

## Urinary Prolactin Concentrations in the Female Bottlenose Dolphin (*Tursiops truncatus*)

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### Abstract

This long-term project provides the first look at serial prolactin concentrations in the female bottlenose dolphin (*Tursiops truncatus*) during known reproductive states using non-invasive urine sampling. Prolactin concentrations were detectable during anestrus, estrous, pregnancy, lactation, and when non-mothers cohabit with mother-calf dyads. Prolactin concentrations were higher during pregnancy than during the luteal phase of the estrous cycle indicating a possible role in pregnancy maintenance. A prolactin rise at the end of gestation suggests an involvement in pre-parturition mammary gland development, and concentrations were higher in months 1 through 5 of lactation vs months 15 through 18. Prolactin concentrations were found to be elevated in both nulliparous and parous non-mothers when they cohabitated with mother-newborn dyads, and lactational anestrus was observed. Spontaneous lactation occurred in a nulliparous female when cohabitating with a mother-newborn calf dyad that was positively correlated with elevated prolactin concentrations. There was no positive correlation between northern meteorological seasons and prolactin concentrations suggesting that dolphins may not use photoperiod in reproduction regulation as is observed in horses and sheep. Methodologies such as mammary gland ultrasound and testing urinary prolactin in pregnant females to help predict healthy vs unhealthy pregnancies could be used by field researchers conducting wild dolphin health assessments. This information is of particular importance when investigating the long-term effects of the Deepwater Horizon oil spill on bottlenose dolphin reproduction in the Gulf of Mexico.

**Key Words:** reproduction, prolactin, prl, pregnancy, ultrasound, lactation, nursing, allomother, behavior, bottlenose dolphin, *Tursiops truncatus*

### Introduction

Prolactin (prl) is a remarkable polypeptide hormone that controls a diverse amount of physiological processes in response to a wide array of physiological and environmental stimuli (Freeman et al., 2000; Bernichtein et al., 2010). For the purposes of this article, prl's role in lactation, reproduction, pregnancy, and maternal behavior will be the main focus.

Prl is secreted primarily from lactotropes in the anterior pituitary. The release is continuous but inhibited by dopamine from the hypothalamus (Leong et al., 1983; Neill & Nagy, 1994). Lactation is prl's most well-known role in mammals where it induces mammogenesis, synthesizes lactogenesis, and maintains galactopoiesis (Freeman et al., 2000). As young dolphins suckle their mothers' nipples, dopamine inhibition is reduced, and prl secretion is released in a series of bursts from the pituitary (Leong et al., 1983; Neill & Nagy, 1994; Bodnár et al., 2002). Prl is released 2 to 5 min after suckling starts; and if nursing continues for 20 to 30 min, prl concentrations may reach 60 times higher than baseline in humans (Leong et al., 1983).

In addition to lactation, prl plays a varied role in female reproduction. In the estrous cycle of the rat (*Rattus norvegicus domestica*), prl secretion appears low until a preovulatory surge occurs similar in timing to the luteinizing hormone (LH) surge (Butcher et al., 1974; Terry et al., 1977; Freeman, 1994). There has been an observation of a slight mid-cycle increase in prl in non-human primates (Vekemans et al., 1977; Aidara et al., 1981). During the breeding season of sheep (*Ovis aries*), GnRH stimulates prl release (Henderson et al., 2008); and in mares (*Equus caballus*), prl appears to play a role in follicular maturation and ovulation (Bennett-Wimbush et al., 1998; King et al., 2008). Prl is correlated with day length and

regulation of reproduction in seasonally breeding animals (Fitzgerald et al., 2000).

Estrogen can stimulate prl production and secretion in various species (del Pozo & Brownell, 1979). In the rat, estrogen is a critical factor in producing the proestrous prl surge (Nicoll et al., 1962; Lieberman et al., 1978; Lawson et al., 1993; Christensen et al., 2011). In the pig (*Sus scrofa domestica*), prl surges at estrus are induced by estrogen (Stevenson et al., 1981); and in the elephant (*Loxodonta africana*), there is a prl rise during the follicular phase that may be estrogen dependent (Bechert et al., 1999). In the mare, a rise in prl coincides with a decline in estradiol that occurs before ovulation (King et al., 2008). Prl can serve as a luteotropic agent in the rat (Bouilly et al., 2012), dog (*Canis lupus familiaris*; Okkens et al., 1990), and spotted seal (*Phoca largha*; Ishinazaka et al., 2002).

In the rat, prl maintains luteal production of progesterone and the integrity of the corpus luteum (CL) (Freeman et al., 2000). In mares, a late luteal-phase prl surge may be associated with luteolysis (Roser et al., 1987). It can also be a luteostatic agent in some marsupials by limiting progesterone synthesis and secretion from the CL (Hinds & Tyndale-Biscoe, 2012). In humans, there is no evidence that prl plays a role in luteal development (Bouilly et al., 2012); however, in human pregnancies, prl concentrations rise markedly at delivery (Kletzky et al., 1985).

It is well documented that suckling during lactation can inhibit folliculogenesis and ovarian function in most mammalian species (Rolland et al., 1975; Wang et al., 1980; Dorrington & Gore-Langton, 1981; McNeilly et al., 1982; Jakubowski & Terkel, 1985; Bouilly et al., 2012). This is due in part to prl's interference with follicle-stimulating hormone (FSH) on estrogen synthesis (Dorrington & Gore-Langton, 1981) but primarily through suppression of LH secretion (McNeilly et al., 1982). Ovarian follicles may develop during lactation, but the increased prl concentrations from suckling can inhibit follicle maturation and result in an anovulatory state (Dorrington & Gore-Langton, 1981). In a study of captive lactating bottlenose dolphins (*Tursiops truncatus*), post-partum return to estrus occurred between 413 to 673 d (West et al., 2007). Studies of wild dolphins have shown that dolphins can continue lactation through ovulations and pregnancies (Yoshioka et al., 1989; West et al., 2000, 2007), which enables the dolphin to maintain 3-y calving intervals (Wells et al., 1987).

Maternal behavior in many species has been found to be induced and maintained by prl (Freeman et al., 2000; Grattan, 2002). The maternal brain undergoes structural and functional modifications during pregnancy and lactation to

ensure the demands of lactation can be met and to exhibit the appropriate behaviors to feed and care for the offspring (Pi & Voogt, 2000; Grattan, 2002). It has also been found that prl is associated with allomothering (i.e., when an individual other than the genetic parent provides care for conspecific young) in several bird and mammal species (Ziegler, 2000; Schradin et al., 2003; Soltis et al., 2005). Allomothering behavior has been observed in wild and captive dolphins where non-genetic mothers escort a calf (Tavolga, 1966; Gurevich, 1977; Leatherwood, 1977; Wells, 1991; Mann & Smuts, 1998; Simard & Gowans, 2004), while the genetic mother engages in social or resting behaviors (Mann & Smuts, 1998). Non-gravid, non-lactating dolphins in captivity have been documented to spontaneously lactate for a non-conspecific orphaned calf (Gaspar et al., 2000) and have been induced to lactate after cohabitation with orphaned conspecific calves (Ridgway et al., 1995). Howells et al. (2009) observed a wild multiparous female adopt an orphaned calf and care for it for 2 y. Captive dolphins have also been observed to spontaneously lactate for non-orphaned calves with which they cohabitate (Carzon et al., 2019).

Urinary prl has proven to be a reliable and non-invasive method to study prl dynamics in humans and primates (Keely & Faiman, 1994; Soltis et al., 2005; Leañes-Miranda et al., 2008). No information about concentrations of dolphin prl was found in the literature, but in one histological study, prl was identified in the bottlenose dolphin pituitary gland (Cowan et al., 2008). Development of a prl assay in bottlenose dolphin urine would provide a non-invasive methodology to understand prl's role in reproduction and lactation. The objectives of this study included development of a urinary prl assay and to analyze those concentrations during anestrus, estrous, pregnancy, lactation, and when non-mothers cohabitate with mother-calf dyads using non-invasive urine sampling.

## Methods

### Dolphins

Bottlenose dolphins used in the study consisted of seven females from two different facilities (Table 1). Dolphins #1, #2, and #7, located at Facility 1, were housed in a 3,141,892 L manufactured seawater pool, maintained at 21° to 23°C year-round. These dolphins received a diet of frozen thawed herring (*Clupea harengus*), capelin (*Mallotus villosus*), sardine (*Sardina pilchardus*), and squid (*Loligo opalescens*) for combined daily totals of 8.62, 7.26, and 8.16 kg, respectively. Dolphins #3, #4, #5, and #6, located at Facility 2, were housed in a 2,717 L manufactured seawater

**Table 1.** Bottlenose dolphins (*Tursiops truncatus*) are listed with details related to their location (facility), sex birth date, weight, and reproductive history during this study

Dolphin	Facility	Sex	Birth date	Weight (kg)	Reproductive history
1	1	F	May 1997	176	Nulliparous
2	1	F	March 2000	159	Parous
3	2	F	July 1979	184	Parous
4	2	F	July 1986	189	Parous
5	2	F	September 2004	137	Nulliparous
6	2	F	August 1979	196	Parous
7	1	F	November 1975	191	Parous

pool maintained at 22°C year-round. These dolphins were fed a combined diet of 10.4 and 5.9 kg, respectively, of whole frozen thawed herring and capelin daily.

Five dolphins (#1, #2, #4, #5, and #6; Table 1) were deemed non-gravid, non-lactating, and anestrus before moving them into a pool with a mother–calf dyad. Dolphins #1 and #2 lived with a newborn calf and its mother for 4 mo; Dolphin #6 lived with two newborn calves and their mothers for 4 mo; Dolphin #5 moved into a pool with two mother–calf dyads when the calves were 3 mo old and lived with the group for 3 mo; and Dolphin #4 moved into a pool with an 18-mo-old calf and its mother and remained with the dyad for 2 mo.

#### *Behavioral Conditioning and Data Collection*

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

*Urine Collection*—The bottlenose dolphins were trained to urinate on cue. For Dolphins #1, #2, and #7, each dolphin was partially out of the water so that the genital slit was dry. The dry ledge was 7 to 10 cm higher than the water surface. 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. For Dolphins #3 through #6, urine collection was achieved by placing the dolphin in a ventral position, and the genital slit was wiped clean with a dry cloth. A sterile plastic 25-ml syringe was used to draw up the urine once it free-flowed from the urethra. An average of 5 to 10 ml of urine was collected from all study dolphins. Urine was immediately frozen at -17°C (Dolphins #1, #2, and #7) and -70°C (Dolphins #3–#6) in 3 to 5 ml aliquots in plastic storage vials. Urine for this study was

collected in the morning (0800 to 1100 h) unless noted.

*Milk Expression*—For milk expression, bottlenose dolphins were positioned as for urine collection. The genital slit and mammary slits were wiped clean with gauze. A gentle pressure was applied to the left and right mammary glands, located approximately 6 to 8 cm anterolateral to the anterior line of the genital slit, while gently massaging toward the mammary slit. If milk was present, it would be expressed from the mammary slit with the first or second application of pressure to the mammary gland.

*Ultrasonography*—A Sonosite Titan portable ultrasound machine with a 5-2 MHz curvilinear transducer (Sonosite Inc., Bothell, WA, USA) was used for the real-time B-Mode trans-abdominal ultrasound imaging. Images were digitally captured using the internal digital memory of the machine or by using an Archos 605 Audio/Visual Player (Archos Inc., Greenwood Village, CO, USA). For all bottlenose dolphins, ultrasound exams were conducted as needed, and exams typically lasted 3 to 5 min. For ovary and fetus visualization, the dolphins were trained to float laterally and stationary at the water's surface. The blowhole was submerged, but the dolphin could lift her head and take a breath as needed. To visualize the ovaries and uterus, the ultrasound transducer was placed between the junction of the rectus abdominus muscle and the hypaxialis lumborum muscle (Brook, 2001). For mammary gland ultrasound, the dolphins were trained to float ventral up and remain stationary at the water's surface. The blowhole was submerged, but the animal could maneuver to a lateral position and lift her head to take a breath as needed. To visualize the mammary gland parenchyma, the transducer was placed transversely on the dolphin approximately 2 cm anterolateral to the anterior line of the genital slit and moved cranially until the parenchyma was identified. A transverse diameter measurement

was taken in the area of the medial parenchyma to record changes in size.

#### *Hormone Testing*

Bottlenose dolphins can experience long-term anestrus (> 1 y) even when not post-partum or lactating (Sawyer-Steffan et al., 1983; Yoshioka et al., 1986). In this study, anestrus was determined by no elevation in urinary progesterone (P) and no follicular development on the ovary. Additionally, dolphins categorized as anestrus were also non-gravid, non-lactating, and not living with a mother–calf dyad.

*Progesterone and Estradiol*—P and estradiol-17 beta (E2) were measured using an Electrochemiluminescent Immunoassay (ECLIA) tested with the Elecsys 2010 instrument (Roche Diagnostics, Mannheim, Germany) at Clinical Pathology Laboratory in Las Vegas, Nevada (USA) as previously reported (Muraco et al., 2009). Urine was shipped frozen to the laboratory from each facility.

*Prl Assay*—Bottlenose dolphin urinary prl concentrations in urine were determined by a modified homologous, double antibody RIA according to the methods of Roser et al. (1984). Briefly, purified equine prl (supplied by A. F. Parlow, National Hormone and Peptide Program, Torrance, CA, USA) was used for standards (0.125 to 32 ng/mL, AFP-7730B) and for iodination (AFP-8794B) by the iodogen method (Matteri et al., 1987). Primary antibody was rat-anti-equine-prl antibody (Parlow AFP-361687R; 1:38,000), and the second antibody was goat anti-rat IgG (Antibodies, Inc., Davis, CA, USA; 1:300). Sensitivity of the assay was 0.125 ng/mL with an intra-assay coefficient of 4.02% ( $n = 8$ ) and an inter-assay coefficient of 10.86% ( $n = 3$ ). For prl assays, concentrations of urinary hormones and metabolites were expressed as ng of prl per mg Cr excreted (Tausky, 1954).

#### *Seasonality and Prl*

Bottlenose dolphins can give birth in any season in aquaria (Urian et al., 1996; Muraco et al., 2010), and they display a broad, diffuse, and flexible reproductive seasonality in the wild giving birth in all seasons with a slight increase of births in spring and fall (Wells et al., 1987; Urian et al., 1996; Thayer et al., 2003). Prl concentrations were examined during all four seasons for anestrus dolphins (#1, #2, and #6) to examine potential seasonal prl changes. Estrous cycles were analyzed (Dolphins #1, #2, #3, and #4) using serial P and E2 concentrations. Ovarian ultrasound was also utilized to further differentiate the follicular and luteal phases (Dolphins #3 and #4).

#### *Pregnancy and Lactation*

To determine pregnancy, P was analyzed for elevated concentrations, and uterine ultrasound was used to verify uterine fluid and fetal development. For this study, pregnancy was considered to have begun 20 d after ovulation.

Daily observations were conducted on post-partum study bottlenose dolphins (#2, #3, #4, and #7; data from Dolphin #2 are included as a non-mother cohabitating with a mother–calf dyad and later nursing her own calf) and calves for nursing duration and frequency. Observations consisted of one to two people watching the animals through underwater observation windows or at the water's surface 24 h/d for the first 15 d of the calves' lives. Recorded observations included all occurrences of nursing behavior and scheduled counts of calf respirations taken over 5-min intervals. Dolphin calf nursing behavior has been described as suckling bouts which include multiple 5-s nipple latches within a 1- to 5-min period, followed by an interval phase where no suckling occurs (Eastcott & Dickinson, 1987). This study defines successful nursing as a latch when the calf places the rostrum on either the left or right mammary slit with the mouth open and tongue wrapped around the nipple resulting in milk being expelled from the mother. A latch duration starts when the calf's rostrum touches the mammary slit and ends when the calf's rostrum separates from the mammary slit. Nursing behaviors not considered a latch (i.e., when a calf attempts to nurse from areas other than the mammary slit) are not included in this study's data. For calves of Dolphins #3 and #4, each latch was timed using a stopwatch and recorded onto a spreadsheet for duration. Nursing data for this study included latch duration and number of latches over a 24-h period. Non-mothers (Dolphins #1, #2, #5, and #6) cohabitating with a mother–calf dyad were periodically checked for milk production through manual stimulation of the mammary glands during routine exams. These exams were conducted to identify if the calf was receiving milk when latching onto the non-mothers.

#### *Statistics*

Arithmetic means and standard deviations are presented as  $M \pm SD$ ; and data were tested for normality before calculating for comparisons, correlations, or regression. The  $p$  values for prl and estradiol were calculated using student paired  $t$  tests, and  $p$  values < 0.05 were considered significant. Correlations between estradiol and prl were tested with Pearson's Correlation Coefficient and presented as  $r = \pm$ . Polynomial linear regression and studentized residual levels in regression diagnostics were used to identify prl outliers during the follicular phase. Calculations were done using SYSTAT software (Systat Software, Inc., Chicago, IL, USA).

### Results

Prl was detectable in urine from all seven study dolphins (range: 0.12 to 3.79 ng prl/mg Cr). The lowest level was recorded during the follicular phase, and the highest level was recorded during the last trimester of pregnancy. Prl  $M \pm SD$  for each reproductive state, lactation, and cohabitation are presented (Table 2). Mean prl concentrations were similar across all categories, while the maximum prl values were highest for pregnancy, lactation, and cohabitation, respectively. Mean and maximum prl concentrations during each studied phase are graphed (Figure 1). During lactation, prl means were highest in months 1 through 5. Mean prl during each reproductive state and lactation are presented (Figure 2).

### Prl and Reproductive State

**Anestrus**—Three dolphins (#1, #2, and #6) were identified as being anestrus (no estrous cycle, non-gravid, not post-partum, or lactating) during a time period when they were not cohabitating with a mother–calf dyad. These anestrus data were used as the baseline for statistical comparisons. Concentrations of

prl during anestrus were significantly higher compared to follicular (Dolphins #1, #2, #3, and #4) and luteal (Dolphins #1, #2, and #3) phases ( $p = 0.022$  and  $p < 0.001$ , respectively) across the study (Figure 2). Additionally, prl concentrations were significantly higher during anestrus and non-cohabitation (Dolphins #1, #2, and #6) when compared to Dolphins #1, #2, #4, #5, and #6 during cohabitation with a mother–calf pair for the first 30 d ( $p < 0.001$ ). Prl levels during anestrus and non-cohabitation (Dolphins #1, #2, and #6) were significantly lower than months 1 through 5 of lactation (Dolphins #2, #3, and #4;  $p < 0.001$ ; Figure 3) and days 60 to 127 of cohabitation with a mother–calf pair (Dolphins #4 and #5;  $p = 0.008$ ; Figure 2). Anestrus statistical data were derived from Dolphins #1, #2, and #6 when they were not cohabitating with mother–calf dyads; however, it should be noted that all study dolphins (#1–#7) experienced anestrus (no estrous cycle) when cohabitating with a mother–calf dyad or when post-partum and lactating.

**Estrous Cycle**—Estrous cycles were studied in Dolphins #1, #2, #3, and #4. Estrous cycles occurred

**Table 2.** Study phases (reproductive state, lactation, and cohabitation) with the number of dolphins and urine prl samples indicated per phase

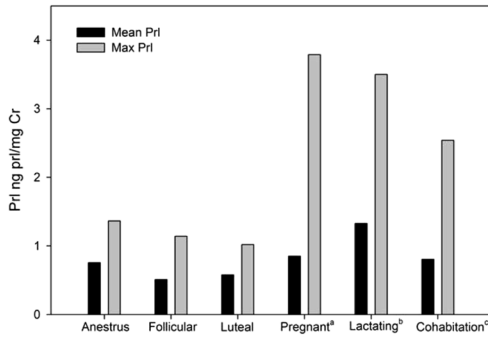
Study phase	Dolphin ( <i>n</i> )	Urine (prl <i>n</i> )	Mean (prl/mgCr)	SD
Anestrus <sup>a</sup>	3	37	0.75	0.26
Follicular	4	61	0.51	0.23
Luteal	3	41	0.57	0.21
Pregnancy first trimester <sup>b</sup>	2	28	0.69	0.18
Pregnancy second trimester	2	6	0.82	0.33
Pregnancy third trimester	3	25	0.95	0.61
Lactation 1 mo post-partum	1	3	0.78	0.22
Lactation 2 mo post-partum	3	16	1.10	0.30
Lactation 3 mo post-partum	3	8	1.85	0.97
Lactation 4 mo post-partum	3	3	1.45	0.28
Lactation 5 mo post-partum	1	1	2.44	0.00
Lactation 15 mo post-partum	1	5	0.98	0.18
Lactation 16 mo post-partum	2	4	0.84	0.44
Lactation 17 mo post-partum	2	4	0.80	0.34
Lactation 18 mo post-partum	1	2	0.41	0.15
Cohabitate <sup>c</sup> days 1-30	4	84	0.70	0.40
Cohabitate days 31-60	4	83	0.75	0.39
Cohabitate days 61-127	1	16	1.05	0.49
Cohabitate with older calf <sup>d</sup>	1	6	0.21	0.04

<sup>a</sup>No progesterone elevation and no follicular development

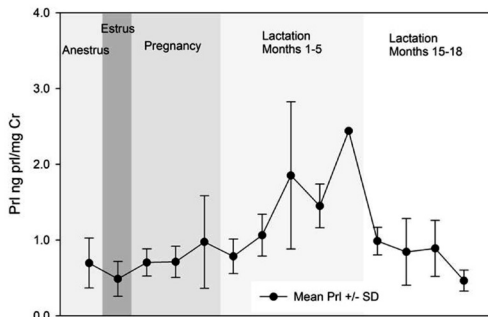
<sup>b</sup>Each trimester consists of 4 mo

<sup>c</sup>Non-gravid, non-post-partum female cohabitates with calf < 3 mo and the calf's mother

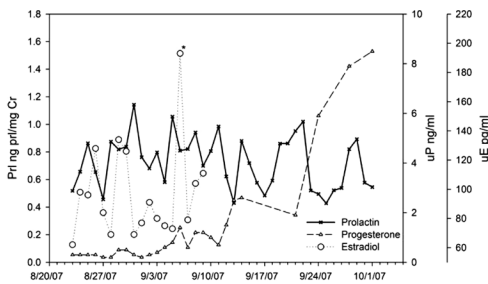
<sup>d</sup>Non-gravid, non-post-partum female cohabitates with 18-mo-old calf and the calf's mother



**Figure 1.** Prolactin (Prl) means and maximum concentrations during studied phases: <sup>a</sup>pregnant – months 1-12 ( $n = 62$ ;  $0.85 \pm 0.45$ ); <sup>b</sup>lactating – months 1-5 ( $n = 28$ ;  $1.32 \pm 0.70$ ); and <sup>c</sup>cohabitation – days 1-127 ( $n = 183$ ;  $0.76 \pm 0.42$ ).



**Figure 2.** Prl means during reproductive states and lactation



**Figure 3.** Prl, estradiol-17 beta (E2), and progesterone (P) during the estrous cycle of Dolphin #2. \*Ovulation.

synchronously in September 2007 for Dolphins #1 and #2 with ovulation occurring 17 and 14 d, respectively, following removal from a mother–calf dyad. Dolphin #4 cohabitated with Dolphin #3 and her nursing 16-mo-old calf for 2 mo. At 18 mo, the calf was weaned, and Dolphins #3 and #4 synchronized estrous in May 2009, with Dolphin #3

ovulating 22 d following the weaning of her calf and Dolphin #4 ovulating 30 d following the calf removal. Follicular phases averaged 10 d, and luteal phases averaged 20 d as previously reported (Robeck et al., 1998; Muraco et al., 2009, 2010). A conceptive estrous cycle detailing prl, E2, P, and ovulation from Dolphin #2 is graphed in Figure 3.

**Follicular Phase**—Mean prl concentrations were significantly lower during the follicular phase (Dolphins #1, #2, #3, and #4) vs anestrus (Dolphins #1, #2, and #6) ( $p = 0.022$ ), and when cohabitating with a mother–calf dyad (Dolphins #1, #2, #4, #5, and #6;  $p = 0.005$ ). Prl concentrations were lower, although not significantly, when nursing a 15- to 18-mo-old calf (Dolphins #3 and #7;  $p = 0.073$ ). However, during the follicular phase (Dolphins #1, #2, #3, and #4), prl rises were detected and are presented in Table 3 and Figures 4 through 6.

**Luteal Phase**—There was a slight increase in prl means in the luteal phase (Dolphins #1, #2, and #3) vs the follicular phase (Dolphins #1, #2, #3, and #4), although it was not significant ( $p = 0.296$ ). However, there was a significant difference between the luteal phase (Dolphins #1, #2, and #3; first 20 d following ovulation) and the first trimester of pregnancy (Dolphins #1 and #2; 21 to 120 d following ovulation) ( $p = 0.003$ ).

**Prl and Estradiol**

Mean E2 from Dolphin #2 was calculated during anestrus ( $n = 10$ ;  $73.70 \pm 17.84$  pg/mL), follicular phase ( $n = 17$ ;  $99.23 \pm 32.01$  pg/mL), cohabitation with a mother–calf dyad ( $n = 6$ ;  $79.66 \pm 16.37$  pg/mL), and during the final 17 d of pregnancy ( $n = 17$ ;  $139.70 \pm 91.64$  pg/mL). Estradiol was significantly higher in the follicular phase vs anestrus ( $p = 0.030$ ) and during the last trimester of pregnancy vs anestrus ( $p = 0.055$ ). There was no significant difference in estradiol concentrations between the follicular phase and the last trimester of pregnancy ( $p = 0.133$ ) or between cohabitation and anestrus ( $p = 0.860$ ). Correlations detected between E2 and prl during anestrus were

**Table 3.** Prolactin (prl) rises detected during the follicular phase

Dolphin #	Day(s) of estrous*
1	-8, -1
2	-6
3	-7, -4
4	-5

\*Days of the follicular phase are listed as negative numbers leading up to ovulation day 0.

positive; during cohabitation were neutral; and during follicular phase were negative (Figure 7). Correlations during the final 17 d of pregnancy were negative (Figure 8).

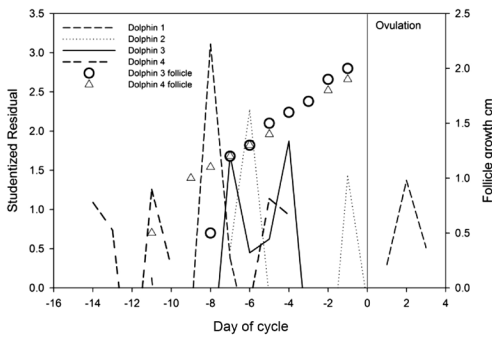
*Prl and Pregnancy*

Pregnancy was confirmed in Dolphins #2, #3, and #4 when a live fetus was visualized on ultrasound (Muraco & Kuczaj, 2015). Dolphin #1 aborted placental tissue positive for maternal and paternal DNA (Duffield & Chamberlin-Lea, 1990) after 3 mo of

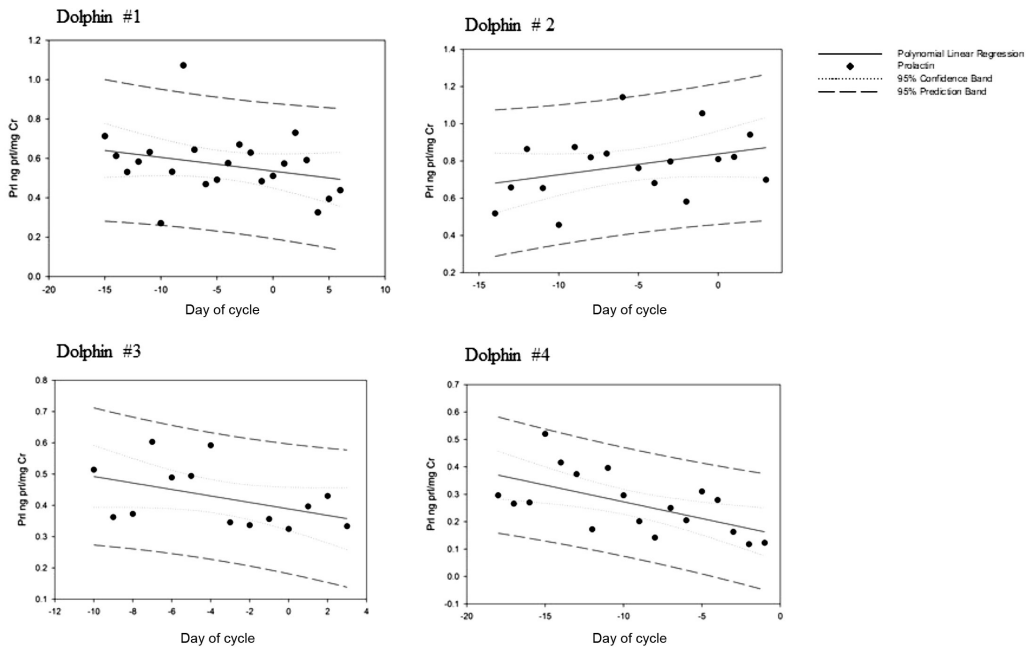
pregnancy. Prl means increased with each trimester of pregnancy (Table 2). The last month of pregnancy showed the highest prl means ( $n = 3; 1.07 \pm 0.04$  ng prl/mg Cr; Figure 2). There was a significant difference in prl concentrations ( $p < 0.001$ ) the first 55 d following ovulation between the pregnancies of Dolphin #1 (abnormal pregnancy) and Dolphin #2 (normal pregnancy) (Figure 9).

*Prl and Lactation*

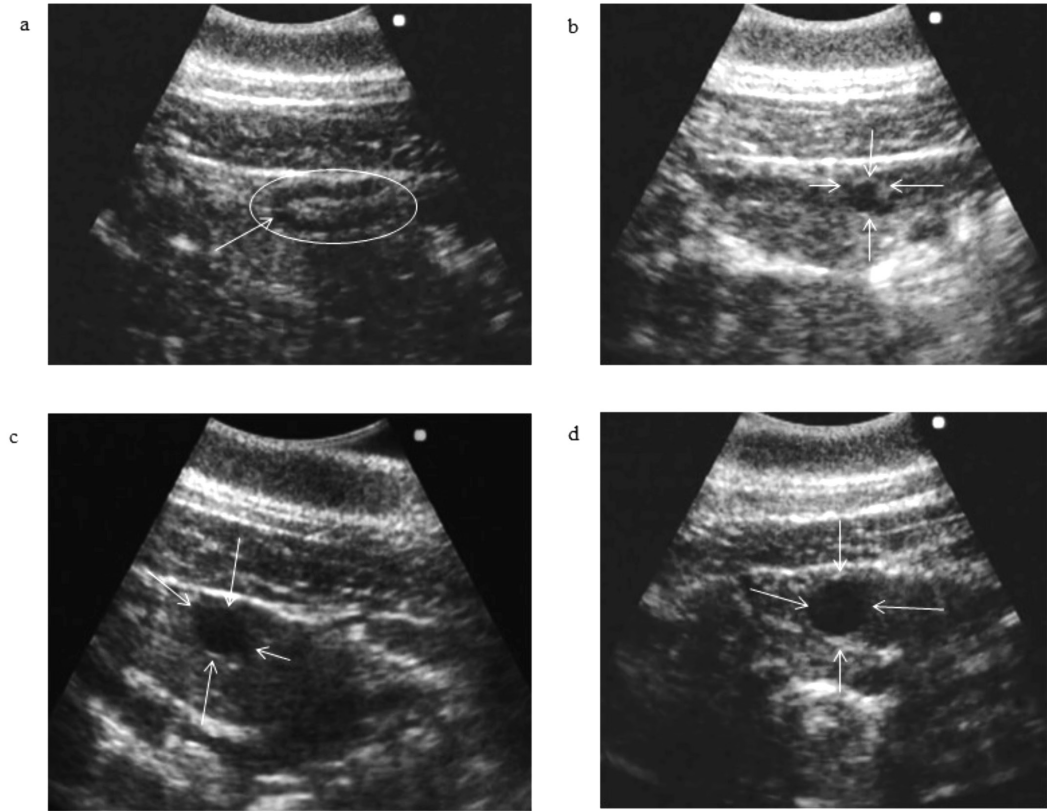
*Post-Partum*—Post-partum mothers (Dolphins #2, #3, #4, and #7) all cared for and nursed their calves. Prl means were examined during early (months 1-5) and late (months 15-18) post-partum lactation. No samples were collected or analyzed during months 6 through 14 of lactation. Prl was higher in months 1 through 5 ( $n = 31; 1.34 \pm 0.67$  ng prl/mg Cr) vs months 15 through 18 ( $n = 15; 0.82 \pm 0.33$  ng prl/mg Cr) of lactation ( $p = 0.08$ ). During the first 5 mo, mean prl concentrations were highest during month 3 ( $n = 8; 1.85 \pm 0.97$  ng prl/mg Cr); however, a higher singular level from month 5 ( $n = 1; 2.44$  ng prl/mg Cr) was recorded (Table 2). Figure 2 shows mean prl concentrations during early (months 1-5) and late (months 15-18) lactation and how it compares to anestrus, estrous, and pregnancy.



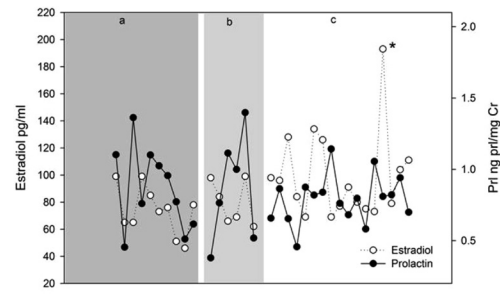
**Figure 4.** Follicle growth and studentized prl rises during the follicular phase



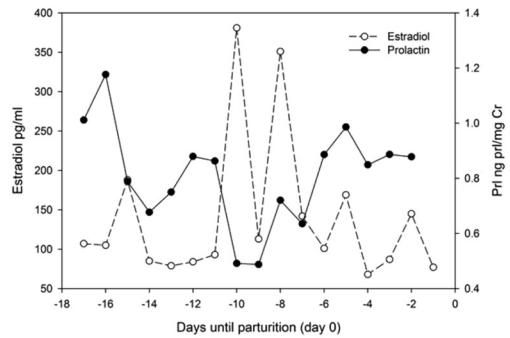
**Figure 5.** Identification of prl outliers using polynomial regression during the follicular phase



**Figure 6.** Ultrasound of follicular dynamics in Dolphin #3 during prl rises; white circle indicates ovary, and white arrows show new follicle developing. (a) Day -9 – start of follicular phase; prl 0.3623 ng/mg Cr; (b) day -7 – dominant follicle 1.2 cm dia; prl 0.60 ng/mg Cr; white arrows > follicle; (c) day -4 – dominant follicle 1.6 cm dia; prl 0.59 ng/mg Cr; and (d) day -1 – perioviulatory follicle 2.0 cm dia; prl 0.35 ng/mg Cr.

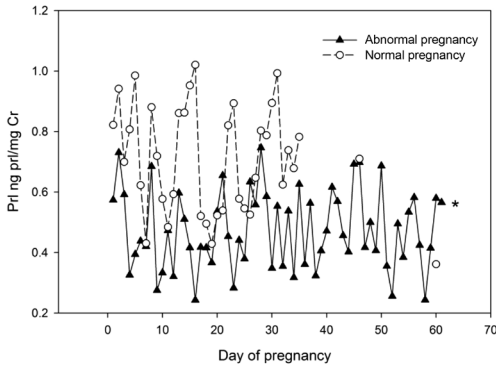


**Figure 7.** Estradiol and prl during anestrus, cohobitation, follicular phase, and ovulation: (a) anestrus ( $r = 0.3450$ ); (b) cohobitation with mother–calf pair ( $r = 0.0714$ ); and (c) follicular phase and ovulation ( $r = 0.1111$ ). \*Ovulation.



**Figure 8.** Estradiol and prl in the final days leading to parturition ( $r = 0.4232$ )





**Figure 9.** Prl concentrations during the first trimester of pregnancy in a normal and abnormal pregnancy. \*Abortion after abnormal pregnancy.

*Prl and Nursing*

Data collected from Dolphins #3 and #4 and their calves included total number of latches in a 24-h period as well as each latch duration. Nursing latch duration from the two calves in the study averaged 3 to 7 s with over 100 latches in a 24-h period (Figure 10).

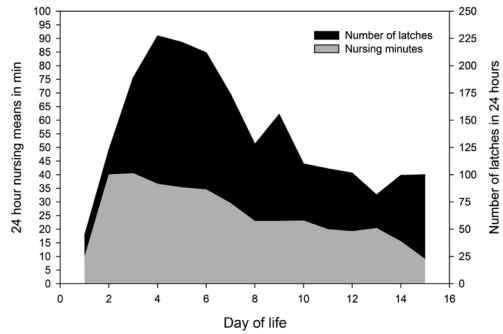
*Prl and Lactation in Non-Mothers*

Milk was manually expressed in Dolphin #2 after 3 mo of cohabitation with a calf, but Dolphin #2 was not observed nursing the calf. Milk production was not identified in Dolphins #1, #4, #5, or #6. Prl concentrations rose markedly when non-mothers cohabitated with mothers and young calves of less than 6 mo for at least 60 d (Figure 11).

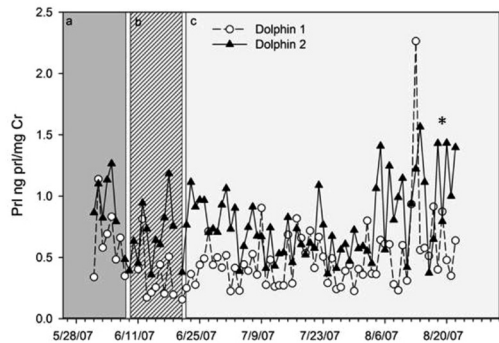
*Prl and Cohabitation*

Dolphins #1, #2, #4, #5, and #6 were all opportunistically observed interacting with mother-calf dyads through group synchronized swimming and escorting the calves without the mothers. Observations were recorded in daily husbandry record keeping.

Cohabitation with calves from newborn to 6 mo had prl concentrations significantly higher than cohabitation with an older calf of 16 mo ( $p = 0.050$ ). Prl means for dolphins cohabitating with newborn calf/calves for 3 mo (Dolphins #1 and #2) and 4 mo (Dolphin #6) are as follows: Dolphin #1 ( $n = 63$ ;  $0.49 \pm 0.29$  ng prl/mg Cr), Dolphin #2 ( $n = 63$ ;  $0.78 \pm 0.30$  ng prl/mg Cr), and Dolphin #6 ( $n = 43$ ;  $1.01 \pm 0.42$  ng prl/mg Cr). Dolphin #5 cohabitated with two 3-mo-old calves for 3 mo ( $n = 14$ ;  $1.06 \pm 0.61$  ng prl/mg Cr), and Dolphin #4 cohabitated with one 18-mo-old calf for 2 mo ( $n = 6$ ;  $0.21 \pm 0.04$  ng prl/mg Cr). Figure 11 shows serial prl concentrations from Dolphins #1 and #2 before and after cohabitation with a calf.



**Figure 10.** Minutes of nursing and number of latches in a 24-h period during the first 15 d of life for two calves



**Figure 11.** Prl concentrations during cohabitation with a mother-calf pair: (a) no calf; (b) calf born in adjacent pool to Dolphins #1 and #2; and (c) Dolphins #1 and #2 cohabitate with newborn calf and calf's mother. \*Milk expressed from Dolphin #2.

*Prl and Seasonality*

Anestrus occurred during each season, and prl concentrations were compared using paired *t* tests. There was no significant difference in anestrus prl levels between seasons. Prl sample means during each season are as follows: winter ( $n = 2$ ;  $0.710 \pm 0.269$ ;  $p = 2.286$ ), spring ( $n = 20$ ;  $0.744 \pm 0.266$ ;  $p = 0.124$ ), summer ( $n = 15$ ;  $0.795 \pm 0.270$ ;  $p = 0.149$ ), and fall ( $n = 2$ ;  $0.640 \pm 0.057$ ;  $p = 0.481$ ).

*Mammary Gland Ultrasound*

The bottlenose dolphin has paired mammary glands with external openings located in two indented slits on either side of the genital groove. Connective tissue septa divide the parenchyma into lobules (Ridgway, 1972). Swollen dolphin mammary glands are easily visualized in lactating post-partum mothers anterolateral to the genital groove. Mammary gland ultrasound was performed on Dolphins #3, #5, and #6.

**Table 4.** Mammary gland state and size during pregnancy, lactation, and post-weaning estrous cycle

Stage	Gland state <sup>a</sup>	Size (cm) <sup>e</sup>	Prl $\pm$ SD ng prl/mg Cr
4 mo pregnant	Resting	1.2	0.06 $\pm$ 0.18
11 mo pregnant	Intermediate	3.2	0.96 $\pm$ 0.62
12 mo pregnant (6 d before parturition)	Lactating	3.8	0.8 $\pm$ 0.12 <sup>b</sup>
3 mo lactation	Lactating	4.8	1.8 $\pm$ 0.97
18 mo lactation	Lactating	5.0	0.42 $\pm$ 0.16
9 d post weaning (no suckle/nursing)	Lactating	4.0	0.47 <sup>c</sup>
15 d post weaning, day -7 (pre-ovulation)	Intermediate	3.9	0.60 <sup>c</sup>
28 d post weaning, day +6 (post-ovulation)	Intermediate	3.0	0.52 <sup>d</sup>

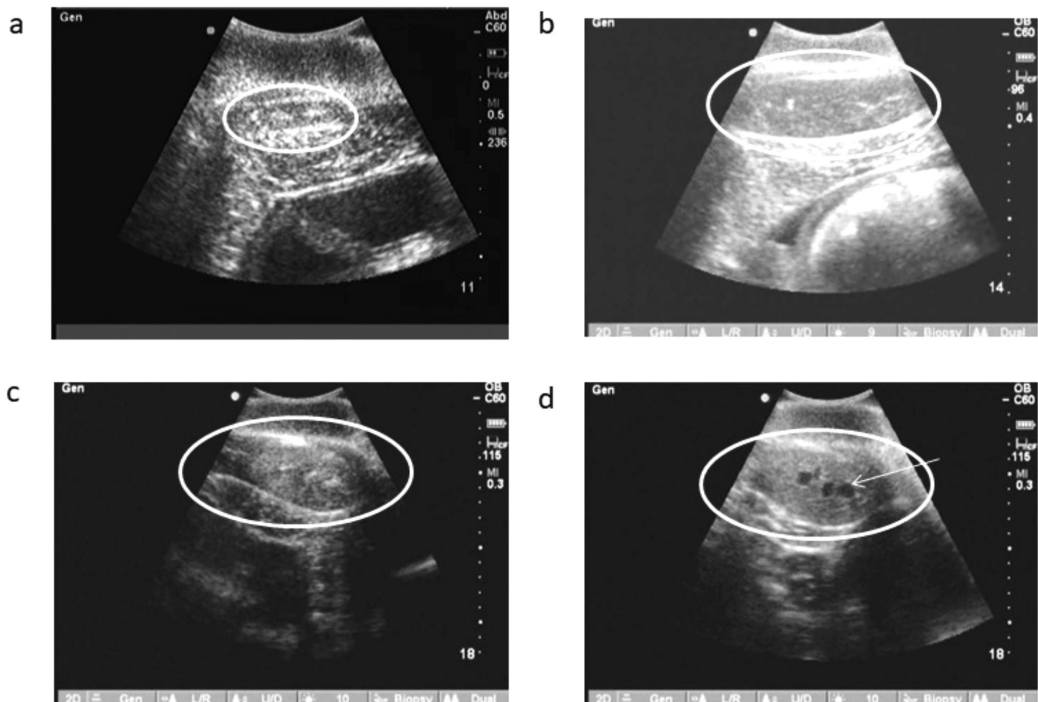
<sup>a</sup>Resting = involution of gland; intermediate = glandular lobules developed but not as developed as in lactation; and lactating = milk secreting (Flock & Winter, 2006)

<sup>b</sup>Prl mean final 7 d before parturition in Dolphin #2

<sup>c</sup>Prl level for the same day as ultrasound in Dolphin #3

<sup>d</sup>Prl level 8 d post-ovulation in Dolphin #3

<sup>e</sup>Medial parenchyma cross-sectional diameter



**Figure 12.** Ultrasound of the mammary parenchyma: (a) resting – 4 mo pregnant; homogenous and small; only hyperechoic ligaments and border visible; (b) lactating – 1 wk before parturition; homogenous and hypoechoic with small anechoic lactiferous ducts and hyperechoic ligaments; (c) lactating – 18 mo post-partum; homogenous echogenicity with small anechoic lactiferous ducts and hyperechoic ligaments and parenchyma border; and (d) lactating – no suckle or nursing for 2 d; multiple large anechoic lactiferous ducts. White circles indicate mammary gland, and white arrow indicates lactiferous ducts.

Dolphin #3 was monitored with ultrasound to identify changes in mammary gland parenchyma size and echogenicity during various stages of lactation concurrent with prl concentrations. Table 4 and Figure 12 show prl means and parenchyma size during resting, intermediate, and lactating stages.

The parenchyma of a healthy mammary gland appears as a homogenous structure of average echogenicity with anechoic blood vessels and lactiferous ducts (Flock & Winter, 2006). The non-lactating mammary parenchyma in the dolphin is difficult to visualize, appearing with a homogenous echogenicity and hyperechoic border. Distinct hyperechoic ligaments can be visualized in the transverse view, and these were used to identify the location of the mammary gland in non-lactating dolphins. The lactating mammary parenchyma was easy to visualize, appearing with a hypoechoic homogenous echogenicity with small anechoic lactiferous ducts and hyperechoic ligaments and border. Immediately following weaning of an 18-mo-old calf, lactiferous ducts filled due to the lack of nursing (Figure 12d).

Dolphins #5 and #6 were monitored via ultrasound for signs of mammary development while cohabitating with a calf, but development and lactation was not detected.

## Discussion

### Pregnancy

Prl concentrations rose as pregnancy progressed with peaks occurring during the last month. This differs from findings in rats for which prl concentrations remain low during the latter half of pregnancy (Amenomori et al., 1970; Morishige et al., 1973) but is similar to humans for whom there is an increase in prl as pregnancy progresses with a peak at delivery (Hwang et al., 1971; Kletzky et al., 1985). Based on ultrasound, the bottlenose dolphin mammary gland enlarges and changes in echogenicity during the last month of pregnancy; thus, increased prl concentrations are most likely involved in lobuloalveoli development (Harris et al., 2006).

Prl concentrations during the first trimester were higher than during the luteal phase and higher in normal healthy pregnancies vs the abnormal/unhealthy pregnancy. This suggests that prl may play a role in CL maintenance during pregnancy such as has been found in ferrets (*Mustela putorius furo*) and dogs (Murphy, 1979; Concannon et al., 2009).

### Lactation

Bottlenose dolphin mother-calf nursing behaviors have been well documented in the literature (Eastcott & Dickinson, 1987; Wells et al., 1987;

Cockcroft & Ross, 1990; Peddemors et al., 1992; Reid et al., 1995; Mann & Smuts, 1998; Mann, 1999), and dolphins have been compared to primates in terms of long-term young dependency (Ofstedal, 1997). Wild dolphin mothers will typically nurse calves for 3 y (Wells et al., 1987; Ofstedal, 1997), although it has been documented to continue for over 5 y (Wells et al., 1987; Mann, 1999). The need to teach young how to forage in a challenging environment may influence lactation duration in cetaceans (Mann et al., 2000).

During the first 2 wks of life, neonate dolphins in this study nursed in 3 to 7 s intervals totaling over 30 min and over 100 nipple latches in a 24-h period (Figure 11). However, after the first 2 wks, total nursing durations drop off dramatically (Eastcott & Dickinson, 1987; Peddemors et al., 1992; Reid et al., 1995). By month 3, nursing durations may be less than 7 min in a 24-h period (Peddemors et al., 1992). Interestingly, prl concentrations were higher in months 3 through 5 of post-partum lactation than months 1 and 2 when nursing durations were more frequent (Table 2 & Figure 3). During the first 20 d post-partum, milk is spontaneously expressed into the water; but as lactation continues, spontaneous milk discontinues (Peddemors et al., 1992).

Peddemors et al. (1992) also noted that after the first month of nursing, calf suckling was often preceded by the calf massaging the mammary glands with the dorsal fin or top of the melon, and this occurred more often before the first suckle of each bout. It has been suggested that the massaging behavior may be required to initiate milk let-down as lactation stages progress (Cockcroft & Ross, 1990). In piglets, mammary massage prior to suckling causes an increase in prl concentrations (Algers et al., 1991). It is possible that the end of spontaneous milk flow after the first month and the increase and improvement of dolphin calf mammary massage could account for increased prl concentrations in months 3 through 5.

Additionally, numerous studies have been conducted on the milk composition of the bottlenose dolphin and show a high level of variance in composition depending on the stage of lactation (Eichelberger et al., 1940; Ackman et al., 1971; Yablokov et al., 1974; Pervaiz & Brew, 1986; Peddemors et al., 1989; Ridgway et al., 1995; Ofstedal, 1997; West et al., 2007). As lactation stages progress, there is a tendency toward higher fat and protein and less water content (West et al., 2007).

Overall, mean prl concentrations were higher during the first 5 mo of post-partum lactation vs months 15 through 18. Captive bottlenose dolphin calves in this study began eating fish as a supplement to nursing at approximately 6 mo of age and by 18 mo were receiving 3% of body weight in

fish. This may reduce the amount of nursing by an older calf and may also subsequently lead to lower prl concentrations.

#### *Cohabitation*

When non-mothers cohabitated with mother-calf dyads, they were assumed to allomother in some aspect. Opportunistic observations in daily husbandry records indicated that non-mothers interacted positively with mother-calf pairs and/or calves alone. Prl concentrations rose markedly when non-mothers cohabitated with mothers and young calves of less than 6 mo for at least 60 d. One 7-y nulliparous study female (Dolphin #2) began lactating after approximately 3 mo of cohabitation with her mother and her mother's newborn calf. No observations were made of the non-mother nursing the calf, although she was observed escorting the calf. This is consistent with Mann & Smuts' (1998) finding that wild nulliparous inexperienced female dolphins were more likely to allomother than parous females. As with primates (Ziegler, 2000; Schradin et al., 2003; Soltis et al., 2005), prl appears to be associated with maternal behavior in dolphins in both the mother and associated non-mothers. Additionally, based on the nulliparous female that began lactating, prl is most likely involved in spontaneous and/or induced lactation in dolphins.

#### *Estrous Cycle, Seasonality, and Anestrus*

During the follicular phase, all four dolphins experienced a rise in prl between days -8 and -5, and two dolphins experienced a second rise on days -4 and -1 (Table 3). The prl rise was positively correlated with selection of a dominant follicle in Dolphin #3 (Figure 7). This suggests that prl may play a role in follicular maturation as seen in horses (Bennett-Wimbush et al., 1998; King et al., 2008). Prl was negatively correlated with estradiol during the follicular phase; however, Figure 8 shows that the prl rise at day -6 in Dolphin #2 came the day after a rise in estradiol. It is possible that the rise in prl was in part due to a previous rise in estradiol.

Prl is an important regulator in seasonal-breeding species. There is a clear seasonal correlation of prl in horses and sheep, with elevated prl during the breeding season of horses and low prl concentrations during the breeding season of sheep (Gerlach & Aurich, 2000). In aseasonal domestic cattle, there is an annual prl rhythm related to photoperiod and temperature with peaks in the summer (Tucker, 1982; Petitclerc et al., 1983). In contrast, there is no correlation between prl and photoperiod in humans or domestic pigs (Reinberg et al., 1978; Ravault et al., 1982; Martikainen et al., 1985). Dolphins do not appear to have an annual prl rhythm and may not use photoperiodic cues for reproductive regulation.

This study showed an association between weaning and a return to estrous similar to pigs (Armstrong & Britt, 1985) as well as a similar "male effect" wherein boar contact with weaned sows can advance estrus by several days (Pearce & Pearce, 1992; Langendijk et al., 2000). The male effect in terrestrial mammals involves an external chemical stimulus that immediately modulates the activity of the hypothalamic gonadotrophin-releasing hormone pulse generator (Hamada et al., 1996). After spending 3 mo with a mother-calf dyad, Dolphins #1 (non-lactating) and #2 (lactating non-mother) were moved into a social group with an adult male with whom they had previously been living separately. Ovulation occurred in 14 (Dolphin #2) and 17 (Dolphin #1) d, and both animals conceived from natural breeding. This quick time to ovulation in the presence of a mature male following weaning is similar to the male effect seen in pigs. In contrast, Dolphins #3 (lactating mother) and #4 (non-lactating) lived in a social group with no adult male following the weaning of Dolphin #3's calf. Ovulation occurred 22 and 30 d, respectively, following weaning, and both dolphins conceived from artificial insemination. This slower post-weaning ovulation could be the result of not having contact with a mature male. Prl concentrations were elevated above basal levels (Table 2) due to lactation or cohabitation with a calf prior to three of the four studied estrous cycles (Dolphins #1, #2, and #3). Removal of the animals from the respective calves resulted in a drop in prl and a return to estrous (Figures 8 & 12). Dolphin #4 did not have markedly elevated prl concentrations when living with the 16-mo-old calf prior to removal from the calf, and she took the longest time to ovulation at 30 d.

There was also a natural paired estrous synchrony between Dolphins #1 and #2 and Dolphins #3 and #4. Natural estrous synchrony irrelevant to seasonality has been documented in elephants (Rasmussen & Krishnamurthy, 2000; Weissenböck et al., 2009), and typically pheromonal and/or odorous cues are the mechanisms by which synchronicity is achieved (McClintock, 1998; Stern & McClintock, 1998; Rasmussen & Schulte, 1999). There have been suggestions that dolphins may have the ability to use chemoreception in communication (Yablokov, 1961; Yablokov et al., 1974), and dolphins have the ability to taste salty, sweet, sour, and bitter, and are most sensitive to bitter (Nachtigall & Hall, 1984; Nachtigall, 1986). Dolphin urine, semen, blood, feces, prostate glandular extract, and perianal glandular secretions were analyzed using gas chromatography and mass spectrometry in a study by the Naval Ocean Systems Center to identify chemoreceptively active compounds (Ceruti et al., 1985). That study found that dolphin urine contained

compounds that were sweet, sour, and bitter, and that one or more of these compounds could account for the dolphin's ability to detect urine during behavioral taste studies (Kuznetsov, 1990). It is unknown how, or to what extent, dolphins may utilize this sense in their daily life, and it is also unknown how dolphins signal reproductive condition to one another. Further study is warranted.

The positive correlation between prl and estradiol during anestrus is interesting and suggestive that the prl rhythm during anestrus could be resultant of an estradiol rhythm (del Pozo & Brownell, 1979). Anestrus in dolphins could be similar to swine for which estradiol provides a negative feedback and prevents FSH and LH release (Almond & Dial, 1990).

All study females remained anestrus while lactating or when cohabitating with a mother-calf dyad, and none of the females had access to a mature male during that time period. Previous studies that showed a return to estrous while lactating (Yoshioka et al., 1989; West et al., 2000, 2007) all involved the lactating females having or gaining access to mature males. This may be further evidence of a possible male effect on female dolphin reproduction.

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