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Measuring fecal progestogens as a tool to monitor reproductive activity in captive female bottlenose dolphins (*Tursiops truncatus*)

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Abstract

The objective was to develop and test radioimmunoassays (RIAs) to measure fecal progestogens (P) and estrogens (E) to monitor ovarian activity in the bottlenose dolphin (*Tursiops truncatus*). Fecal samples were collected at least once a week for 20 mo from three peripubertal female bottlenose dolphins. Blood samples were collected at least once a month to compare serum and fecal steroid concentrations. Moreover, random fecal samples from three pregnant females, one lactating female, and one sexually mature female receiving oral altrenogest treatment were also collected. Fecal samples were collected behaviorally with a probe to avoid water contamination and extracted with petroleum ether (for P analysis) or diethyl ether (for E analysis). When possible, vaginal cytology and ovarian ultrasonography were used to monitor the estrous cycle. The RIA for fecal P had good reproducibility and negligible matrix effect. In addition, when fecal samples (N = 25) were extracted with ethanol, the results with the two methods of extraction were highly correlated (r = 0.923). Therefore, extraction of fecal samples with petroleum ether represented a valid alternative to other, more time-consuming methods of determining fecal P concentrations. In the absence of luteal activity, fecal P concentrations were consistently < 10 pmol/g feces, although they never decreased below 10 pmol/g during pregnancy. Thus, the threshold to confirm the presence of an active corpus luteum was provisionally set at 10 pmol/g. Around the onset of puberty, luteal phases appeared shorter and irregular in the bottlenose dolphin, as in other mammalian species. Additional HPLC-MS studies should be performed to identify predominant P metabolites to be used as fecal indicators of luteal activity in this species.
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1. Introduction

The endocrine status of dolphins kept in controlled environments is usually monitored by sampling serum and urine [1]. In addition, reproductive status can be monitored by ultrasonography [2]. However, not all animals are trained for urine collection, and not all facilities are able to conduct ultrasonographic examinations on a regular basis. Additionally, although frequent blood samples can be obtained, daily blood sample collection may be stressful and result in the loss of this particular voluntary behavior.

Because the capture of wild marine mammals is strictly regulated, the demand for captive-born dolphins

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has increased during recent years. Monitoring the reproductive status of these animals is of the utmost importance to optimize management and implement effective reproductive programs. Noninvasive methods (e.g., analysis of fecal steroid concentrations) are used successfully in many domestic and exotic species [3–10] to assess reproductive status, including monitoring corpus luteum (CL) activity, pregnancy, and adrenal gland activity [11]. Furthermore, these noninvasive techniques and baseline fecal hormone concentrations may be applied to studies of wild animals, for which blood or urine collection would be very difficult [7].

In addition to progesterone (P4) per se, the analysis of progesterone metabolites in feces has also been used to monitor ovulation and pregnancy in various species [3,7,10,12–14]. Several studies demonstrated that progesterone undergoes several metabolic processes, resulting in the fecal excretion of various 5-α and 5-β pregnanes [3,10], as well as 20-α and 20-β pregnanes [8,9].

Several studies describing methodologies for the extraction and measurement of reproductive hormones from fecal samples of marine mammals have been conducted, such as in right whales (*Eubalaena glacialis*) [4,7] and sea otters (*Enhydra lutris*) [5]. However, we are not aware of any study measuring fecal reproductive hormones in dolphins. Schroeder et al. [15] and Robeck et al. [16] reported that a serum P4 concentration ≥3 ng/mL is commonly considered an indicator of ovulation (luteal activity), pregnancy, or pseudopregnancy in the bottlenose dolphin. If this concentration persists or increases for 6 consecutive weeks, it is generally considered indicative of pregnancy. High plasma P4 concentrations for >6 wk might also indicate a persistent luteal cyst (Brook F, personal communication; Lacave G, unpublished observation). In addition to hormone measurement, vaginal cytology has been used to stage the estrous cycle in several animal species [17–20], including dolphins [21,22].

The objectives of this study were to develop radioimmunoassay (RIA) methods for measuring fecal progestogens and estrogens to noninvasively monitor ovarian activity in the bottlenose dolphin and to test these methods in animals in various physiologic states.

2. Materials and methods

2.1. Animals

Eight female bottlenose dolphins (*Tursiops truncatus*) housed in three European facilities (A: Mediterraneo Marine Park, Malta; B: Oltremare, Italy; C: Zoomarine,

Portugal) were used. These dolphins were maintained in public displays in outdoor pools. Five females performed shows, two were in swimming programs, and one was in both shows and swimming programs (Table 1). Husbandry management was similar among facilities. Diets consisted of frozen fish, including herring (*Clupea harengus*), capelin (*Mallutus villosus*), sprat (*Sprattus sprattus*), blue whiting (*Micromesistius poutassou*), mackerel (*Scomber scombrus*), and squid (*Loligo opalescens*) and were formulated to meet individual animal requirements [23].

Bottlenose dolphins are considered to have a seasonal polyestrous cycle. Females reach sexual maturity at approximately 10 yr of age. Although it is reported that the youngest captive bottlenose dolphin calved at 4 yr of age [16], most animals calve for the first time between 7 and 10 yr. The length of gestation is approximately 12 mo, and lactation, during which estrus is usually suppressed, can last up to 2 yr [16].

2.2. Sample collection

Fecal and serum samples and vaginal smears were collected at various time intervals and frequencies (Tables 1 and 2), according to the possibilities offered by the three establishments, and were used to monitor the following physiologic conditions: peripubertal period, pregnancy, and lactational anestrus. Blood was collected at least once a month and occasionally more frequently, according to the medical protocol adopted at each facility. When possible, blood and feces were collected on the same day. Vaginal cytology samples were collected from Females 001, 002, and 003 between 0900 and 0930 and were routinely monitored throughout the years at various time intervals (Table 2). The frequency of fecal sampling was scheduled according to the feeding requirements and the training sessions of the animals (Table 1), with all samples collected between 1600 and 1700.

For peripubertal Females 001, 002, and 003, sampling of feces and serum began before puberty and lasted for 20 mo. Fecal samples were collected at least once a week and up to four times a week. In addition, ovarian activity was monitored in these females with a SonoSite 180 Plus (Sonosite, Inc., Bothell, WA, USA) ultrasonographic scanner, with 3.5-MHz transducer. For Female 004, fecal samples were randomly collected from 1 mo before the establishment of the pregnancy to the fifth month of pregnancy. For pregnant Females 007 and 008, fecal samples were collected once every 7 or 10 d, respectively, after pregnancy had been confirmed by serum P4 and ultrasonographic monitoring. Pregnancies of Females

Table 1
Characteristics of the captive bottlenose dolphin females used in this study.

Animal	Facility	Date of birth	Sexual stage	Fecal sample collection	Activity	Body weight (kg)		Body length (cm)	
						Initial	Final	Initial	Final
001	B	1996–1997[*]	Peripubertal	Oct 2005 to Jun 2007	Swim program	117	141	218	230
002	B	1997–1998[*]	Peripubertal	Oct 2005 to Jun 2007	Swim program	123	144	228	237
003	B	2000[*]	Prepubertal	Oct 2005 to Jun 2007	Swim and show	109	123	210	225
004	A	1994	Pregnant	Jun 2005 to Dec 2005	Show	—	—	—	—
005	A	1976[*]	Lactating	Jun 2005 to Dec 2005	Show	—	—	—	—
006	C	1984[*]	Sexually mature	Jun 2006 to Apr 2007	Show	180	169	—	—
007	C	1968[*]	Pregnant	Jun 2006 to Apr 2007	Show	230	204[†] 210[‡]	—	—
008	C	1980[*]	Pregnant	Jun 2006 to Apr 2007	Show	210	190[†]	n.a.	n.a.

Facilities: A, Mediterraneo Marine Park, Malta; B, Oltremare, Italy; C, Zoomarine, Portugal.

n.a.: data not available.

[*] Estimated date of birth for wild-caught dolphins.

[†] Body weight at approximately 1 mo after parturition.

[‡] Body weight at the end of the fecal sampling period.

Table 2
Frequency of vaginal cytology sample collection from captive bottlenose dolphin Females 001, 002, and 003. The days from the beginning of observations are in parentheses.

Animal	Every other day	Three times weekly	Daily
001	06 Oct to 28 Oct 05 (0–22) 03 Dec to 11 Dec 05 (58–66)	01 Feb to 13 Mar 06 (118–158)	26 Mar to 30 Apr 06 (171–206) 16 May to 22 May 06 (222–228) 01 Jun to 07 Jul 06 (238–274) 01 Oct to 06 Nov 06 (360–396) 26 Mar to 29 Apr 07 (536–570)
002	06 Oct to 28 Oct 05 (0–22) 03 Dec to 11 Dec 05 (58–66)	01 Feb to 13 Mar 06 (118–158)	26 Mar to 30 Apr 06 (171–206) 16 May to 22 May 06 (222–228) 01 Jun to 07 Jul 06 (238–274) 01 Oct to 06 Nov 06 (360–396) 26 Mar to 29 Apr 07 (536–570)
003	06 Oct to 28 Oct 05 (0–22) 03 Dec to 11 Dec 05 (58–66)	01 Feb to 09 Mar 06 (118–154)	26 Mar to 30 Apr 06 (171–206) 17 May to 22 May 06 (223–228) 01 Jun to 07 Jul 06 (238–274) 02 Oct to 11 Oct 06 (361–370) 26 Mar to 19 Apr 07 (536–560)

007 and 008 were monitored with an Aloka SSD-900 ultrasonographic scanner (Aloka Co. Ltd, Leca do Balio, Portugal), using a convex sectorial 3.5-MHz transducer to measure fetal development. Lactating Female 005 was monitored through fecal samples opportunistically for 6 mo. Female 006 was sexually mature but was placed on oral altrenogest (Regu-mate; Intervet Inc., Millsboro, DE, USA) to prevent cycling. Feces from this animal were sampled twice a week for 10 mo.

Fecal samples were collected by voluntary behavior. The dolphin was asked to assume a ''belly-up'' position and feces were collected directly from the rectum with a ''Levins tube'' (Pennine Healthcare, Derby, UK), with a diameter of 4 mm, inserted 10 to 20 cm into the rectum.

After collection, the fecal samples were placed in a plastic container and kept frozen at –23 °C until analyzed for progestogens and estrogens.

Blood was collected by voluntary behavior into Vacutainer tubes (Becton Dickinson Italia, Buccinasco-Milan, Italy) from venipuncture of the tail fluke (superficial vein or artery) or the periarterial venous rete [24]. Within 10 min after collection, the blood was centrifuged ($1500 \times g$ at 4 °C) to separate the serum, which was kept frozen at -23 °C prior to analysis.

To collect samples for vaginal cytology, the dolphins were trained for a ventral lay-out presentation. The genital area was dried with gauze, and samples were collected using a sterile culture swab (Sterilin; Barloworld Scientific, Milan, Italy) that was inserted into the vaginal opening approximately 8 cm and rotated one complete circle before removal. The swab was then rolled in a tube containing 1 mL Hartmann solution, and the liquid containing the cells was smeared onto a slide and air-dried. Once the smears were dry, they were fixed and stained with the rapid hematoxylin-eosin stain (DiffQuik; Mertz-Dade, Milan, Italy) as modified by Muraco et al. [21]. One hundred cells were counted to stage the estrous cycle. Cells were classified as basal, parabasal, intermediate, and superficial (keratinized), as described by Muraco et al. [21].

All procedures described here were performed in accordance with the Animal Welfare Act of Malta, the European directive 1999/22/CE, and the EAAM standard for establishments housing bottlenose dolphins (http://www.eaam.org/housing_standards/).

2.3. Hormone solvent extraction

Progestogens (P) were extracted from fecal samples by petroleum ether, whereas diethyl ether was used to extract estrogens (E). Fecal samples were thawed and thoroughly mixed until homogeneous. Samples (100 mg) were placed in Pyrex extraction tubes with 8 mL of either petroleum ether or diethyl ether, according to the hormone to be analyzed. Samples were then mixed vigorously for 10 min at room temperature. Tubes were centrifuged ($1500 \times g$, 4 °C) for 5 min, and the aqueous phase was frozen at -20 °C for 1 h. The organic phase was transferred to fresh tubes and evaporated under nitrogen current. The dry extracts were dissolved in 0.5 mL (P) or 0.25 mL (E) of RIA buffer (61 mM di-sodium hydrogen orthophosphate, 40 mM sodium di-hydrogen orthophosphate, 154 mM sodium chloride, and 0.1% BSA, pH 7.2). Fecal samples obtained from Females 001 and

002 (N = 25) were also extracted with ethanol, as described by Brown et al. [25].

Progesterone (P4) was extracted from serum by petroleum ether, whereas diethyl ether was used to extract 17β-estradiol (E2). Serum samples were thawed, and 0.2 mL (P4) or 0.5 mL (E2) aliquots were placed in Pyrex extraction tubes with 8 mL of solvent and mixed vigorously for 10 min at room temperature. Tubes were centrifuged ($1500 \times g$, 4 °C) for 10 min, and the aqueous phase was frozen at -20 °C for 1 h. The organic phase was transferred to fresh tubes and evaporated under nitrogen current. The dry extracts were carefully dissolved in RIA buffer (P4, 0.2 mL; E2, 0.25 mL).

2.4. Hormone analysis

Sex steroid concentrations in both fecal and serum extracts were analyzed by solid-phase microtiter RIAs [26,27]. Briefly, 96-well microtiter plates (Optiplate; Perkin-Elmer Life and Analytical Sciences, Shelton, CT, USA) were coated with a goat anti-rabbit γ-globulin serum diluted 1:1000 in 0.15 mM sodium acetate buffer pH 9 and incubated overnight at 4 °C. The plates were carefully washed with RIA buffer, followed by the addition of 0.2 mL of either rabbit anti-P4-carboxy-methyloxime-BSA serum (working dilution 1:8000) or rabbit anti-E2-6-carboxymethyloxime serum (working dilution 1:15,000). After an overnight incubation at 4 °C, the antiserum solutions were decanted, plates were washed with RIA buffer, and standards, quality controls, and unknown extracts (P, 0.05 mL; E, 0.1 mL) were added in duplicate. For P analysis, the standard curve was made by serially diluting (2.5 to 320 pg/well) a P4 preparation (Sigma, Milan, Italy) in RIA buffer. [1,2,6,7-³H]Progesterone (Perkin-Elmer Life and Analytical Sciences; specific activity, 97 Ci/mmol) was used as the tracer at concentration of 30 pg well^{-1} 10 μL^{-1}. For E analysis, the standard curve was made by serially diluting (1.5 to 200 pg/well) an E2 preparation (Sigma) in RIA buffer. The tracer [2,4,6,7-³H]estradiol (Perkin-Elmer Life and Analytical Sciences; specific activity, 72 Ci/mmol) was used at 30 pg well^{-1} 10 μL^{-1}. The total volume of the reaction mixture was adjusted to 0.2 mL with RIA buffer and was incubated overnight at 4 °C. Finally, the reaction mixture was decanted, and the plate was carefully washed. Bound radioactivity was β-counted (Top-Count; Perkin-Elmer Life and Analytical Sciences).

Cross-reactions for the progesterone antibody were progesterone, 100%; 11α-hydroxyprogesterone, 77%; 11β-hydroxyprogesterone, 65%; 17α-hydroxyprogesterone, 2.9%; 20α-hydroxyprogesterone, 0.01%; and

20β-hydroxyprogesterone, <0.001%. Cross-reactions for the estrogens antibody were 17β-estradiol, 100%; estrone, 2.5%; estriol, 1.2%; and <0.007% for the other tested steroids. The detection limit of the assays as calculated by the software Riasmart (Perkin-Elmer Life and Analytical Sciences) was 2.5 pg/well for P and 1.5 pg/well for E. The repeatability of the RIA for P was assessed in samples containing high and low P concentrations (approximately 12 and 2 pmol/g). The intra-assay coefficients of variation were 5.8% and 4.9%, and the interassay coefficients of variation were 6.2% and 11.7% for the high and low P samples, respectively. The repeatability of the E RIA was studied in samples containing high and low E concentrations (approximately 6 and 0.5 pmol/g). The intra-assay coefficients of variation were 6.3% and 5.5%, and the interassay coefficients of variation were 4.2% and 8.0% for the high and low E samples, respectively.

The progestogen RIA was validated for bottlenose dolphin feces and serum extracts by parallelism and recovery tests. Parallelism was assessed by calculating the regression curve between the observed hormone concentrations and the reciprocal of the dilution factors in serially diluted (1:2 to 1:32) fecal and serum extracts. Recovery was expressed as the regression curve obtained between observed and expected hormone concentrations measured in fecal and in charcoal-treated steroid-free serum samples spiked with known amounts of hormone (range: 0.16 to 5.09 pmol/100 mg in feces, 0.032 to 0.636 pmol/100 L in serum) and extracted as described earlier. The relationship between fecal P concentrations measured after ethyl ether or ethanol extraction was studied by the Pearson's correlation test [28].

3. Results

3.1. RIA validation

In the RIA for fecal P concentrations, the intercept of the parallelism curve ($[H_{obs}] = 14 \times (1/fd) - 0.32$;
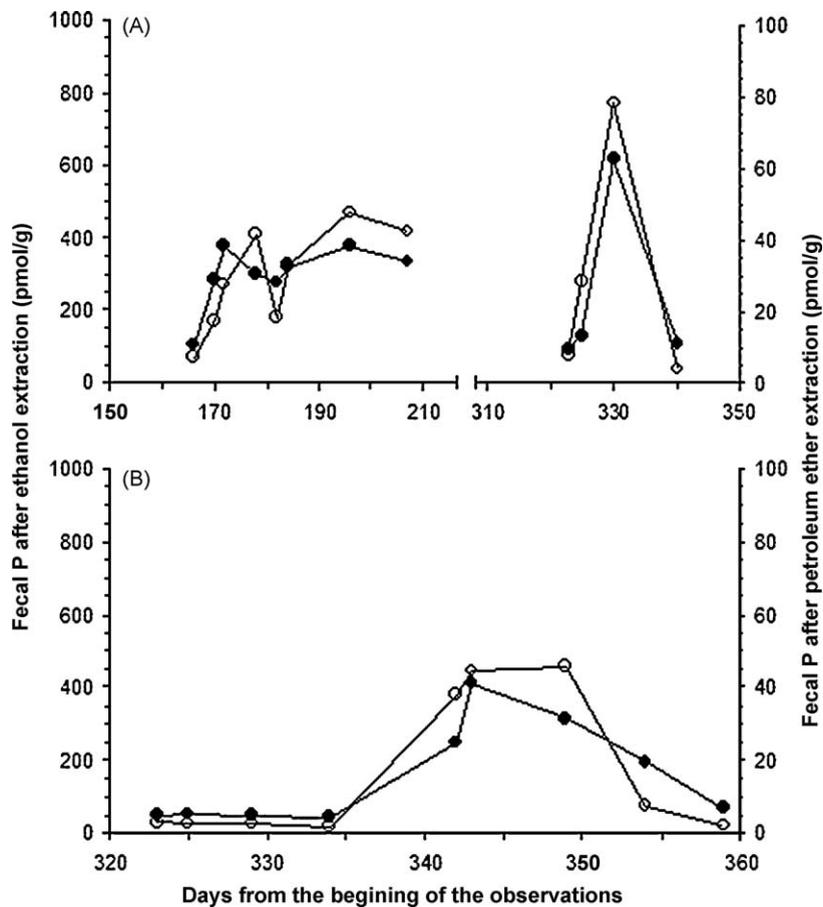

Fig. 1. Comparison between fecal progestogen (P) concentrations in captive bottlenose dolphins obtained after petroleum ether (open circles) or ethanol (solid circles) extraction in cycling Females (A) 001 and (B) 002.

$R^2 = 0.99$) was not significantly different from 0, indicating a good degree of parallelism. Both the intercept and the angular coefficient of the recovery test curve ([H_{obs}] = 0.94 × [H_{exp}] − 0.59; R^2 = 0.98) were not significantly different from 1, indicating good correspondence between observed and expected hormone concentrations. However, there was a slight underestimation of fecal P concentrations. For estrogens, RIA parallelism was very good ([H_{obs}] = 18.5 × (1/fd) − 0.08; R^2 = 0.99), and the recovery test ([H_{obs}] = 0.75 × [H_{exp}] − 0.67; R^2 = 0.99) indicated a slight underestimation of fecal E concentrations, particularly at lower concentrations.

3.2. Relationship between the two extraction methods

Progestogen concentrations measured after ethanol extraction averaged 16-fold higher than those measured after petroleum ether extraction. However, fecal hormone concentrations measured after the two methods of extraction were highly correlated (r = 0.923), and the fecal P profiles obtained had acceptable congruence (Fig. 1).

3.3. Hormone concentrations

3.3.1. Peripubertal period

Female 003 was monitored for 20 mo; no sign of ovarian activity was detected by either vaginal cytology (N = 80) or ultrasonography, suggesting that she did not become pubertal within this time interval. In particular, mean (±SD) superficial (62.8 ± 17.1%) and intermediate (37.0 ± 16.9%) cells and rare parabasal and basal cells were observed on vaginal cytology, whereas ovarian follicles were never detected ultrasonographically. In fecal samples (N = 136), P varied between 0.3 and 9.8 pmol/g (mean, 1.6 ± 1.7), whereas E varied between 0.3 and 32.8 pmol/g (mean, 7.4 ± 14.0). During the same time interval, P4 in serum samples (N = 29) varied between 0.04 and 1.0 nmol/L (mean, 0.3 ± 0.2).

At the beginning of the study, between mid-October 2005 and mid-February 2006 (0 to 130 d after the start of observations), Females 001 and 002 did not show any signs of ovarian activity on ultrasonography or vaginal cytology and thus were considered prepubertal. Vaginal cytology of the two animals (Female 001, N = 20; Female 002, N = 22) showed a majority of keratinized cells (85.9 ± 13.2%). Basal cells were not observed during this period, and a few parabasal cells were present in three samples only. During this interval, fecal P remained <10 pmol/g in both subjects.

After Day 170 from the beginning of the observation, vaginal cytology, plasma and fecal hormone concentrations (Table 3), and fecal hormone profiles (Fig. 1 and 2) substantially changed in Female 001, whereas Female 002 had remarkable hormonal changes starting 342 d after the beginning of the study. Female 001 had periodic elevations of fecal P concentrations >10 pmol/ g beginning 139 d after the onset of the observation period, suggesting the onset of ovarian cyclicity. The presence of at least three consecutive fecal samples with P concentrations >10 pmol/g and an interval between two consecutive samples ≤4 d were adopted as criteria to define a luteal phase. Based on these criteria, there were four nonconsecutive luteal phases, lasting approximately 10, 10, 12, and 14 d, respectively. Three follicular phases prior to each luteal phase were identified comparing hormonal findings, ultrasonography, and vaginal cytology (Table 3) and were estimated to last 8, 14, and 17 d. Due to the lack of consistent vaginal cytology and ultrasound data, the time interval with fecal P <10 pmol/g observed before the luteal phase between 234 and 244 d was not classified as a follicular phase. During follicular phases, the diameter of the dominant ovarian follicle was ≥0.5 cm, and the percentage of keratinized cells observed on vaginal cytology was greater than that during luteal phases. Keratinized cells, considered as the mean of the median values recorded during the three follicular and luteal phases presented above, were 55.8% (follicular phase) and 40.5% (luteal phase), respectively.

Female 002 had a single elevation of fecal P between 342 and 350 d after the beginning of the observation period (Figs. 1 and 2), and fecal P varied between 42.8 and 50.9 pmol/g (N = 3). During the rest of the study, fecal P was 1.8 ± 1.3 pmol/g (N = 159). On Day 324, a follicle 0.42 cm in diameter was detected ultrasonographically, suggesting a possible follicular phase. No further ultrasonographic evaluations were performed at that time. Elevation of fecal P was considered indicative of a luteal phase lasting approximately 8 d.

3.3.2. Pregnancy

All pregnant females gave birth to healthy calves. Fecal samples from Females 004, 007, and 008 were collected during various stages of pregnancy, with the intent to monitor the trend of fecal P throughout gestation. Mean (±SD) fecal P and E concentrations were 54.5 ± 41.8 pmol/g and 5.2 ± 5.8 pmol/g, respectively, from 48 fecal samples collected during pregnancy from these three females.

In Female 004, which conceived after natural mating, fecal samples were collected once a month

Table 3
Mean (±SD) data regarding ovarian activity in peripubertal captive bottlenose dolphin Female 001.

Time interval[*] (d)	Vaginal cytology [b]				Fecal P (pmol/g)	N[†]	Serum P4 (nmol/L)	N[†]	Fecal E (pmol/g)	N[†]	Serum E2 (pmol/L)	N[†]	Diagnosis
	B (%)	P (%)	I (%)	K (%)									
158–166	0	0	23 ± 4.9	78.0 ± 4.9	2.0 ± 1.1	5	n.a.	0	4.0	1	n.a.	0	Follicular
170–184	0 ± 0.4	3.0 ± 3.5	37.0 ± 10.8	60.0 ± 14.1	30.0 ± 11.6	5	17.7	1	3.0 ± 2.2	4	101.1	1	Luteal
234–244	1.0 ± 1.7	3.7 ± 2.9	57.3 ± 5.0	38.0 ± 9.2	29.6 ± 9.8	5	18.6	1	0.7	1	164.1	1	Luteal
246–260	0	1.8 ± 2.7	60.8 ± 19.5	37.4 ± 20.5	3.4 ± 1.6	5	0.9 ± 1.0	2	1.4 ± 1.6	4	212.7 ± 23.6	2	Follicular
263–275	1.2 ± 1.9	3.2 ± 1.3	66.0 ± 10.5	29.5 ± 11.6	30.6 ± 11.7	4	18.6	1	0.9 ± 0.3	4	196.4	1	Luteal
367–384	0.6 ± 0.1	1.1 ± 2.6	41.3 ± 13.7	58.4 ± 13.8	4.9 ± 2.9	7	n.a.	0	1.2 ± 0.6	6	n.a.	0	Follicular
386–396	0.2 ± 0.45	2.6 ± 2.6	63.2 ± 16.4	34.0 ± 17.3	29.8 ± 14.6	5	32.8	1	1.1 ± 1.3	4	79.5	1	Luteal

B, basal cells; P, parabasal cells; I, intermediated cells; K, keratinized cells. n.a.: data not available.
[*] Interval relative to the start of observations.
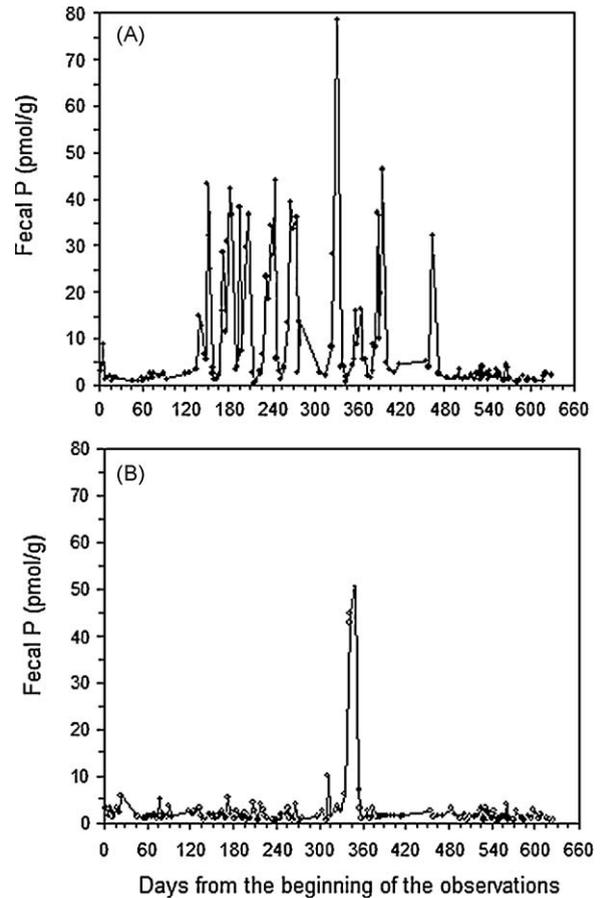[†] N: Number of measurements performed during the corresponding time interval.

Fig. 2. Fecal progestogen (P) concentrations in captive bottlenose dolphin Females (A) 001 and (B) 002. At the beginning of the observation period, these dolphins were 8 to 9 and 7 to 9 yr old, respectively.

during the first 5 mo of pregnancy: fecal P and E varied between 15.3 and 42.6 pmol/g (mean, 21.3 ± 10.5) and 0.9 and 2.1 pmol/g (mean, 1.5 ± 0.5), respectively. In Female 007, which was artificially inseminated, fecal samples were collected weekly during the last 4 mo of pregnancy; fecal P and E ranged between 27.0 and 176.9 pmol/g feces (mean, 67.6 ± 46.4) and between 0.5 and 14.8 pmol/g (mean, 4 ± 4.4), respectively. In Female 008, which mated naturally, fecal samples were collected every 10 d during the last 9 mo of pregnancy after serum P measurements and ultrasonographic confirmation of pregnancy. Fecal P (Fig. 3) varied between 12.6 and 197.7 pmol/g feces (mean, 52.7 ± 39.7), whereas fecal E ranged from 0.5 to 18.4 pmol/g feces (mean, 6.7 ± 6.7).

3.3.3. Anestrus and lactation

Female 005 was lactating, and fecal P ranged from 0.6 to 8.9 pmol/g feces (mean, 3.4 ± 2.4). Female 006

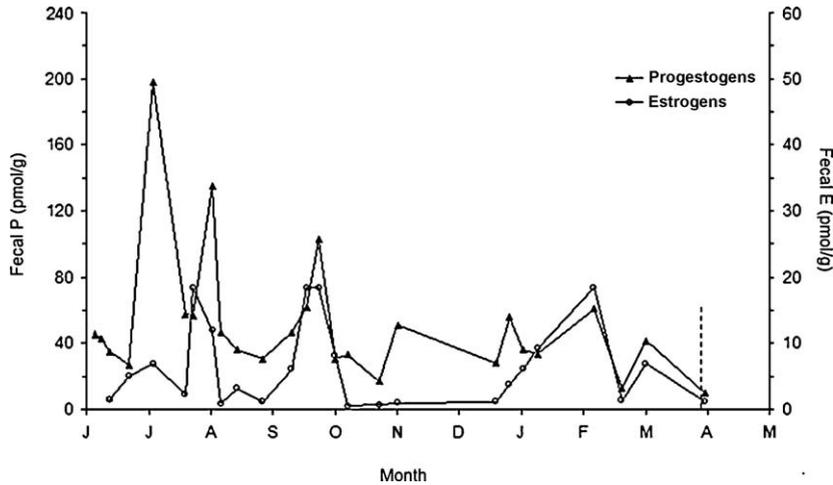Fig. 3. Fecal progestogen (P; solid triangles) and estrogen (E; open circles) concentrations in bottlenose dolphin Female 008 during pregnancy. The dotted line indicates the time of parturition.

was undergoing treatment with altrenogest (Regu-mate; Intervet) 0.044 mg/kg orally, once daily, and fecal P varied between 0.8 and 3.9 pmol/g feces (mean, 1.8 ± 0.7). In Female 007, fecal sample collection continued during the postpartum period from Day 8 postpartum for 6 mo, every 2 wk, and fecal P varied between 1.6 and 5.4 pmol/g feces (mean, 2.7 ± 1.4). In Female 008, fecal P was 9.4 pmol/g on a sample of feces collected 20 d after parturition (Fig. 3).

4. Discussion

This study demonstrated the efficacy of the RIA techniques in detecting fecal P and E in female bottlenose dolphins. Despite the small number of animals used, numerous fecal samples were analyzed, thus providing considerable information regarding fecal concentrations of sex steroids. The P and E assays had good reproducibility and negligible matrix effects.

The use of petroleum ether led to the extraction of mostly nonpolar (unconjugated) progestogens, which were present in lower concentrations in feces of bottlenose dolphins. In fact, ethanol extraction permitted observation of greater variations of fecal progestogens in cycling animals that were presumably most represented by polar (conjugated) metabolites. Nevertheless, the results obtained by the two extraction methods were highly correlated, and fecal progestogen profiles were parallel. Thus, we inferred that a substantial amount of polar progestogens was present in feces of bottlenose dolphins, which facilitated monitoring of luteal function.

The sampling method adopted for captive dolphins is minimally invasive, non-stressful for the animals, and easily conducted under voluntary behavior control.

Furthermore, this method might be used to monitor the reproductive status of wild dolphins, as already reported for the right whale [7]. This experiment was performed under controlled conditions, as fecal samples were collected directly from the rectum (with the aid of a probe) to avoid pool water contamination and were immediately frozen to reduce hormone degradation [29]. Because progestogens are extremely polar, they should not leach from fecal samples deposited in water. In that regard, based on preliminary data, at least 50% of progestogens were retained in samples after they were thoroughly mixed in water (data not shown). The main limitation to use of this method to monitor reproductive activity in wild dolphin populations is the ability to correctly identify individual samples. In right whales, individual animals were identified using photographs taken just before or after defecation [7]. However, bottlenose dolphins are more active than right whales, and they tend to swim in groups, thus rendering individual identification by direct observation difficult. Perhaps this could be overcome by development of identification methods based on genetic profiles using microsatellite loci and fecal samples as a source of DNA [30].

Measurement of sex steroids in fecal samples can be used to monitor the reproductive status of captive female bottlenose dolphins. In particular, measurement of fecal P may be useful to monitor estrous cyclicity. Several studies examining serum P4 concentrations as indicators of luteal activity have been performed. In anovulatory female bottlenose dolphins, serum P4 averaged 0.9 ± 0.1 nmol/L [31], whereas Kirby [32] and Atkinson et al. [33] reported that a serum P4 concentration ≤3.18 nmol/L (=1 ng/mL) indicated

sexual immaturity in small delphinid species. At the beginning of the current study, Female 003 was estimated to be 5 yr old. Schroeder [34] reported that puberty in female bottlenose dolphins is estimated to begin at 5 to 7 yr of age, whereas sexual maturity is achieved at 7 to 10 yr of age. Serum P4 concentrations in Female 003 were comparable with those reported by Sawyer-Steffan et al. [31] and Atkinson et al. [33] for prepubertal females; thus, we considered her prepubertal throughout the study. For this dolphin, fecal P was always <10 pmol/g.

As reported by Robeck et al. [16,35], lactation can suppress estrus. During lactation, if the total suckling time exceeds the minimum threshold duration of stimuli required to suppress estrus, the animal does not return to estrus. This threshold effect may be attributed either to decreased suckling stimuli or to an innate time clock that reduces the hypothalamic inhibitory effects of suckling stimuli after a certain postpartum period. Female 005 was lactating throughout the study, thus she was likely anestrus. During the experimental period, fecal P reached a maximum of 8.9 pmol/g, supporting this assertion.

Female 006 was receiving oral altrenogest, which mimics the action of endogenous progesterone in suppressing gonadotropin release, blocking ovarian follicular development and estrus. During treatment, fecal P reached a maximum of 3.9 pmol/g.

Finally, both Females 007 and 008 were monitored for several days after parturition, and in both cases their fecal P concentrations were consistently <10 pmol/g. There were no differences in fecal P concentrations among lactating females, not pregnant, or lactating adult females and prepubertal females, in agreement with results reported by Rolland et al. [7] for fecal P in right whales. Unfortunately, fecal E concentrations could not be evaluated for lactating and anestrous females in this study, due to the small amount of feces collected at most sampling times.

Considerable fluctuations of fecal P were observed during pregnancy, even though fecal P concentrations were never <10 pmol/g. However, fecal P concentrations measured in bottlenose dolphins during pregnancy showed a pattern similar to that observed in serum P4 in bottlenose dolphins [36] and killer whales (range, 31.8 to 95.4 nmol/L) [37,38]. In killer whales, serum P4 decreased between the fifth and eight months of pregnancy (with individual variation), perhaps due to a switch from CL and placental production of P4 [38]. The fecal P variations observed in the current study may reflect fluctuations in P4 production, or modifications of intestinal P metabolism during pregnancy. On the basis of these observations, the fecal P threshold to

discriminate between an active and nonfunctional CL was set at 10 pmol/g.

In this study, two dolphins were followed during the transition from the prepubertal to the postpubertal period. At the beginning of the study, fecal P never exceeded 10 pmol/g in Females 001 and 002, which were classified as prepubertal at that time on the basis of ovarian ultrasonography [2] and vaginal cytology. Varela et al. [39] reported a predominance of intermediate cells in vaginal cytology of anestrus bottlenose dolphins. In other mammals in which vaginal cytology is an important clinical tool in staging the estrous cycles (e.g., the dog), anestrus is characterized by a predominance of parabasal and intermediate cells, with possible presence of a few neutrophils [18]. In the current study, basal and parabasal cells were rarely present, whereas superficial and intermediate cells were predominant in prepubertal females. As suggested by Varela et al. [39], this may represent a fundamental difference between dogs and dolphins in vaginal cytology. During the prepubertal period, fecal E could not be measured in Female 001; therefore, the effect of estrogen on the vaginal cytology presentation cannot be excluded. In addition, ectopic estrogen production may have been responsible for the high percentage of superficial and intermediate cells. Indeed, although estrogens are produced mainly in the ovary, smaller amounts are produced in other tissues such as the liver, adrenal glands, muscle, and nervous tissue [40].

Using various diagnostic tools to monitor cycling females, our goal was to test if the RIA methods were useful to monitor the phases of the estrous cycle. Three follicular phases were identified through fecal hormone analysis, lasting 8, 14, and 17 d. These findings were similar to those of Robeck et al. [41], who reported follicular phases of 4 to 14 d. During follicular phases, fecal P remained consistently <10 pmol/g, whereas a slight increase in fecal E was observed. However, fecal E was measured in few samples, and the minimal variations in fecal E between samples was considered physiologically nondiagnostic. Perhaps the antiserum used to measure fecal E was very specific for E2 and not suitable to detect many of E metabolites present in feces. Thus, although the E assay validation performed quite well, we concluded that the fecal E concentrations were far from conclusive and suitable, at present, to assess reproductive status in female bottlenose dolphins.

When ovarian follicles were detected ultrasonographically, vaginal cytology was clearly associated with ovarian follicular growth, showing an increase of superficial cells up to 75%. Later, superficial cells

decreased and intermediate cells increased. Muraco et al. [21] monitored vaginal cytology changes in bottlenose dolphins and reported that ovulation occurred when keratinized cells reached 80% to 100%. In the current study, when ovulation occurred, the percentage of superficial cells was ≤80%. When the percentage of keratinized cells decreased, fecal P and serum P4 concentrations increased to values ≥10 pmol/g and ≥9.5 nmol/L, respectively, indicating a luteal phase. Furthermore, follicular and luteal phases were defined when prolonged collection of feces and vaginal cytology were performed and inversion in the predominance of keratinized cells above intermediate cells was observed.

Integrating fecal P, serum P4, and vaginal cytology data, we identified four luteal phases in Female 001, lasting 10, 10, 12, and 14 d, respectively. During these time intervals, fecal P and serum P4 were always >10 pmol/g and >9.5 nmol/L, respectively. The luteal phases observed in our study seemed shorter than those observed by Robeck et al. [38] (range, 16 to 23 d). This may be due to the sampling frequency and the criteria adopted in our study to estimate the luteal phase length, which may have led to the underestimation of its duration. More likely, luteal phases are shorter and less regular around the onset of puberty in bottlenose dolphins, as in other mammalian species (e.g., killer whales) [35].

In conclusion, extraction of fecal samples with petroleum ether represented a valid alternative to other more laborious and time-consuming methods for measuring P in bottlenose dolphins. More work with cycling animals is required to confirm the threshold for the discrimination of an active CL, and HPLC-MS studies should be performed to identify dominant P and E metabolites in bottlenose dolphin feces as indicators of ovarian activity.

Acknowledgments

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