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Diagnosing pregnancy in free-ranging dugongs using fecal progesterone metabolite concentrations and body morphometrics: A population application

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ABSTRACT

Assessing reproductive status and monitoring reproductive rates is important in the effective management of vulnerable marine mammal species such as the dugong (*Dugong dugon*). Knowledge of the reproductive physiology of this species is limited, and determining reproductive parameters (e.g., sexual maturation, pregnancy, and reproductive senescence) has been restricted by a lack of non-lethal methods for assessing reproductive status in free-ranging individuals. The aim of this study was to develop a method to identify pregnant individuals in a wild dugong population. Using an enzymeimmunoassay, we quantified concentrations of fecal progesterone metabolites (fP) in 322 dugongs, including confirmed pregnant females ($n = 10$), presumed non-pregnant adult females ($n = 25$), juvenile females ($n = 24$), sub-adult females ($n = 41$), adult females of unknown pregnancy state ($n = 63$), and males of all sizes ($n = 159$). External body morphometrics of each dugong were measured, and confirmation of pregnancy in adult female dugongs was determined by ultrasonography or observation of subsequent neonates. Concentrations of fP were different between sexes and reproductive size classes ($P < 0.001$), and ~30-fold higher in confirmed pregnant dugongs (2017–7760 ng/g) compared to presumed non-pregnant females (30–221 ng/g), juvenile females (29–195 ng/g), and males (24–261 ng/g) ($P < 0.001$). Body measures of maximum and anal girths, and teat length were all greater in confirmed pregnant females than presumed non-pregnant females (all $P < 0.05$). We evaluated a Discriminant Function Analysis (DFA) to provide a model for predicting pregnant and non-pregnant dugongs. Cross-validated results showed that the DFA correctly classified 100% of pregnant and non-pregnant females using fP concentrations, body length, fineness ratio (an index of body shape), and teat length (a female reproductive trait). Using the DFA model, we classified the pregnancy status of all female dugongs and identified a total of 30 females as pregnant and 133 females as non-pregnant from the sampled population over the sample period. Pregnant dugongs in the Moreton Bay population are characterized by fecal progesterone metabolite concentrations > 1000 ng/g, body length ⩾ 260 cm, maximum girth ⩾ 215 cm, anal girth ⩾ 126 cm, and teat length ⩾ 5 cm long. In summary, analysis of fP concentrations in combination with body morphometrics may be used to diagnose pregnancy in free-ranging dugongs, and provides a new tool to monitor breeding rates of wild sirenian populations.

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1. Introduction

Accurate estimates of pregnancy rate for wild populations, an index of recruitment and reproductive success, are essential to studies of mammalian population dynamics. The ability to diagnose pregnancy in a species allows evaluation of critical life history parameters including age at sexual maturity, inter-calving interval, frequency of pregnancy, gestation period, seasonality of reproduction, population fecundity rate, and capacity, all of which influence population viability. Quantification of reproductive parameters that can monitor and evaluate changes in population size are important to population ecology, wildlife management, and conservation biology [13,58].

The dugong (*Dugong dugon*) is listed as vulnerable to extinction throughout its global range [25], and the development of effective programs for its conservation and management has been impeded by a lack of knowledge on its reproductive biology [42]. Until now, reproductive status and pregnancy rates of wild dugongs have been determined by gross and histopathological examination of the

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reproductive tract and gonads collected postmortem from strandings, traditional harvests, and incidental bycatch [28,40,42,43]. Necropsy examination, however, is often constrained by opportunistic and limited sampling, as well as being prone to biases spatially, demographically, and even temporally [28,40,43]. Relying on necropsy for determination of pregnancy rates in populations restricts studies to areas where dugong carcasses are readily available, such as on some indigenous hunting grounds [28,40,43]. Non-lethal alternatives to accurately determine reproductive status of free-ranging dugongs are required to establish and monitor life history parameters for live wild populations at local and regional levels.

Measuring endocrine function of the gonads is a practical approach for investigating the reproductive status of live animals, because all aspects of reproduction are mediated through hormonal signals. Patterns of reproductive hormone secretions in female mammals can be used to detect and characterize physiological events such as pregnancy e.g., [21,24]. Progesterone is one of the primary hormones secreted by the healthy mammalian ovary, and plays an important role in establishing and maintaining pregnancy [3]. In mammals, maternal progesterone concentrations increase markedly during pregnancy, especially when primary production switches from the corpus luteum (ovary) to the placenta [19]. Pregnancy is therefore associated with blood progesterone concentrations greater than those observed during the non-pregnant luteal phase in most mammals [21,24]. However, due to innate logistics, regular blood collection to measure progesterone is only feasible in relatively rare circumstances when investigating free-ranging marine mammals, such as during haul-out with pinnipeds [17,23], or when sampling relatively small numbers of animals during health assessments [7,8]. In fully aquatic and cryptic marine mammals, such as dugongs, blood collection from large numbers of individuals is impractical [35], and doing so to assess pregnancy rate at a population level is not feasible on a routine basis. However, sampling excreta, such as feces, that contain steroid hormones eliminated from circulation, can also provide insights into reproductive state [38]. For wildlife endocrinologists, the relative ease of collecting samples has made the application of fecal hormone analysis a widely and routinely used technique for noninvasive reproductive monitoring of many captive and free-ranging mammals (reviewed by [38,55]). Reproductive steroid hormones are removed from the bloodstream, metabolized by the liver, excreted with the bile into the rectum, and then accumulate in the feces until defecation [55]. The concentration of hormone metabolites in feces has been shown to reflect circulating blood concentrations, albeit at concentrations that are often two to four orders of magnitude higher than that of the parent steroid in the blood [38]. Moreover, unlike blood, fecal samples are less affected by episodic fluctuations or pulses of hormone secretion, and probably represent the overall reproductive status of an animal more accurately than a single serum sample [20].

Accurate pregnancy detection through measuring fecal progesterone metabolite concentrations has been successful in a number of domestic and captive wildlife species, as well as free-ranging wildlife species [22,38,55]. Whilst fecal progesterone assays have been used extensively in terrestrial mammals, they have not been widely applied to marine mammals (reviewed by [2]). In captive situations, fecal progesterone metabolite concentrations have been used to successfully monitor pregnancy in sea otters (*Enhydra lutris*) [37] and bottlenose dolphins (*Tursiops truncatus*) [5], and have been trialed in Florida manatees (*Trichechus manatus latirostris*) [36]. Beyond captivity, the practical utility of collecting feces has provided a means of reliably detecting pregnancy in free-swimming North Atlantic right whales (*Eubalaena glacialis*) [53], and remains the only study investigating pregnancy in a wild marine mammal population using fecal samples.

The aim of this study was to develop a reliable method to determine pregnancy in free-ranging dugongs. Specifically, our objectives were to: (1) validate an enzymeimmunoassay for measuring fecal progesterone metabolites (fP) in wild dugongs sampled as part of a population tagging program [33]; (2) compare fP concentrations between sexes, size classes, and reproductive status; (3) compare body morphometrics between sexes, size classes, and reproductive status; and, (4) determine if fP and external body morphometrics could be used to accurately differentiate pregnant from non-pregnant females. This work is part of a broader study examining reproductive status, life history, and population dynamics of the vulnerable dugong in Australian waters. As the first study investigating pregnancy in live dugongs applied at a population-level, our results will provide a foundation for future studies of population demography and dynamics.

2. Methods

2.1. Sample collection

Free-ranging dugongs were sampled in Moreton Bay in southeast Queensland, Australia (latitudes 27°20.09′–27°24.87′S; longitudes 153°21.26′–153°23.84′E), between July 2005 and June 2011 across all seasons, as part of a long-term population mark-recapture program [33]. Dugongs of both sexes and all ages (except neonate calves) were captured using an open-water technique [32] and restrained at the water surface for a short period (5–6 min). During restraint, each dugong was sexed, gene-tagged [10,32], and physically tagged to confirm individual identity [33]. Total body length was measured in a straight line from snout to fluke notch, and girth measures were taken at the peduncle, anal, maximum (umbilical), and axillar positions along the body [32,33]. Female dugongs have a prominent external mammary teat located against the body under each pectoral flipper; this trait is also present in males but is rudimentary. Unstretched teat length was measured from the medial base of insertion to the extremity using a short ruler. A small (~4 g) fecal sample, uncontaminated by seawater, was collected by inserting a soft latex tube (cut-down equine yearling stomach tube with 8 mm diameter) into feces held in the distal part of the rectum. Fecal samples were transferred into zip-lock plastic bags and kept on ice before being frozen and stored at −20 °C until processing and analysis.

In addition to in-water sampling, 66 dugongs of both sexes ($n = 37$ females and $n = 29$ males) and all size classes (except neonates) were sampled during out-of-water health assessments over the austral late fall to early winter periods, 19–23 May 2008 ($n = 13$), 11–17 May 2009 ($n = 17$), 11–17 June 2010 ($n = 20$) and 30 May–3 June 2011 ($n = 16$). Upon capture, each dugong was sexed, tagged, and measured as described above. The dugong was then lifted clear of the water onto the rear deck of a research vessel for a comprehensive medical examination [35]. For each of these dugongs, blood samples were collected as soon as possible after capture to minimise the effects of stress on blood hormone concentrations. Blood was collected from the brachial arteriovenous plexus accessed via the palmar medial or lateral surface of the pectoral flipper at the proximal aspects of the ulna and radius. The flipper was scrubbed numerous times with Betadine® solution and alcohol prior to collection. Blood was drawn using a 21 gauge 3.8 cm/1.5 in needle (or 5 cm/2 in needle in the case of large adults >270 cm body length, or for a lateral draw) fitted to a 20 cm (14 in) extension set with Luer® fitted Vacutainer® collar and collected into a 10 ml red-top clot-activator Vacutainer® tube. Within 30 min of collection, tubes were centrifuged (3000 rpm for 10–15 min, 1790 g) onsite to separate the serum, and aliquots were frozen in cryovials until analysis.

Sampled dugongs were grouped into reproductive size classes based on body length, following the classification of Marsh et al. [42]. Using histological examination of gonads to confirm sexual maturity [41] and relative body length growth, Marsh et al. [42] differentiated three size classes of dugongs: dugongs at body lengths <220 cm were considered likely to be reproductively immature (juvenile), showing small and undeveloped reproductive organs; dugongs ⩾250 cm body length were probably mature (adult), showing developed and/or active reproductive organs; and dugongs between 220–249 cm body length were of variable reproductive status (subadult).

2.2. Independent confirmation of pregnancy

Verification of female reproductive status was conducted using transabdominal ultrasonography. A portable image ultrasound system (Logiq book XP, GE Healthcare) was used to scan the caudal abdominal region of each female dugong for evidence of pregnancy. Still images and digital video of scans were made of the abdomen using a 2.5 MHz probe with focal distance set between 20 and 25 cm. Those females showing evidence of a fetus were confirmed pregnant, and those showing no fetus were presumed to be non-pregnant.

We also used database records of recaptured dugongs over the six year period July 2005 to June 2011 to identify periods of pregnancy or non-pregnancy in individual females. To increase the likelihood of recapturing dugongs previously sampled for feces, we undertook intensive sampling of dugongs during each austral summer calving period (November–March) using a remote biopsy method to obtain skin for genetic identification [34]. This technique allowed us to rapidly obtain skin samples (~0.3 g) from a large number of adults with and without attendant calves, as well as from the calves. Genotype and sex matching against a panel of 26 microsatellites [10,45] was then used to identify adult females with or without calves from whom feces had been sampled in the previous year. The presence of a newborn calf suggested that any fecal sample obtained from the mother within the preceding year was collected during her 14-month gestation [28,43]. Conversely, females showing no signs of an attendant calf at >14 months post-fecal sampling were assigned a probable non-pregnant status. These independent methods of identifying pregnancy in females could not account for false assessment due to spontaneous abortion, perinatal mortality, or undetected embryogenesis.

2.3. Steroid extraction and analysis

Fecal samples were stored frozen (−20 °C) and then extracted using a modification of the method by Wasser et al. [59]. Fecal samples were oven-dried at 55 °C overnight and then pulverized until homogeneously mixed. Steroid hormones were extracted from feces by adding 4.0 ml of 80% methanol (Crown Scientific, Brisbane, Australia) to 0.20 ± 0.01 g aliquot of dried fecal sample in a glass scintillation vial (Sigma–Aldrich, New South Wales, Australia). Samples were sealed with a screw cap, vortexed for 30 s until homogenized, and then put on a rotating mixer at room temperature overnight (minimum of 12 h) to solubilize the steroid hormones from the fecal mass. Following rotation, samples were centrifuged for 15 min at 2000 rpm and the methanol supernatant was decanted into a clean glass scintillation vial for storage at −20 °C until hormone analysis.

A single-antibody progesterone enzymeimmunoassay (EIA) was used to quantify progestagens in serum and fecal extracts, as described by Munro and Stabenfeldt [47]. In brief, microtiter plates (Nunc, Thermo-Fisher Scientific, Victoria, Australia) were coated with 50 μl of antibody (1:8000 CL425; C. Munro, UC Davis, California, USA) per well. Plates were sealed tightly with an acetate sealer and incubated at 4 °C overnight. The following day, plates were washed five times with 250 μl of wash solution and drained to remove antiserum not bound to the plate. Dugong samples (undiluted serum or fecal extract diluted 1:10–1:400), standards (0.78–200 pg/50 μl; Sigma–Aldrich, New South Wales, Australia), and controls, all made up in standard assay buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.15 M NaCl, 0.1% albumin bovine serum, pH 7.0), were loaded as 50 μl volumes into designated wells on the plate. Then 50 μl of antigen conjugated to horseradish peroxidase (1:40,000; C. Munro, UC Davis, California, USA) was added to each well. Following incubation at room temperature for 2 h, the plates were washed and 100 μl of the color substrate solution (0.4 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), 1.6 mM H_2O_2, 0.05 M citric acid, pH 4.0) was added to each well. Plates were sealed, shaken for 5 min and left at room temperature until color developed and the optical density of the zero wells (assay buffer only) reached 1.0. The plates were read using a microplate reader (optical density 405 nm) with Revelation® software (Dynex MRX II, Q-Lab, Australia). All samples, controls, and standards were assayed in duplicate. Fecal progesterone metabolite concentrations were expressed as ng/g dry weight. The assay was biochemically validated for dugongs by demonstrating (1) parallelism between serially diluted extracts (1:1–1:512) and the standard curve with significant correlation ($R^2 = 0.99$, $P < 0.001$), and (2) significant (107%, $n = 4$) recovery of exogenous progesterone added to dry fecal material before extraction and analysis. Quality control in the assay was monitored by measuring progesterone concentration of high (30% binding) and low (70% binding) control samples on each plate ($n = 17$ assays). Inter-assay coefficients of variation were 10.5 ± 3.8% (high control) and 16.3 ± 0.4% (low control). Intra-assay coefficient of variation was 8.3 ± 0.1%. The progesterone monoclonal antibody CL425 used to quantify serum progesterone and fP had the following cross-relativities: 100% progesterone, 55% 5α-pregnan-3,20-dione, and <0.1% pregnanediol, androstenedione, and corticosterone (see [21]).

2.4. High performance liquid chromatography (HPLC) analysis

The number and relative proportions of progestagen metabolites in dugong fecal extracts were determined by reverse phase HPLC (Microsorb C-18 Column; Rainen Inc., Massachusetts, USA) using modifications of the method described by Monfort et al. [46]. Before HPLC, samples were passed through a C-18 matrix column (Spice Cartridge, Rainen, Inc.) and eluted with 5 ml of 80% methanol to remove contaminants (sample loss was <10%). For separation of metabolites, a pooled fecal extract was eluted with a gradient of 20:80 to 32:68 acetonitrile/water over 15 min, increasing to 50% over 50 min and then to 100% over 55 min. One-milliliter fractions were collected over a 120-min period (1 ml/min flow rate). A total of 5000 dpm of tritiated progesterone was added to the fecal extract before HPLC to determine the elution pattern of native steroid. HPLC fractions of fecal extract were taken to dryness, reconstituted in assay buffer and immunoreactivity quantified by EIA.

2.5. Statistical analysis

Data on sex, body morphometrics, and reproductive status through ultrasound detection of a fetus and/or calf association were integrated with hormone analysis results for each dugong. A total of 359 fecal samples were collected from free-ranging dugongs in Moreton Bay. This sample set comprised 322 samples from different individual dugongs of both sexes, with an additional 37 samples from 35 recaptured individuals identified through physical and/or genetic tags (J.M. Lanyon, unpubl. data). For those dugongs repeatedly sampled during the project, we used the data from the first

fecal sample collection only in statistical analyses (n = 322). All data were analyzed using SPSS® statistical software (version 19.0 for Macintosh, SPSS Inc.). We performed a linear regression to evaluate the relationship between matched serum and fecal progesterone concentrations. Hormone data were $\log_{10}$-transformed, and residuals for each model were analyzed to confirm that assumptions of normality and homoscedasticity were met. To examine the effect of reproductive status on hormone metabolite values, we compared fP values according to sex, reproductive size class (juvenile, subadult, and adult), and female reproductive status (pregnant and presumed non-pregnant) using one-way ANOVA; a *post hoc* Tukey HSD test was used to locate differences. To assess the relationship between fP concentration and various measurements of girth, body length, and body length to girth ratios, a linear correlation analysis was performed with the log-transformed concentrations. Body morphometrics were used to calculate the fineness ratio for each dugong as a simple measure of overall shape of a streamlined body [1,15]. Fineness ratio was calculated following Webb [60]: Fineness ratio = body length/maximum body diameter; where maximum body diameter = maximum girth/π. Fineness ratio is a dimensionless number, and body length and maximum girth were measured in cm. We performed two-tailed Pearson correlations to evaluate the relationship between fP concentrations and body morphometrics in males and females. One-way ANOVAs were used to determine whether body morphometrics varied between reproductive groups (juvenile female, juvenile male, adult male, confirmed pregnant, and presumed non-pregnant adult female); a *post hoc* Tukey HSD test was used to locate differences. All results were presented as mean ± standard error, as well as range of minimum and maximum values. Differences were considered significant if $P < 0.05$.

To examine hormonal and morphometric changes with pregnancy, we performed a Principal Components Analysis (PCA) on the correlation matrix of fP concentration ($\log_{10}$-transformed ng/g) and morphological indices of growth (body length in cm), body form (fineness ratio), and female reproductive trait (teat length in cm) for all dugongs. Prior to performing PCA, the suitability of the data for factor analysis was assessed. Inspection of the correlation matrix revealed the presence of many coefficients of 0.3 and above. The Kaiser–Meyer–Olkin value was 0.6 and Bartlett's Test of Sphericity reached statistical significance ($P < 0.001$), supporting the factorability of the matrix. The PCA generated a new set of standardized uncorrelated variables, with the number of factors to be retained guided by eigenvalues >1 and inspection of the scree plot.

Finally, we performed a Discriminant Function Analysis (DFA) to provide a convenient and efficient means of diagnosing pregnancy in dugongs. Using this approach, a linear classification function was calculated for each group (pregnant and non-pregnant), which allocated individual cases to groups based on a combination of variables. Collinearity was detected by calculation of a tolerance for each variable in the analysis. We developed combinations of multivariate models to determine those variables most useful (i.e., statistically reliable and practical for researchers) in predicting memberships of pregnant versus non-pregnant dugong groups. Inconsistencies in the classification of confirmed pregnant females were used to demonstrate how well each model performed, and a cross-validation technique (jackknife procedure) validated the results [44]. Analyzing all sampled female dugongs, the linear classification function based on fP concentration, body length, fineness ratio, and teat length was used to assign each individual to its appropriate pregnancy status.

3. Results

A total of 322 individual free-ranging dugongs were sampled in Moreton Bay (159 males and 163 females). Based on body length measurements and previously reported reproductive size classification methods by Marsh et al. [42], 53 dugongs were classified as juvenile (<220 cm body length) and likely to be immature; 182 as adult (⩾250 cm body length) and probably mature; and 87 as subadult (220–249 cm body length) of uncertain reproductive status. This sample included 66 dugongs that were sampled out-of-water and from which blood was collected: 42 adults, 15 subadults, and 9 juveniles.

Fecal samples of female dugongs (n = 163) were collected from 98 adults, 41 subadults, and 24 juveniles. Sampled adult females included 10 confirmed pregnant and 25 presumed non-pregnant, with 63 adult females of unknown pregnancy status. Five pregnant females were confirmed by detecting a fetus using ultrasonography (Fig. 1) and a further five through genetic-matching of the same individual sighted with a newborn attendant calf in the season following sample collection. Twenty-three adult females were presumed non-pregnant because no discernible fetus was detected using ultrasonography. A further two samples were classed as being from non-pregnant females based on recapture records; two adult females were sighted without an attendant calf at 15 months and 19 months after sampling, and were presumed to be non-pregnant when fecal sampling occurred during the mating season (September–November; [12]).

Progesterone EIA analysis of HPLC fractions of dugong fecal extracts identified 10 immunoreactive peaks, of which 10% was associated with the tritiated progesterone reference tracer (fractions 67–69). About 36% of progestagen immunoactivity was associated with five more polar, as yet unidentified, metabolite peaks (fractions 12–14, 23–27, 40–42, 46, 58, 62–63, and 65). The remaining immunoactivity (53%) was associated with three less polar peaks, all eluting within 15 fractions of progesterone metabolites.

Matched serum and fecal samples were collected from 66 dugongs, including five pregnant adult females, 23 non-pregnant adult females, five subadult females, four juvenile females, and 29 males. Close correspondence was found between serum progesterone and fP concentrations (R^2 = 0.83, $P < 0.001$; Fig. 2). Serum progesterone concentration was generally low in dugongs (0.15 ± 0.05 ng/ml) and often at undetectable levels in samples from presumed non-pregnant females (0.04 ± 0.01 ng/ml, range 0–0.23 ng/ml), subadult females (0.03 ± 0.03 ng/ml, range 0–0.13 ng/ml), juvenile females (0.09 ± 0.05 ng/ml, range 0–0.18 ng/ml), and males (0.03 ± 0.01 ng/ml, range 0–0.22 ng/ml). The highest

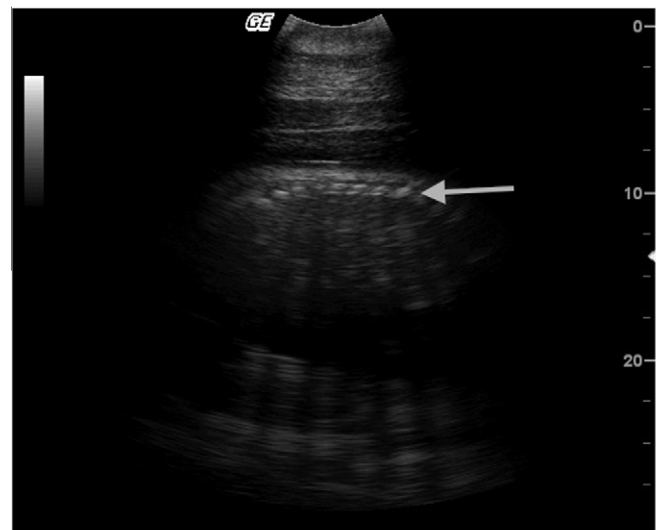

Fig. 1. Ultrasound still image showing a sagittal cross-section of a fetus in the uterine horn of a free-ranging dugong. An arrow marks the ribs of the fetus, with acoustic shadowing from the bone structure seen below. Scale on the right in cm.

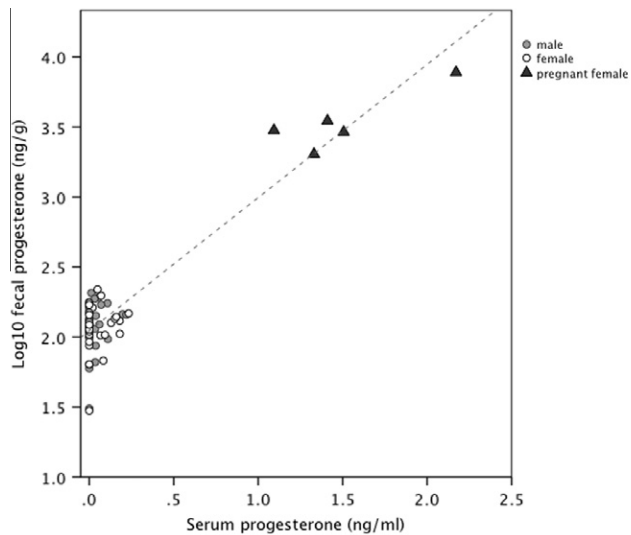

Fig. 2. Comparison of serum (ng/ml) and fecal progesterone metabolite concentrations (log_{10}-transformed ng/g) in individual live free-ranging dugongs (total $n = 66$), for biological validation. Males ($n = 29$) represented by closed grey circles, females ($n = 32$) by open white circles, and confirmed pregnant females ($n = 5$) by closed black triangles. Dotted line represents the linear regression equation: $y = 0.95x + 2.05$.

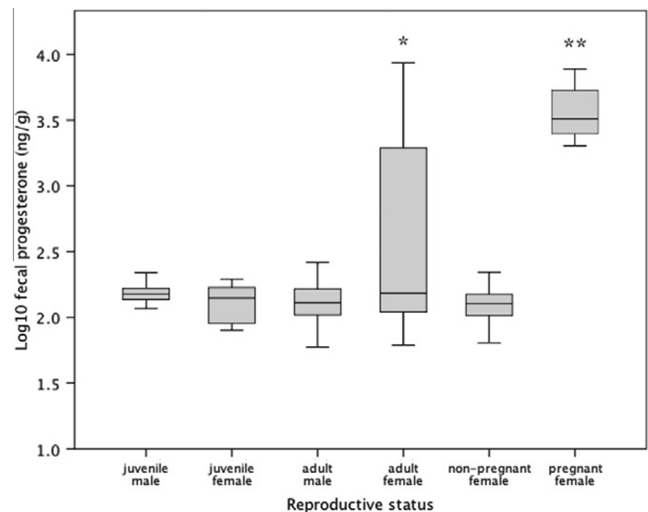

Fig. 3. Differences in fP concentrations (log_{10}-transformed ng/g) in dugongs, according to sex, reproductive size class (juvenile, <220 cm body length; and adult, ⩾250 cm), and pregnancy status (presumed non-pregnant, $n = 25$; and confirmed pregnant, $n = 10$). Adult female boxplot represents females of unknown pregnancy state ($n = 65$). For boxplots, the line inside the box indicates the median value, the height of the box encompasses the distance between the 25th and 75th quartiles, and the whiskers delineate extreme observations. Asterisks denote significantly different groups at $P < 0.05$.

serum progesterone concentrations of the 66 dugongs sampled for blood were recorded in five females (1.50 ± 0.18 ng/ml, range 1.09–2.17 ng/ml) whose pregnancies were all confirmed by ultrasound (Figs. 1 and 2). Serum progesterone concentrations of these pregnant females were on average 38 times higher than non-pregnant female dugongs ($t_{26} = -15.59$, $P < 0.001$).

Fecal progesterone metabolite concentrations according to sex, reproductive size class, and confirmed pregnant and presumed non-pregnant status are given in Table 1. Female dugongs (all females averaged) had significantly higher fP concentrations (817 ± 133 ng/g, range 29–8658 ng/g) compared to males (139 ± 4 ng/g, range 24–261 ng/g; $t_{320} = -5.32$, $P < 0.001$). Confirmed pregnant females were readily identifiable by fP higher than all other female (presumed non-pregnant adult, adult of unknown pregnancy status, subadult, and juvenile), and male groups (adult, subadult, and juvenile) ($F_{7,314} = 33.21$, $P < 0.001$; Fig. 3). Mean fP values were 30-fold greater in confirmed pregnant females (3947 ± 601 ng/g) than presumed non-pregnant females (129 ± 9 ng/g; Table 1). Importantly, there was no overlap in the range of fP between confirmed pregnant and presumed non-pregnant female reproductive states (Fig. 3), and a ninefold difference from the highest concentration recorded in non-pregnant females (221 ng/g) to the lowest measured in pregnant animals (2017 ng/g). Adult females of unknown reproductive status had intermediate fP concentrations ($P < 0.001$), as this group likely contained mature females in both pregnant and non-pregnant reproductive states. No significant differences in fP levels were detected between non-pregnant female, juvenile female, or male dugongs ($P > 0.05$; Fig. 3).

Reproductive status had a significant effect on fineness ratio which is a measure of body shape ($F_{4,165} = 34.27$, $P < 0.001$; Fig. 4). Pregnant females (fineness ratio 3.9) and all juvenile dugongs

Table 1
Summary of fP concentration (ng/g) and body morphometrics, i.e., body length (cm), axillar girth (cm), maximum girth (cm), anal girth (cm), peduncle girth (cm) and teat length (cm) of juvenile (<220 cm body length), subadult (220–249 cm) and adult dugongs (⩾250 cm) of each sex, as well as presumed non-pregnant and confirmed pregnant females. Mean values are displayed with standard error, minimum and maximum values in parentheses, and sample sizes (n).

Sex/reproductive status	fP (ng/g)	Body length (cm)	Axilla girth (cm)	Maximum girth (cm)	Anal girth (cm)	Peduncle girth (cm)	Teat length (cm)
Female							
Juvenile ($n = 24$)	132 ± 10 (29–195)	203 ± 2 (183–217)	132 ± 2 (109–151)	164 ± 3 (120–189)	94 ± 3 (68–117)	46 ± 1 (38–68)	0.7 ± 0.1 (0.5–2)
Subadult ($n = 41$)	150 ± 8 (33–257)	238 ± 1 (222–249)	152 ± 1 (136–171)	182 ± 1 (159–197)	114 ± 2 (91–143)	53 ± 0.5 (48–61)	1.3 ± 0.2 (0.5–5)
Adult ($n = 63$)	1288 ± 267 (61–8658)	275 ± 2 (253–312)	173 ± 2 (149–219)	210 ± 2 (182–241)	125 ± 1 (105–154)	60 ± 0.5 (51–69)	4.9 ± 0.3 (0.5–11)
Non-pregnant ($n = 25$)	129 ± 9 (30–221)	271 ± 3 (255–305)	173 ± 2 (159–210)	207 ± 2 (187–225)	125 ± 2 (105–160)	60 ± 1 (52–73)	3.4 ± 0.5 (0.5–8)
Pregnant ($n = 10$)	3947 ± 601 (2017–7760)	276 ± 4 (254–290)	176 ± 3 (163–190)	219 ± 4 (203–243)	133 ± 4 (120–155)	61 ± 1 (56–67)	6.5 ± 0.5 (5–9)
Male							
Juvenile ($n = 29$)	147 ± 8 (35–218)	199 ± 3 (147–220)	133 ± 2 (116–154)	164 ± 2 (136–185)	93 ± 3 (66–121)	46 ± 1 (35–59)	0.6 ± 0.0 (0.5–1)
Subadult ($n = 46$)	144 ± 7 (34–238)	237 ± 1 (221–249)	154 ± 2 (135–188)	182 ± 2 (160–204)	107 ± 2 (83–130)	53 ± 0.5 (45–62)	0.6 ± 0.1 (0.5–1.5)
Adult ($n = 84$)	134 ± 5 (24–261)	268 ± 1 (252–299)	168 ± 1 (141–188)	197 ± 1 (172–220)	121 ± 1 (83–168)	61 ± 0.5 (53–74)	0.9 ± 0.1 (0.5–2)

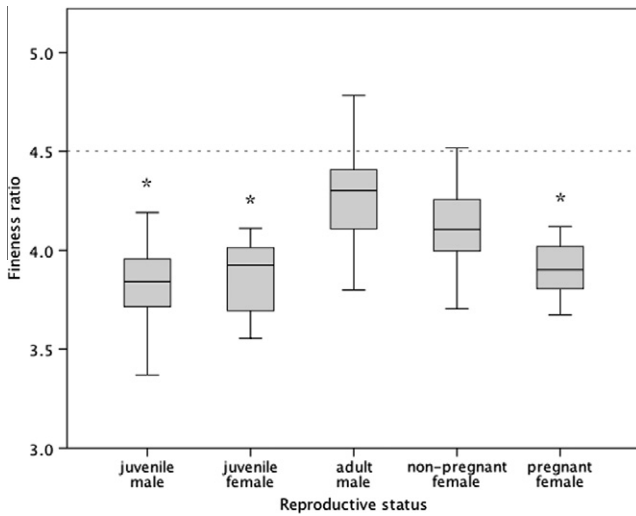

Fig. 4. Differences in fineness ratio (body length / maximum body diameter) in dugongs, according to sex, reproductive size class (juvenile, <220 cm body length; and adult, ⩾250 cm), and pregnancy status (presumed non-pregnant, $n = 25$; and confirmed pregnant, $n = 10$). Optimum fineness ratio for streamlined body form in marine animals is achieved at close to 4.5, represented by horizontal dotted line [60]. For boxplots, the line inside the box indicates the median value, the height of the box encompasses the distance between the 25th and 75th quartiles, and the whiskers delineate extreme observations. Asterisks denote significantly different groups at $P < 0.05$.

(3.8) had a more rotund body form, and therefore, significantly lower fineness ratios than the more slender and presumed non-pregnant females (4.1) and adult males (4.3). Body length was positively and significantly correlated with fP in females ($r = 0.36$, $P < 0.001$), but this was not the case for males ($r = -0.14$, $P = 0.09$). Body length and maximum girth were strongly correlated in all dugongs regardless of sex ($r = 0.84$, $P < 0.001$). Hence, to investigate possible changes in girth morphometrics with pregnancy, we restricted our analysis to females of the adult size class (⩾250 cm). In adult females ($n = 98$), fP concentrations increased with each of axillar girth ($r = 0.27$, $P = 0.01$), maximum girth ($r = 0.41$, $P < 0.001$; Fig. 5), and anal girth ($r = 0.21$, $P = 0.04$), but there was no significant relationship with peduncle girth ($r = 0.21$, $P = 0.05$). The relationship between fP concentration and the ratio of body length: maximum girth was significant and negative ($r = -0.29$, $P = 0.004$), indicating that adult females with higher fP concentrations were likely to have proportionally larger girth in the umbilical region compared to their body length. Pregnant females had maximum girth measures on average 12 cm larger (219 ± 4 cm) and anal girth measures 8 cm larger (133 ± 4 cm) than presumed non-pregnant individuals (207 ± 2 cm and 125 ± 2 cm, respectively; $t_{33} = -3.26$, $P = 0.003$ and $t_{33} = -2.00$, $P = 0.05$, respectively; Table 1), though there were no differences in axillar and peduncle girth measures ($t_{33} = -0.59$, $P = 0.56$ and $t_{33} = -0.95$, $P = 0.35$, respectively; Table 1). Females had significantly larger teat lengths (3.2 ± 0.2 cm) than male dugongs (0.8 ± 0.1 cm; $t_{208} = -7.08$, $P < 0.001$). Teat length was generally 3 cm longer in confirmed pregnant (6.5 ± 0.5 cm) than presumed non-pregnant dugongs (3.4 ± 0.5 cm; $t_{33} = -4.99$, $P < 0.001$; Table 1, Fig. 6). Longer teats were significantly correlated with higher fP concentrations in female dugongs ($r = 0.47$, $P < 0.001$).

PCA matrix of component loadings showed the correlation between the original measurements and two principal components (eigenvalues >1), with PC 1 and PC 2 explaining 81% of the variation (Table 2). Confirmed pregnant females were significantly different to presumed non-pregnant and juvenile females (Fig. 7), and to adult and juvenile males based on both PC 1 and PC 2 (PC 1: $F_{4,165} = 158.50$, $P < 0.001$; PC 2: $F_{4,165} = 89.09$, $P < 0.001$). Presumed non-pregnant females were similar to adult males (both PC 1 and PC 2, $P > 0.05$), but were significantly different to juvenile females and males ($P < 0.001$).

For pregnant and non-pregnant dugongs, the overall DFA performed on four variables ($\log_{10}$ fP concentration, body length, fineness ratio, and teat length) showed a significant discrimination between female groups (Wilk's $\lambda = 0.06$, $\chi^2 = 85.31$, $P < 0.001$), and contributed to 100% of the total variance (eigenvalue = 14.67, canonical correlation = 0.97). The classification matrix revealed that reproductive status was correctly assigned to all individuals of known pregnancy state with a classification rate of 100%. Fecal progesterone metabolite concentration was identified as the

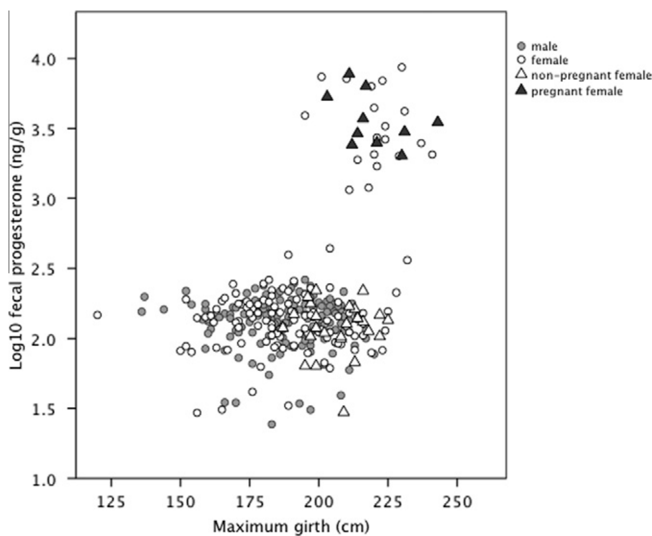

Fig. 5. Comparison of fecal progesterone metabolite concentrations ($\log_{10}$-transformed ng/g) as a function of maximum girth (cm) in pregnant and non-pregnant female, and male dugongs. Males ($n = 159$) represented by closed grey circles, females of unknown pregnancy status ($n = 128$) by open white circles, presumed non-pregnant females ($n = 25$) by open white triangles, and confirmed pregnant females ($n = 10$) by closed black triangles.

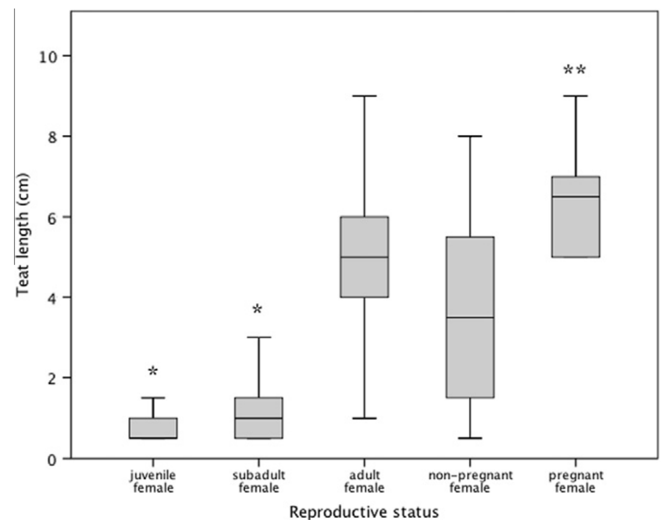

Fig. 6. Differences in teat length (cm) in female dugongs, according to reproductive size class (juvenile, <220 cm body length; subadult, 220–249 cm; and adult, ⩾250 cm) and pregnancy status (presumed non-pregnant, $n = 25$; and confirmed pregnant, $n = 10$). For boxplots, the line inside the box indicates the median value, the height of the box encompasses the distance between the 25th and 75th quartiles, and the whiskers delineate extreme observations. Asterisks denote significantly different groups at $P < 0.05$.

Table 2
Principal Component Analysis matrix showing the factor loadings of each measured variable ($\log_{10}$ fecal progesterone metabolite concentration (ng/g), body length (cm), fineness ratio, and teat length (cm)) and in which direction they contribute towards PC 1 and PC 2 in dugongs.

Principal component	PC 1	PC 2
% of variance	48.7	32.6
Log_{10} fecal progesterone (ng/g)	0.60	−0.65
Body length (cm)	0.85	0.33
Fineness ratio	0.36	0.85
Teat length (cm)	0.86	−0.24

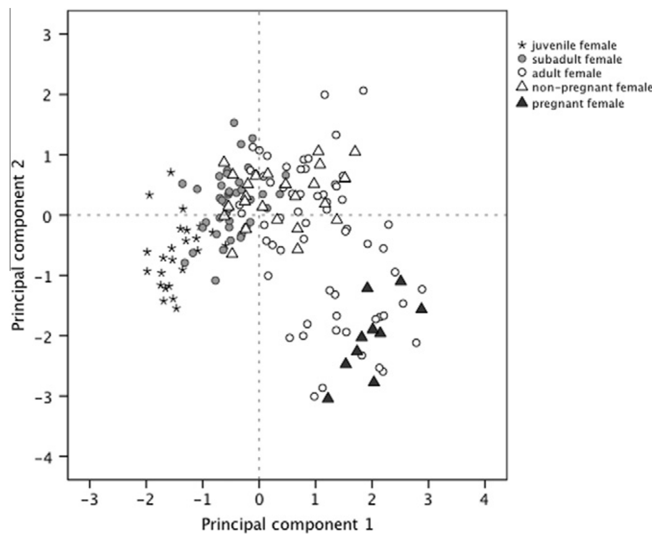

Fig. 7. Component matrix of the Principal Component Analysis, showing the factor scores of each female dugong by reproductive size class (juvenile, <220 cm body length; subadult, 220–249 cm; and adult ⩾250 cm) and pregnancy status (presumed non-pregnant and confirmed pregnant). Juvenile females (n = 24) represented by stars, subadult females (n = 41) by closed grey circles, adult females of uncertain reproductive status (n = 63) by open white circles, presumed non-pregnant females (n = 25) by open white triangles, and confirmed pregnant females (n = 10) by closed black triangles.

variable with the greatest contribution to the discrimination between groups with the highest coefficient (0.97) and loading (0.94) for this function. Because the function relied strongly on fP concentrations, we performed another DFA containing only the variables of body length, fineness ratio, and teat length to determine the accuracy of classification based on female body morphometrics alone. Using this model, a significant discrimination was still reported between pregnant and non-pregnant dugongs (Wilk's λ = 0.49, χ^2 = 22.52, P < 0.001), and explained 100% of the variance (eigenvalue = 1.04, canonical correlation = 0.72). However, the DFA model without fP values did affect classification accuracy, with the jackknife validation procedure showing 80% of pregnant females (8 out of 10 confirmed pregnant females) and 80% of non-pregnant females (20 out of 25 presumed non-pregnant females) correctly classified. Classifying all female dugongs of unknown pregnancy status in our study (n = 128) using the DFA model with all four variables (i.e., fP, body length, fineness ratio, and teat length), we predicted 20 additional individuals as *pregnant* (these were all adult females) and 108 females as *non-pregnant* (comprising 43 adults, 41 subadults, and 24 juveniles).

A total of 30 dugongs were identified as pregnant from the sampled population in Moreton Bay (31% of all adult females (n = 98) sampled; Fig. 8) based on the discriminant function model. For all pregnant dugongs identified using DFA, the range of fP concentrations was 1150–8658 ng/g (mean 3809 ± 393 ng/g, n = 30), and 29 to 439 ng/g for identified non-pregnant females (141 ± 5 ng/g, n = 133). The smallest female diagnosed as pregnant was 253 cm body length and two females were 254 cm body length, although most (90% of pregnant females, n = 27) of the identified pregnant females were ⩾260 cm (Fig. 8). Pregnant dugongs were characterized by the following body morphometrics: axillar girth ⩾172 cm, maximum girth ⩾215 cm, anal girth ⩾126 cm, and teat length ⩾5 cm, based on lower 95% confidence intervals.

Pregnant females were indentified in all seasons: summer (n = 5), fall (n = 2), winter (n = 6), and spring (n = 17). There was no seasonal effect on fP concentrations in pregnant females ($F_{3,26}$ = 0.12, P = 0.95) with similar mean fP levels measured in summer (3505 ± 310 ng/g, range 2420–4197 ng/g), fall (2949 ± 42 ng/g, range 2907–2991 ng/g), winter (4135 ± 959 ng/g, range 2017–7760 ng/g), and spring (3885 ± 614 ng/g, range 1150–8658 ng/g). Spring had the highest variation in fP concentrations between pregnant females with a range of 7509 ng/g, compared to ranges of fP in summer (1777 ng/g), fall (84 ng/g), and winter (5743 ng/g). Season had no significant effect on body girth measures (P > 0.1). For example, maximum girths of pregnant females were similar in summer (231 ± 6 cm, range 195–231 cm), fall (223 ± 9 cm, range 214–231 cm), winter (219 ± 5 cm, range 211–243 cm), and spring (219 ± 2 cm, range 201–241 cm). Similarly, fineness ratio of pregnant females were not statistically significantly different across seasons ($F_{3,26}$ = 2.08, P = 0.13), although spring showed the highest variation in body morphometrics of pregnant females (e.g., maximum girth range = 40 cm, fineness ratio range = 0.8), compared to other seasons (summer = 36 cm, 0.3; fall = 17 cm, 0.2; winter = 32 cm, 0.4).

4. Discussion

In this study, we applied progestagen analysis to demonstrate that the quantification of progesterone metabolite concentrations in fecal samples (fP) may be used successfully to diagnose the pregnancy status of free-ranging dugongs, a vulnerable species. Previously, the determination of pregnancy rate, an important parameter in studies of population demography, has been made from postmortem examination of dugong carcasses from wild populations [43]. The data presented here are the first to quantify differences in hormone levels between pregnant and non-pregnant dugongs, and provide a reliable means of diagnosing pregnancy in a live sirenian species. All of the female dugongs examined in this study could be assigned to their appropriate reproductive status based on increased fP concentrations and also on physical changes (body morphometrics and teat length) accompanying gestation, suggesting that our measures represent an accurate indication of pregnancy in live wild dugongs.

Pregnant dugongs were distinguishable from other non-pregnant females by significantly higher fP concentrations (mean 30-fold increase). The wide variation in fP values among adult female dugongs probably represents a range of mature reproductive states, including females in both follicular and luteal phases of the estrous cycle, as well as individuals at different stages of pregnancy. In male dugongs, the range of fP concentrations was similar to those of non-pregnant and juvenile females, and we expect other hormones, such as androgens [12], to show more significant sex differences in dugongs. Dugong fP concentrations showed no overlap between pregnant females (>1000 ng/g) and individuals in all other sex and reproductive size classes, including non-pregnant adult females (<500 ng/g) sampled within and outside the breeding season, providing a strong discrimination of pregnancy status. The significant elevation in fP concentrations in pregnant dugongs is consistent with hormonal changes accompanying pregnancy in other marine mammals, including captive bottlenose dolphins (mean 16-fold)

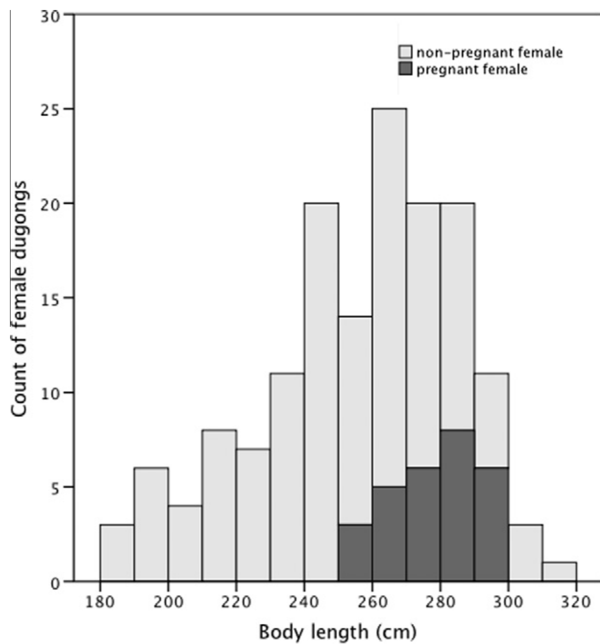

Fig. 8. Size distribution (in 10 cm increments of body length) of identified pregnant (black bars; $n = 30$) and non-pregnant (grey bars; $n = 133$) female dugongs, as predicted by Discriminant Function Analysis using fecal progesterone metabolite concentration, body length, fineness ratio, and teat length.

[5] and free-ranging right whales (mean > 600-fold) [53]. Furthermore, serum progesterone concentrations in pregnant dugongs were much higher (mean 38-fold) than those found in non-pregnant adult females, as well as other sex and reproductive size classes, which suggests that the chosen assay measures biologically relevant changes in hormone expression.

Circulating concentrations of progesterone in dugongs were generally low in this study, and this is indicative of low progesterone production, similar to their phylogenetic relatives the Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephant [11,24], rock hyrax (*Procavia capensis*) [27], and West Indian manatee (*Trichechus manatus*) [56]. Pregnant dugongs had serum progesterone concentrations >0.3 ng/ml (maximum level in non-pregnant dugongs), which is similar to the threshold level of 0.4 ng/ml serum progesterone for detecting pregnancy in manatees (mean 1.2 ± 1.3 ng/ml) using the methodology reported by Tripp et al. [56]. However, in contrast to our fecal hormone study, the application of fP concentrations to diagnose pregnancy in the Florida manatee (*Trichechus manatus latirostris*) found minimal variation in fP levels between pregnant and non-pregnant individuals using a different analysis technique, and fecal hormone results were deemed physiologically non-diagnostic [36]. It is probable that the progesterone antibody used in the manatee fecal study [36] was too specific to the primary steroid progesterone and not able to detect many of the progesterone metabolites in feces [55]. Progesterone is extensively metabolized in the mammalian gut and excreted in the feces as numerous structurally-similar steroid molecules (metabolites) with varying antibody affinities [38,55,59]. HPLC analysis in this study confirmed that some progesterone was present in dugong fecal extracts, although the majority of screened fractions were unidentified metabolites. Therefore, the use of a broad-spectrum antibody (CL425) in this study permitted the quantification of a significant elevation in fP metabolite concentrations associated with pregnancy in dugongs. This antibody has also proved efficacious in pregnancy studies of right whales [53] and other mammalian herbivores [21,55]. As highlighted by Garrott et al. [18], the critical factor in any immunoassay is the specificity and sensitivity of the antibody employed to the contents of the matrix analyzed. The utility of our method was further demonstrated by the strong correlation between progesterone concentrations in serum and fecal samples collected concurrently. This association is physiologically significant as it suggests that circulating concentrations in the blood of dugongs are reflected in the feces for this species (though dependent on intestinal transit time; 6–7 days in adult dugongs [30]), and that fecal hormone analysis may be reliably used in pregnancy determination. Measurement of fP using a broad specificity antibody, as used here, has the potential to detect pregnancy in other sirenian species from which fecal samples can be obtained, e.g., Florida manatees [36] and Amazonian manatees [48], with useful applications for captive management (reviewed by [38]).

Pregnant female dugongs had significantly different body morphometrics than non-pregnant adult females and males, with the development of a fetus presumably accounting for this change in shape. Similarly, length and maximum width measures taken from aerial photographs of migrating gray whales (*Eschrichtius robustus*) showed that near-term pregnant females were proportionally wider than other adult and juvenile whales [51]. Confirmed pregnant females had girth measures that were larger by 12 cm and 8 cm around the maximum (umbilicus) and anal positions, respectively. The lack of difference in axillar and peduncle girths between pregnant and non-pregnant females was not unexpected due to the nature of the anatomy of these areas. The axillar measurement encompasses the girth intersecting the axillae at the xiphoid process where the ribs are attached to the sternum; hence body expansions with fetal development may be expected to occur caudally from this position where the ribs are floating. The peduncle anatomy comprises caudal vertebrae and attached locomotory musculature, and body changes with pregnancy would be less likely to affect the peduncle girth. With pregnancy, bulging in the lumbar region from the urogenital opening towards the axillar position would be expected as the fetus develops and as parturition approaches, which was reflected in the greater girth measures at maximum and anal positions of pregnant dugongs.

These morphometric changes with pregnancy were also evident in a lower fineness ratio for pregnant females (mean 3.9) compared to non-pregnant females (4.1) and adult males (4.3). The optimum fineness ratio that results in minimum drag with maximum accommodation for volume is 4.5 [60], with killer whales (*Orcinus orca*) considered to have a streamlined body close to the optimal hydrodynamic design for efficient locomotion [15]. A streamlined form of optimal fineness ratio is associated with a minimum drag coefficient [6,14], and is considered a common morphological adaptation of active aquatic animals to reduce energy cost of swimming [1]. Most marine mammals have body shapes with fineness ratios between 3.3 and 8.0 [15]. A lower fineness ratio indicates a more rotund body form with increased resistance, such as pregnant dugongs in this study, which had deeper bodies relative to their length presumably due to gestational changes in the uterus and carriage of a large fetus (full-term size 100–130 cm, 20–30 kg; [42]). Adult males and non-pregnant adult female dugongs were significantly more slender than pregnant animals, and showed fineness ratios closer to optimum for minimum drag. Furthermore, Fish [15] suggested that a fineness ratio near the optimal value might also aid in thermoregulation by limiting surface area and heat loss, which are important in thermally sensitive sirenians, and this function may therefore be compromised during pregnancy.

The increased body volume found in pregnant females may also alter locomotor abilities and maneuverability, such that pregnant dugongs generally have slower swim speeds during pursuit compared to other females (F. Mingramm, pers. comm., October 2011). Further, indigenous dugong hunters report that pregnant animals have a unique diving behavior, which is easily

distinguished from other dugongs ([28], C. Repu, pers. comm., April 2011). Changes in body form and fineness ratio associated with pregnancy, as confirmed in this study, may increase flexural stiffness, and hence limit axial bending during acceleration and surface breathing, thereby altering diving behaviour. As an open-water species, the morphological changes in a female dugong's body form with deviations away from optimal fineness ratio during pregnancy may represent an energetic cost of reproduction [29]. Because swimming is an integral behavior to dugongs, an increase in non-muscle mass associated with pregnancy may reduce performance at critical tasks. As herbivores, dugongs do not require speed and rapid acceleration to catch prey. However, sprints of over 6 m/s reported for dugongs would aid in escape from threats [49], and pregnant dugongs may experience increased resistance to acceleration during fast-start escapes, which may potentially increase their risk to predators and human activities.

A conspicuous trait of pregnant dugongs was elongated axillary teats (>5 cm long), which correlated strongly with high fP concentrations. In mammals, hormones including progesterone help regulate mammary growth and lactation, and the onset of pregnancy stimulates the mammary gland to become distended with fluid [57] so that the teat becomes visibly enlarged. In postmortem examinations of female dugongs, pregnant individuals had histological features characteristic of both proliferating mammary glands and active lactation during gestation [28]. The present study also found a clear sexual difference in teat length of dugongs, with all males having rudimentary bumps less than 1 cm long. Further, pregnant dugongs had teat lengths on average 3 cm longer than non-pregnant females and 6 cm longer than juvenile females. Such small teats in non-breeding females probably reflect inactive mammary glands as shown in immature, ovulating, and resting female sirenians whose reproductive states were confirmed by gonadal examination [39,41]. Mammary glands are ancillary to female reproduction, and teat length is sexually dimorphic in dugongs and confirmed as a good indicator of reproductive status in females.

Using our technique to diagnose pregnancy across a subset of a live population provides a proxy method to determine sexual maturation in female dugongs, which is important for population modeling and management. The rationale of defining sexual maturation based on pregnancy does not account for the possibility of unobserved pregnancy loss and the inability to detect ovulations not resulting in pregnancy. However, in a population study, the distinction between female sexual maturity and first conception is probably of limited relevance [9], especially when sterile estrous cycles are common in dugongs, and females may undergo a number of estrous cycles before conceiving [41]. In the present study, the smallest female identified as pregnant was 253 cm body length, although most pregnant females (90% of 30 pregnant females) were larger than 260 cm. From postmortem studies in other dugong populations, the smallest female with placental scarring (evidence of parity) sampled from Townsville in northern Queensland was 234 cm long [42], which is a size similar to the smallest pregnant female at 229 cm long necropsied in Numbulwar, Northern Territory [4]. In both of these northern tropical populations, parous dugongs were generally larger than 240 cm body lengths; 86% of 21 parous females necropsied in Townsville [42] and 82% of 28 pregnant females necropsied in Numbulwar [4]. The smallest pregnant dugong on record is a female at 205 cm body length hunted in the waters around Mabuiag Island, Torres Strait [28], also in northern Australia. If body size at calving is used to define female sexual maturation [50], female dugongs in Moreton Bay become parous at larger body lengths (>250 cm) than other studied populations in northern Australia [4,28,42]. Moreton Bay supports the southern-most dugong population on the east Australian coast, and it is likely that this subtropical population has a more pronounced seasonality compared to tropical regions in northern Australia. Our study adds to the evidence that reproductive maturity in female dugongs varies among populations, with Moreton Bay at the southern limit of dugong distribution exhibiting the most protracted maturation yet recorded, based on body size. Reproduction in this species is thought to be resource-dependent with spatial as well as temporal variability [43]. Dugongs forage almost exclusively on seagrasses, and animals may be nutritionally limited by the seasonality of seagrass abundance and productivity in subtropical Moreton Bay [52] when compared to seagrass growth in tropical areas [31]. Such dynamics serve to highlight the need to have regional and current information on female population structure for dugong management.

In Moreton Bay, pregnant dugongs were present throughout the year, similar to populations in northern Australia [4,28,42], which was expected given a gestation period of 14 months [28,43]. Considering the predominance of neonatal calves observed during the spring and summer months in Moreton Bay (J.M. Lanyon, unpubl. data) and 14 month gestation [28,43], we predict that most conceptions occur during late winter and spring in this population. Among pregnant females in this study, temporal differences in fP concentration across seasons were not significant. This could be the result of low statistical power (due to small sample sizes, especially over fall) and should be clarified with additional samples collected from pregnant females. However, fP concentrations of pregnant females showed the greatest variability during spring, along with body girth measures and fineness ratios. If most implantations and births occur in spring as predicted, then we would expect the spring season to show a greater range of pregnancy states from female dugongs at early conception to those approaching parturition, which may account for the larger variation in fP observed in these pregnant females.

In this study, the progesterone assay did not detect a significant difference between non-pregnant adult and juvenile females, and the same has been found in other studies investigating hormone metabolites in single samples from wild marine mammal populations [26,53]. We recognize that this outcome may potentially be a result of greater sampling of non-pregnant females during a follicular phase or outside the breeding season, which should be clarified with additional samples. Terrestrial field endocrinologists generally use at least two consecutive fecal samples to ensure accurate discrimination between pregnant and diestrus females in wildlife populations e.g., [16,54,61]. However, this is not usually feasible when studying large populations of free-ranging marine mammals. Although there are potential sources of error inherent in restricting our sampling to one fecal sample, Garrott et al. [18] argue that with an efficient steroid extraction protocol and appropriate antibody specificity, the ability to discriminate between pregnant and non-pregnant animals from a single fecal sample can be greatly improved. Our method reliably diagnosed pregnancy with the addition of body morphometrics in this study, particularly measures of body growth (body length), body form (fineness ratio), and a female reproductive trait (teat length). Based on female morphometrics alone, we were able to predict pregnancy with reasonable accuracy (80% correct classification). The inclusion of body measures together with fP concentrations increased the accuracy of discrimination between pregnant and non-pregnant females to 100%. Such an approach may compensate for the inability to conduct consecutive sampling of hormone concentrations in the same individuals in a free-ranging population. Given the importance of determining pregnancy rates for population management, we believe that our approach using single samples for pregnancy diagnosis will be useful for field researchers.

This paper presents a valuable contribution towards a large-scale assessment of pregnancy in a live population of dugongs. Fecal samples from significant numbers of free-ranging dugongs can be

successfully collected in tandem with mark-recapture population studies. This study demonstrates that enzymeimmunoassay techniques originally developed for terrestrial mammals are also effective in marine mammals and can be applied at a population level. We illustrate the value of fecal steroid hormone analysis in a single sample combined with morphometrics (if possible) as a useful tool to determine pregnancy. The robustness of this approach is verified by statistically significant differences in the concentrations of fP correlated with biological parameters including sex and body morphometric changes. Pregnant dugongs in Moreton Bay were characterized as having fecal progesterone metabolite concentrations >1000 ng/g, generally longer than 260 cm in body length, with an expanded girth greater than 215 cm at maximum (umbilicus) and greater than 126 cm at anal position, and possessing prominent teats distended to a length of greater than 5 cm. The methodology described here will provide biologists with an effective means to non-lethally assess pregnancy status of female dugongs, as well as the frequency of pregnancy and thus potential recruitment to a dugong population. Because demographic models depend upon actual rather than theorized estimates of reproductive potential, our technique may ultimately provide more reliable information for the assessment of population dynamics and management of dugongs, and potentially other vulnerable sirenian species.

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References

[1] B.K. Ahlborn, R.W. Blake, K.H.S. Chan, Optimal fineness ratio for minimum drag in large whales, Can. J. Zool. 87 (2009) 124–131.
[2] R.S. Amaral, Use of alternative matrices to monitor steroid hormones in aquatic mammals: a review, Aquat. Mamm. 36 (2010) 162–171.
[3] C.A. Bedford, J.R.G. Challis, F.A. Harrison, R.B. Heap, The role of oestrogens and progesterone in the onset of parturition in various species, J. Reprod. Fertil. (Suppl. 16) (1972) 1–23.
[4] G.C.L. Bertram, C.K.R. Bertram, The modern Sirenia: their distribution and status, Biol. J. Linn. Soc. 5 (1973) 297–338.
[5] B. Biancani, L. Da Dalt, G. Lacave, S. Romagnoli, G. Gabai, Measuring fecal progestogens as a tool to monitor reproductive activity in captive female bottlenose dolphins (*Tursiops truncatus*), Theriogenology 72 (2009) 1282–1292.
[6] R.W. Blake, Functional design and burst-and-coast swimming in fishes, Can. J. Zool. 61 (1983) 2491–2494.
[7] R.K. Bonde, A.A. Aguirre, J. Powell, Manatees as sentinels of marine ecosystem health: are they the 2000-pound canaries?, Ecohealth 1 (2004) 255–262
[8] G.D. Bossart, Marine mammals as sentinel species for oceans and human health, Oceanography 19 (2006) 134–137.
[9] I. Boyd, C. Lockyer, H. Marsh, Reproduction in marine mammals, in: J.E. Reynolds III, S.A. Rommel (Eds.), Biology of Marine Mammals, Melbourne University Press, Melbourne, Australia, 1999, pp. 218–286.
[10] D. Broderick, J. Ovenden, R. Slade, J.M. Lanyon, Characterization of 26 new microsatellite loci in the dugong (*Dugong dugon*), Mol. Ecol. Notes 7 (2007) 1275–1277.
[11] J.L. Brown, Reproductive endocrine monitoring of elephants: an essential tool for assisting captive management, Zoo Biol. 19 (2000) 347–367.
[12] E.A. Burgess, J.M. Lanyon, T. Keeley, Testosterone and tusks: maturation and seasonal reproductive patterns of live free-ranging dugongs (*Dugong dugon*) in a subtropical population, Reproduction, in press.
[13] G. Caughley, Analysis of Vertebrate Populations, The Blackburn Press, New York, USA, 2005.
[14] S.D. Feldkamp, Swimming in the California sea lion: morphometrics, drag and energetics, J. Exp. Biol. 131 (1987) 117–135.
[15] F.E. Fish, Influence of hydrodynamic-design and propulsive mode on mammalian swimming energetics, Aust. J. Zool. 42 (1993) 79–101.
[16] C.A.H. Foley, S. Papageorge, S.K. Wasser, Noninvasive stress and reproductive measures of social and ecological pressures in free-ranging African elephants, Conserv. Biol. 15 (2001) 1134–1142.
[17] K.J. Gardiner, I.L. Boyd, P.A. Racey, P.J.H. Reijnders, P.M. Thompson, Plasma progesterone concentrations measured using an enzyme-linked immunosorbent assay useful for diagnosing pregnancy in harbor seals (*Phoca vitulina*), Mar. Mamm. Sci. 12 (1996) 265–273.
[18] R.A. Garrott, S.L. Monfort, P. White, K.L. Mashburn, J.G. Cook, One-sample pregnancy diagnosis in elk using fecal steroid metabolites, J. Wildl. Dis. 34 (1998) 126–131.
[19] R.T. Gemmell, A comparative study of the corpus luteum, Reprod. Fertil. Develop. 7 (1995) 303–312.
[20] W. Goymann, Noninvasive monitoring of hormones in bird droppings physiological validation sampling extraction sex differences and the influence of diet on hormone metabolite levels, Ann. N.Y. Acad. Sci. 1046 (2005) 35–53.
[21] L. Graham, F. Schwarzenberger, E. Möstl, W. Galama, A. Savage, A versatile enzyme immunoassay for the determination of progestogens in feces and serum, Zoo Biol. 20 (2001) 227–236.
[22] D.F. Gudermuth, P.W. Concannon, P.F. Daels, B.L. Lasley, Pregnancy-specific elevations in fecal concentrations of estradiol, testosterone and progesterone in the domestic dog (*Canis familiaris*), Theriogenology 50 (1998) 237–248.
[23] R.G. Harcourt, E. Turner, A. Hall, J.R. Waas, M. Hindell, Effects of capture stress on free-ranging, reproductively active male Weddell seals, J. Comp. Physiol. A 196 (2010) 147–154.
[24] J.K. Hodges, Endocrinology of the ovarian cycle and pregnancy in the Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephant, Anim. Reprod. Sci. 53 (1998) 3–18.
[25] IUCN, IUCN Red List of Threatened Species, Version 2011.1, 2011. <www.iucnredlist.org>, Accessed 13 July 2011.
[26] N.M. Kellar, M.L. Trego, C.I. Marks, A.E. Dizon, Determining pregnancy from blubber in three species of delphinids, Mar. Mamm. Sci. 22 (2006) 1–16.
[27] S. Kirkman, E.D. Wallace, R.J. van Aarde, H.C. Potgieter, Steroidogenic correlates of pregnancy in the rock hyrax (*Procavia capensis*), Life Sci. 68 (2001) 2061–2072.
[28] D. Kwan, Towards a sustainable indigenous fishery for dugongs in Torres Strait: a contribution of empirical data analysis and process, Unpublished Ph.D. thesis, James Cook University, Townsville, Queensland, Australia, 2002, p. 282.
[29] R.B. Langerhans, D.N. Reznick, Ecology and evolution of swimming performance in fishes: predicting evolution with biomechanics, in: P. Domenici, B.G. Kapoor (Eds.), Fish Locomotion: an Etho-ecological Perspective, Science Publishers, USA, 2009, pp. 200–248.
[30] J. Lanyon, H. Marsh, Digesta passage times in the dugong, Aust. J. Zool. 43 (1995) 119–127.
[31] J.M. Lanyon, H. Marsh, Temporal changes in the abundance of some tropical intertidal seagrasses in North Queensland, Aquat. Bot. 49 (1995) 217–237.
[32] J.M. Lanyon, R.W. Slade, H.L. Sneath, D. Broderick, J.M. Kirkwood, D. Limpus, et al., A method for capturing dugongs (*Dugong dugon*) in open water, Aquatic Mammals 32 (2006) 196–201.
[33] J.M. Lanyon, H.L. Sneath, J.M. Kirkwood, R.W. Slade, Establishing a mark-recapture program for dugongs in Moreton Bay, south-east Queensland, Aust. Mammal. 24 (2002) 51–56.
[34] J.M. Lanyon, H.L. Sneath, T. Long, Three skin sampling methods for molecular characterisation of free-ranging dugong (*Dugong dugon*) populations, Aquat. Mamm. 36 (2010) 298–306.
[35] J.M. Lanyon, H.L. Sneath, T. Long, R.K. Bonde, Physiological response of wild dugongs (*Dugong dugon*) to out-of-water sampling for health assessment, Aquat. Mamm. 36 (2010) 298–306.
[36] I.L.V. Larkin, Reproductive endocrinology of the Florida manatee (*Trichechus manatus latirostris*): estrous cycles, seasonal patterns and behavior, Unpublished Ph.D. thesis, University of Florida, Gainesville, Florida, USA, 2000, p. 339.

[37] S. Larson, C.J. Casson, S. Wasser, Noninvasive reproductive steroid hormone estimates from fecal samples of captive female sea otters (*Enhydra lutris*), Gen. Comp. Endocrinol. 134 (2003) 18–25.
[38] B.L. Lasley, J.F. Kirkpatrick, Monitoring ovarian function in captive and free-ranging wildlife by means of urinary and fecal steroids, J. Zoo Wildl. Med. (1991) 23–31.
[39] M. Marmontel, Age and reproduction in female Florida manatees, in: T.J. O'Shea, B.B. Ackerman, H.F. Percival (Eds.), Population Biology of the Florida Manatee, US Department of the Interior, National Biological Service, Information and Technology Report 1, 1995, pp. 98–119.
[40] H. Marsh, B.R. Gardner, G.E. Heinsohn, Present-day hunting and distribution of dugongs in the Wellesley Islands (Queensland): implications for conservation, Biol. Conserv. 19 (1981) 255–267.
[41] H. Marsh, G.E. Heinsohn, P.W. Channells, Changes in the ovaries and uterus of the dugong, *Dugong dugon* (Sirenia: Dugongidae), with age and reproductive activity, Aust. J. Zool. 32 (1984) 743–766.
[42] H. Marsh, G.E. Heinsohn, L.M. Marsh, Breeding cycle, life history and population dynamics of the dugong, *Dugon dugon* (Sirenia: Dugongidae), Aust. J. Zool. 32 (1984) 767–788.
[43] H. Marsh, D. Kwan, Temporal variability in the life history and reproductive biology of female dugongs in Torres Strait: the likely role of sea grass dieback, Cont. Shelf Res. 28 (2008) 2152–2159.
[44] K. McGarigal, S. Cushman, S. Stafford, Multivariate Statistics for Wildlife and Ecology Research, Springer, New York, USA, 2000.
[45] M. McHale, D. Broderick, J.R. Ovenden, J.M. Lanyon, A PCR assay for gender assignment in dugong (*Dugong dugon*) and West Indian manatee (*Trichechus manatus*), Mol. Ecol. Resour. 8 (2008) 669–670.
[46] S.L. Monfort, N.P. Arthur, D.E. Wildt, Monitoring ovarian function and pregnancy by evaluating excretion of urinary oestrogen conjugates in semi-free-ranging Przewalski's horses (*Equus przewalskii*), J. Reprod. Fertil. 91 (1991) 155–164.
[47] C. Munro, G. Stabenfeldt, Development of a microtitre plate enzyme immunoassay for the determination of progesterone, J. Endocrinol. 101 (1984) 41–49.
[48] C.C. Nascimento, Avaliação da função reprodutiva de fêmeas de peixe-boi da Amazônia (Trichechus inunguis, Natterer, 1883), mantidas em cativeiro, por meio da extração e dosagem de esteróides fecais [Reproductive assessment in captive females of Amazonian manatees (Trichechus inunguis, Natterer, 1883) by fecal steroid extraction and quantification], Unpublished M.Sc. thesis, University of Sao Paulo, Sao Paulo, Brazil, 2004, p. 113.
[49] M. Nishiwaki, H. Marsh, Dugong dugon (Muller, 1776), in: S.H. Ridgway, R.J. Harrison (Eds.), Handbook of Marine Mammals, Academic Press, London, UK, 1985, pp. 1–31.
[50] W.F. Perrin, S.B. Reilly, Reproductive parameters of dolphins and small whales of the family Delphinidae, Rep. Int. Whal. Comm. Special Issue 6 (1984) 97–134.
[51] W.L. Perryman, M.S. Lynn, Evaluation of nutritive condition and reproductive status of migrating gray whales (*Eschrichtius robustus*) based on analysis of photogrammetric data, J. Cetacean Res. Manage. 4 (2002) 155–164.
[52] A. Preen, Diet of dugongs: are they omnivores, J. Mammal. (1995) 163–171.
[53] R.M. Rolland, K.E. Hunt, S.D. Kraus, S.K. Wasser, Assessing reproductive status of right whales (*Eubalaena glacialis*) using fecal hormone metabolites, Gen. Comp. Endocrinol. 142 (2005) 308–317.
[54] C.C. Schwartz, S.L. Monfort, P.H. Dennis, K.J. Hundertmark, Fecal progestagen concentration as an indicator of the estrous cycle and pregnancy in moose, J. Wildl. Manage. (1995) 580–583.
[55] F. Schwarzenberger, E. Möstl, R. Palme, E. Bamberg, Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals, Animal Reprod. Sci. 42 (1996) 515–526.
[56] K.M. Tripp, J.P. Verstegen, C.J. Deutsch, R.K. Bonde, M. Rodriguez, B. Morales, et al., Validation of a serum immunoassay to measure progesterone and diagnose pregnancy in the West Indian manatee (*Trichechus manatus*), Theriogenology 70 (2008) 1030–1040.
[57] H.A. Tucker, Hormones, mammary growth, and lactation: a 41-year perspective, J. Dairy Sci. 83 (2000) 874–884.
[58] S. Tuljapurkar, H. Caswell, Structured-Population Models in Marine, Terrestrial, and Freshwater Systems, Springer, New York, USA, 1997.
[59] S.K. Wasser, S.L. Monfort, J. Southers, D.E. Wildt, Excretion rates and metabolites of oestradiol and progesterone in baboon (*Papio cynocephalus cynocephalus*) faeces, J. Reprod. Fertil. 101 (1994) 213–220.
[60] P.W. Webb, Hydrodynamics and energetics of fish propulsion, Bull. Fish. Res. Bd Can. 190 (1975) 1–158.
[61] P. White, R.A. Garrott, J.F. Kirkpatrick, E.V. Berkeley, Diagnosing pregnancy in free-ranging elk using fecal steroid metabolites, J. Wildl. Dis. 31 (1995) 514–522.