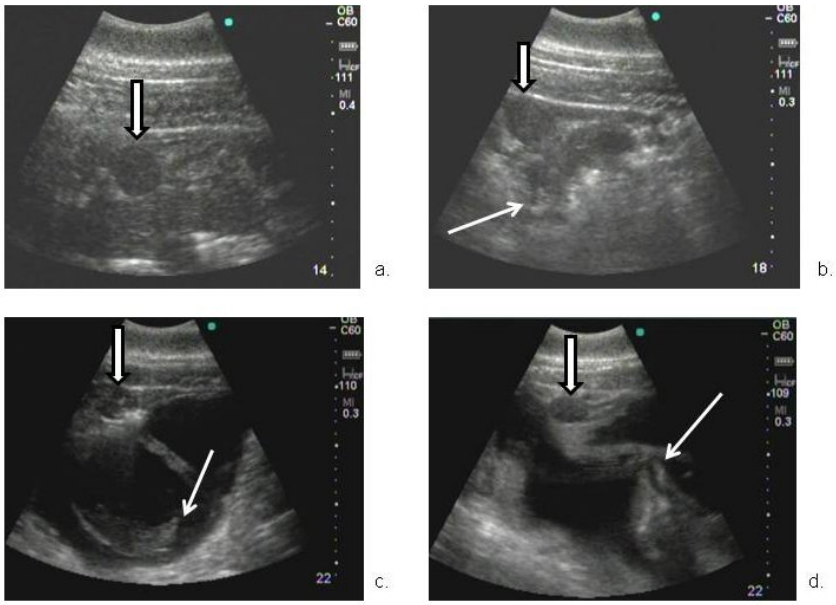

Figure 5. Bottlenose dolphin corpus luteum (CL) appearance during pregnancy from artificial insemination. White arrow with black outline indicates CL. Long white arrow indicates embryo/fetus. a. CL at 22 days post-ovulation. b. CL and embryo at 57 days post-ovulation. c. CL and fetus at 100 days post-ovulation. d. CL and fetus (lateral view of rostrum and skull visible) at 142 days post-ovulation.

Urinary LH was profiled before, during and after the ovulatory LH surge in 9 natural estrous cycles from 8 bottlenose dolphins using the canine LH assay (Table 2). There was no significant score difference between results using nUr vs. cUr. A score of 0-4 was given to the darkness of the color band on the test strip (Fig. 1). The LH surge (score: 3-4) duration from tested urine samples collected 2-4 x per day was 6.050 +/- 1.332 h (n = 6).

Urinary progesterone (uP) was tested the day of the LH, surge and then again at 7-10d, 11-17d and 18-24 d (Fig. 3). Mean uP levels the day of the LH surge were 0.271 +/-0.125, at 7-10 d were 0.7 +/ 0.414 ng/ml, at 11-17 d were 1.188 +/- 0.482, and at 18-24 d were 1.843 +/- 1.488.

Estrous cycles were ovulatory based on the disappearance of the POF on ultrasound and subsequent rises in uP following ovulation (Fig. 2d, 3). Animal 3 ovulated on day 21 following the end of altrenogest treatment and then ovulated again naturally 32 days later based on days between peak size of the POF. No other animals in this study received any altrenogest pre-treatment. Animal 5 cycled twice with 36 days between peak LH levels. 6 of the ovulations occurred on the right ovary and 4 occurred on the left. Of the two animals that experienced 2 successive ovulations, animal 3 ovulated both times on the left side and animal 5 ovulated both times on the right side. The 9 natural cycles occurred in March (animal 4), May (animals 1-2), June (animal 6), July (animal 5), August (animal 5), October (animals 7-8), and December (animal 3). The one altrenogest-induced cycle occurred in November (animal 3).

Discussion

To effectively study the relationship between the LH surge, follicular dynamics and ovulation of the bottlenose dolphin requires careful monitoring and multiple samples taken daily. Identification of these fleeting processes would be impossible to achieve without conditioned dolphins. One benefit to this project was the ability to monitor natural cycles. This allowed for an accurate representation of the estrous cycle without influence of an artificial treatment regimen such as altrenogest. The study showed that the rise and fall of the dolphin ovulatory LH surge can be detected by the canine rapid ICG assay, and scoring the color band intensity can provide a semi-quantitative measurement tool. The POF during the LH surge was a turgid round structure with thickened walls and a mean diameter of 1.9 cm (Fig. 2b). In all the study animals, the POF remained intact for the duration of the LH surge with ovulation occurring 24-48 hours following the last recorded LH surge score. Interestingly, 6 of the ovulations (4 conceptive) occurred on the right ovary and 4 ovulations (1 conceptive) on the left ovary. This finding is different from previously documented results of dolphin ovulations occurring 82% of the time on the left ovary (Robeck et al., 2005). Of the 10 estrous cycles in this study, 4 were conceptive from artificial insemination (AI) and 1 from natural breeding. Of the remaining non-conceptive cycles, 2 were non-bred, 2 were AI and 1 was from a natural breeding. Further study will compare non-conceptive to conceptive cycles to look for potential infertility patterns in the endocrinology

and physiology. Understanding the relationship between LH, POF's, and ovulation will aid natural breeding programs and advance artificial insemination techniques.

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