Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 70 (2008) 1030–1040
Theriogenology
www.theriojournal.com

Validation of a serum immunoassay to measure progesterone and diagnose pregnancy in the West Indian manatee (*Trichechus manatus*)

K.M. Tripp [a,*], J.P. Verstegen [b], C.J. Deutsch [c], R.K. Bonde [d], M. Rodriguez [e], B. Morales [f], D.L. Schmitt [g], K.E. Harr [h]

[a] *University of Florida College of Veterinary Medicine, Aquatic Animal Health Program, 2015 SW 16th Avenue, P.O. Box 100126, Gainesville, FL 32610-0126, USA*
[b] *University of Florida College of Veterinary Medicine (LACS), 2015 SW 16th Avenue, P.O. Box 100136, Gainesville, FL 32610-0136, USA*
[c] *Florida Fish and Wildlife Conservation Commission, Wildlife Research Laboratory, 1105 SW Williston Road, Gainesville, FL 32601, USA*
[d] *U.S. Geological Survey, Florida Integrated Science Center, 2201 NW 40 Terrace, Gainesville, FL 32605-3574, USA*
[e] *Miami Seaquarium, 4400 Rickenbacker Causeway, Key Biscayne, FL 33149, USA*
[f] *El Colegio de la Frontera Sur, Unidad Chetumal, Av. Centenario Km 5.5 s/n, Chetumal, Quintana Roo, Mexico*
[g] *Missouri State University Agriculture Department, 901 South National Avenue, Springfield, MO 65897, USA*
[h] *FVP Consultants, Inc., P.O. Box 67, Bushnell, FL 33513, USA*

Received 2 April 2008; received in revised form 9 June 2008; accepted 9 June 2008

Abstract

The objective was to validate a high-sensitivity chemiluminescent assay of serum progesterone concentrations for pregnancy diagnosis in manatees. Assay analytical sensitivity was 0.1 ng/mL, with mean intra- and inter-assay coefficients of variation of 9.7 and 9.2%, respectively, and accuracy had a mean adjusted R^2 of 0.98. Methods comparison (relative to Siemen's Coat-A-Count RIA) demonstrated $r = 0.98$, Deming regression slope of 0.95, and an intercept of 0.01. Based on ROC analysis, a progesterone concentration $\geq$0.4 ng/mL was indicative of pregnancy. Assay results were not significantly altered by two freeze–thaw cycles of samples. Characteristic progesterone concentrations during pregnancy were Months 1–4 (1.7–4.7 ng/mL), 5–8 ($\sim$1.0 ng/mL), and 10 and 11 (0.3–0.5 ng/mL), whereas two late-pregnant females with impending abortion had progesterone concentrations of 0.1 ng/mL. Among pregnant females, maximum progesterone concentrations occurred in autumn (3.9 $\pm$ 1.8 ng/mL), and were greater during all seasons than concentrations in non-pregnant females (0.1–0.2 ng/mL). Progesterone concentrations were also significantly higher in pregnant females than in non-pregnant females and males. This highly sensitive, specific, and diagnostic assay will be valuable for monitoring pregnancy and abortion in manatees.

Keywords: Manatee; Pregnancy diagnosis; Progesterone; Reproduction; Assay validation

1. Introduction

There is minimal peer-reviewed literature regarding progesterone concentrations in manatees (*Trichechus manatus*) and endocrine-based pregnancy diagnosis has not been possible. This was due in part to the absence of

* Corresponding author. Current address: Save the Manatee Club, 500 N. Maitland Avenue, Maitland, FL 32751, USA.
Tel.: +1 407 539 0990; fax: +1 407 539 0871.
E-mail address: ktripp@savethemanatee.org (K.M. Tripp).

0093-691X/$ – see front matter
doi:10.1016/j.theriogenology.2008.06.024

captive breeding of this species in the United States and concerns related to the capture of pregnant females. Isolation of a specific chorionic gonadotropin, used to diagnose pregnancy in species such as the horse [1,2], has not been attempted. Additionally, whereas pregnancy-specific hormones including prolactin and relaxin have been identified in species such as African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants [3–5], which are close terrestrial relatives of manatees [6,7], similar diagnostic hormones have not been identified in manatees. Furthermore, abdominal ultrasound, which can be used to diagnose pregnancy in many species [8,9], can only be utilized in manatees after the first 10 wk of gestation, and its sensitivity is lowered by the manatee's expansive, gas-filled gastrointestinal tract (M. Rodriguez, unpublished).

The absence of an early pregnancy detection method has caused biologists and clinicians to rely upon visual indications of pregnancy that appear late in the 12-mo gestation [10]. These include highly variable and subjective changes such as distension of the abdomen and vulva, and have left biologists and clinicians without methods to diagnose or monitor early pregnancy [10]. However, the ability to diagnose pregnancy is valuable, particularly for manatees undergoing active medical treatment for such traumatic injuries as watercraft strike and entanglement, because treatment may be altered for pregnant females.

Earlier studies measured serum progesterone in captive manatees [11] and utilized fecal samples to estimate the duration of the manatee estrous cycle as 28–42 d [12]. In the first study, serum progesterone in a captive female was >3.0 ng/mL [11], whereas the second study found that progesterone concentrations in pregnant females exhibited a wide range, wherein lower values could overlap with those of non-pregnant females and males [12]. Although fecal hormone data provided estimates of estrous cycle length and progesterone concentrations, the use of fecal steroid assays was complicated by factors related to sample collection and storage and steroid extraction [13]. The manatee's long gut transit time (approximately 7 d) [14] further contributed to the imprecision of manatee fecal steroid hormone data, making it less suitable for clinical pregnancy diagnosis.

In many domestic mammals, pregnancy is associated with progesterone concentrations greater than those observed during non-pregnant diestrus [9,15–18]. In Asian and African elephants, progesterone concentrations during pregnancy are low in comparison with those of many other species. Nevertheless, in most studies in elephants, concentrations were still greater during pregnancy than diestrus [4,19], although serial sampling was recommended to confirm pregnancy [20]. This observation of low but diagnostic serum progesterone concentrations during pregnancy in the elephant [4,19] suggested that a highly sensitive serum progesterone assay might be useful for diagnosing pregnancy in the manatee.

The objectives of this study were to: (1) analytically and diagnostically validate a chemiluminescent serum progesterone assay for pregnancy diagnosis in healthy manatees; (2) determine the effect of repeated freeze–thaw cycles on serum progesterone concentrations; (3) document progesterone concentrations throughout gestation; (4) identify the progesterone concentration indicative of abortion; (5) identify any seasonal progesterone variation in non-pregnant and pregnant females; and (6) examine differences in progesterone concentrations throughout the manatee population, considering gender, age class, and reproductive status. This information will allow biologists, clinicians, and managers to better assess the reproductive status of free-ranging and captive manatees.

2. Materials and methods

2.1. Blood sampling

Free-ranging manatees were captured using nets deployed from boats [21]. Blood was collected from the brachial vascular bundle during all seasons of the year as part of ongoing, government-led ecological investigations [22–25]. Such health assessments were conducted for free-ranging and captive Florida manatees (*T. manatus latirostris*), as well as free-ranging Antillean manatees (*T. manatus manatus*) in Mexico and Puerto Rico. Serum was separated within 1 h after collection by onsite centrifugation ($3000 \times g$ for 10 min). Samples were then transported on ice and either assayed immediately upon arrival at the University of Florida (UF) or stored at -80 °C for later analysis.

2.2. Sample population

Serum samples from 114 manatees were analyzed, including females ($n = 69$), males ($n = 45$), calves ($n = 9$), subadults ($n = 33$), and adults ($n = 72$). Thirteen replicate samples for each of three females were used to test intra-assay precision and three replicate samples from six manatees were used to assess inter-assay precision. For accuracy, dilution experiments were used to test parallelism and recoverability of progesterone using samples from three manatees. A methods comparison was completed using paired samples from 40 manatees.

Only adult females, defined by a total straight length >265 cm [26], were included in the sample population for diagnostic validation using receiver operating characteristic (ROC) analysis [27]. Additionally, healthy females were used to establish the reference interval for the non-pregnant population, because inflammation associated with disease may affect progesterone concentrations [28]. Health was assessed by field observations (including body condition, heart rate, and respiratory rate) and standard biochemical analysis of serum amyloid A (SAA) [29]. In the pregnant manatee group, three females with SAA > 70 μg/mL (and ≤130 μg/mL) were included, as their inflammatory processes were not severe enough to interfere with delivery of normal healthy calves [30]. No long-term captive females were utilized for diagnostic validation, to minimize confounding factors associated with numerous infertile estrous cycles, such as cystic endometrial hyperplasia, which has been documented in captive elephants [31]. Known reproductive pathology in captive elephants, as well as the observation of ovarian pathology in one long-term captive female and endocrine abnormalities in other long-term captives (K. Tripp, unpublished) suggested that such long-term captive females should be excluded from the present study. Healthy females who were lactating and observed with calves at the time of their capture were included in the non-pregnant population.

Pregnancy was confirmed using physical signs or presence of a newborn calf that indicated an individual had been pregnant on the date of her previous blood sampling. Healthy manatees that had been in a rehabilitation or temporary captive setting were assessed as non-pregnant once they had been in captivity for >7 mo without showing signs of pregnancy or abortion. Additionally, subsequent field observations of previously captured, free-ranging females, showing no signs of pregnancy or a dependent calf at approximately 1 y post-capture, were used to assess females as non-pregnant. This method of pregnancy diagnosis cannot account for false negative assessment due to abortion or perinatal mortality within the free-ranging population.

The first ROC analysis compared progesterone concentrations in non-pregnant females ($n = 30$) and pregnant females throughout gestation ($n = 15$). Two additional ROC analyses compared progesterone values in non-pregnant females ($n = 30$) with values from females in the first (Months 1–6; $n = 5$) and second halves of gestation (Months 7–12; $n = 10$). Serum samples from nine of these female manatees were used to test the effect of freeze–thaw on manatee serum progesterone results.

A single early gestation rehabilitating manatee was sampled for blood 13 times over a 6-mo time period until pregnancy was diagnosed. This female was not known to be pregnant at the time of her capture, and once physical signs of pregnancy appeared, sampling was stopped to minimize danger to the fetus. Additionally, single samples from other females with known gestation dates were used to examine trends in progesterone concentration during early (Months 1–4; $n = 9$), mid (Months 5–8; $n = 8$), and late (Months 9–12; $n = 5$) pregnancy.

Progesterone concentrations associated with abortion were defined using samples available from three late gestation manatees known to have aborted a fetus during their treatment for injuries at a rehabilitation facility. Manatee calves may range in length from 82 to 160 cm or 95–155 cm at birth [10,32], indicating that these fetuses, which were >81 cm in total length, were in the final months of gestation.

Progesterone concentrations for female Florida manatees were evaluated for seasonal variation, since the Florida manatee has been identified as a diffusely seasonal breeder [10]. Seasonal variation in progesterone concentrations was examined for Florida non-pregnant ($n = 21$) and pregnant females ($n = 14$). For non-pregnant females, samples were available from winter ($n = 3$), spring ($n = 1$), summer ($n = 5$), and autumn ($n = 12$). Samples from pregnant females also included winter ($n = 4$), spring ($n = 2$), summer ($n = 4$), and autumn ($n = 4$).

Progesterone concentrations were summarized by age–gender class as follows: female calves ($n = 7$), female subadults ($n = 17$), non-pregnant female adults ($n = 30$), pregnant female adults ($n = 15$), male calves ($n = 2$), male subadults ($n = 16$), and male adults ($n = 27$). Calves were defined by a total straight length ≤245 cm, subadults were 246–265 cm, and adults were >265 cm [26].

2.3. Laboratory analysis

The high-sensitivity and specificity automated chemiluminescent immunoassay analyzer (IMMULITE 1000®: Siemen's Medical Solutions Diagnostics, Los Angeles, CA, USA) uses a polyclonal rabbit anti-progesterone antibody and two reagents (ligand-labeled synthetic progesterone and alkaline phosphatase conjugated to anti-ligand, in buffer; Product no. LKPG1, Siemen's Medical Solutions Diagnostics). This progesterone assay was analytically validated for manatee serum by testing precision, accuracy, and analytical sensitivity, and by completing a methods comparison experiment. Analytical specificity (the assay's specificity for progesterone versus interfering compounds) was established by the manufacturer (DPC Progesterone PILKPG-10, 2006-12-29), but all other parameters were tested at the University of Florida endocrine laboratory. Intra- and inter-assay precision were assessed with repeat sample testing and accuracy was assessed with dilution experiments. Assay analytical sensitivity (the lower limit of detection or LLOD) was established during experiments to test precision and accuracy where the assay's LLOD was challenged. Results generated by the IMMULITE at the University of Florida were compared with a progesterone Coat-A-Count radioimmunoassay (RIA) (Siemen's Medical Solutions Diagnostics) at the Missouri State University's Department of Agriculture. The RIA, with a LLOD equal to 0.05 ng/mL, has been validated for use in elephants (D. Schmitt, unpublished.). Samples across the manatee's physiologic range of progesterone concentrations were included in validation experiments.

Serum samples with a range of progesterone concentrations ($n = 9$) were tested for effects of freeze–thaw upon measured progesterone concentrations in manatee samples. Fresh samples were analyzed within 12 h of collection by venipuncture. These fresh samples were frozen to $-80\ ^{\circ}C$ and the same aliquots were thawed twice within 1 y of their original collection date to assess any changes in measured progesterone concentration. Samples were thawed at room temperature and were refrozen immediately following each assay.

2.4. Statistical analysis

Validation results (precision, accuracy, methods comparison, and ROC) were calculated with EP Evaluator Release 7 (David G. Rhoads Associates, Inc., Kennett Square, PA, USA). Precision was evaluated by coefficients of variation (CV). Accuracy, assessed by linearity, required definition of total allowable error (25%), and linearity was achieved when the dilution results did not differ from the expected results by a percentage greater than the systematic allowable error (50% of the total allowable error budget). Accuracy was further evaluated using adjusted R^2 values resulting from linear regression, where results closer to 1.0 indicate a better relation between the independent and dependent variables. Methods were compared using Deming regression [33] and results were evaluated based upon a total allowable error (TEa) [34] of 25% and the observed correlation coefficient (r). Diagnostic validation using ROC analysis was completed to determine the assay's ability to discern differences between pregnant and non-pregnant manatees [27]. For each ROC analysis, diagnostic sensitivity (test's probability of producing a true positive result) and specificity (test's probability of producing a true negative result), and positive and negative predictive values were determined. Positive predictive value (PPV) is the probability that a positive test result accompanies a true positive condition [# True Positives/(# True Positives + # False Positives)], whereas negative predictive value (NPV) is the probability that a negative test result accompanies a true negative condition [# True Negatives/(# True Negatives + # False Negatives]).

Power calculations ($\alpha = 0.05$) were used to calculate the number of samples required to distinguish non-pregnant and pregnant manatees via ROC analysis (Minitab 14, Minitab Inc., State College, PA, USA). The remaining statistics were completed using SigmaStat (Systat Software, Inc., Point Richmond, CA, USA). A Kruskal–Wallis one-way ANOVA on ranks was used to investigate differences in progesterone concentration between adult non-pregnant Florida manatees (all with SAA $\leq 60\ \mu g/mL$) that were ($n = 10$) and were not ($n = 16$) lactating (mean $\pm$ S.D.) to determine if lactating females could be grouped with other non-pregnant females. Additionally, a one-way ANOVA was used to test differences in progesterone concentration between healthy, non-pregnant, non-lactating female manatees from Mexico ($n = 7$) and Florida ($n = 16$) (different subspecies) to determine if females from Mexico could be included in the study population. Paired t-tests were used to test for significant differences in measured progesterone concentration among samples ($n = 9$) that were frozen and thawed. Fresh samples were first compared to samples that had been freeze–thawed once, and a second test compared fresh samples to samples freeze–thawed twice. Dunn's method (ANOVA on ranks), which provides pairwise significance testing, was also used to test for variation in progesterone concentrations among calves ($n = 9$), subadults ($n = 33$), and adults ($n = 58$). Statistical tests were deemed significant when $P < 0.05$.

3. Results

3.1. Assay analytical and diagnostic validation

CV from precision analyses ranged from 6.2 to 14.5% (Table 1). Accuracy results were linear within

Table 1
Validation of the analysis of progesterone concentrations in manatee serum

Validation test	Range of sample concentration means tested ± S.D. (ng/mL) (n)	Number of replicates	Validation result
Inter-assay precision	0.9 ± 0.2–17.2 ± 0.2 (6)	3	CV min–max: 6.2–14.5%; mean CV: 9.2%
Intra-assay precision	1.2 ± 0.1–7.2 ± 0.5 (3)	13	CV min–max: 7.2–10.9%; mean CV: 9.7%
Accuracy	0.3–4.6 (3)	5 (dilutions)	Adjusted R^2 min–max: 0.93–1.0; mean adjusted R^2: 0.98
Methods comparison	0.1–5.8 (40)	1	$r = 0.98$

the allowable systematic error, with adjusted R^2 values from 0.93 to 1.0. The analytical sensitivity of this assay system for manatee serum was 0.1 ng/mL, based on precision results and dilution curves. The methods comparison produced a Deming regression slope of 0.95 [95% CI: 0.89–1.01] and an intercept of 0.01 [95% CI: −0.06 to 0.09] indicating diagnostically acceptable results. Results for three paired samples evaluated by methods comparison fell outside the region of total allowable error (Fig. 1).

In the Florida manatee, progesterone concentrations in non-pregnant, lactating females (0.2 ± 0.1 ng/mL; n = 10) were slightly higher than those of non-pregnant, non-lactating females (0.1 ± 0.06 ng/mL; n = 16). However, the difference was not statistically significant

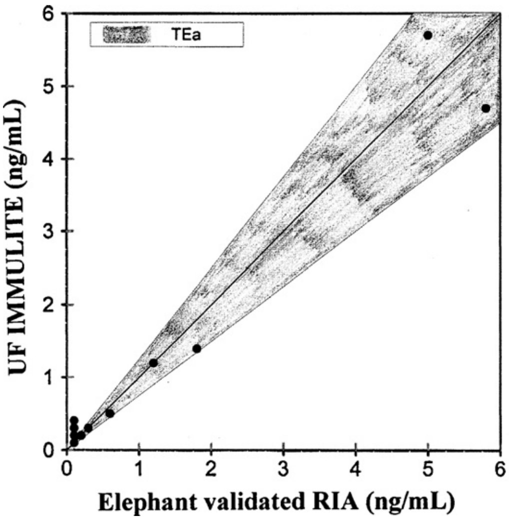

Fig. 1. Scatter plot of progesterone results for matched serum samples from manatees, tested during methods comparison of an RIA validated for use in elephants (x-axis) and the chemiluminescent system (y-axis). The progesterone results were represented by the points on the graph and the shaded area of the plot represented the total allowable error (TEa) for the methods comparison, which was set at 25%. Points outside the shaded region represented results with error that exceeded the defined TEa. A perfect fit between the methods is represented by results that fall along the line in the center of the shaded region.

(P = 0.065) and is not believed to be clinically significant. There was also no statistically significant geographic difference (P = 0.231) in progesterone concentration between non-pregnant, non-lactating females from Mexico (0.1 ± 0.00; n = 7) and Florida (0.1 ± 0.06; n = 16).

Power calculations indicated that a sample size of 15 non-pregnant and 15 pregnant manatees was required to distinguish pregnancy status via ROC analysis when comparing non-pregnant females to females throughout gestation. This ROC analysis indicated that assay sensitivity and specificity as well as PPV and NPV were optimized when 0.4 ng/mL was selected as the threshold for pregnancy diagnosis. At the threshold of ≥0.4 ng/mL, the positive likelihood ratio for pregnancy is 7.0 and this ratio increases to 22.0 at concentrations ≥0.5 ng/mL (Table 2). Power ≥95% was also achieved within ROC analyses for the first and second halves of gestation. For the first half of gestation (range, 1.0–5.6 ng/mL), ROC analysis reported sensitivity, specificity, PPV, and NPV as 100% at a threshold of 1.0 ng/mL. For the second half of gestation (range, 0.2–1.7 ng/mL), sensitivity was 90.0% and specificity was 86.7% at a threshold of 0.4 ng/mL. The associated PPV was 69.2% and the NPV was 96.3%.

3.2. Effects of freeze–thaw

Although there was some variation in progesterone concentration following freeze–thaw, there were no statistically significant differences among the results obtained from fresh samples (2.8 ± 5.2, n = 9) and those that were freeze–thawed once (3.0 ± 5.8, n = 9, P = 0.610) and twice (3.1 ± 5.8, n = 9, P = 0.309).

3.3. Normal gestational changes

In a rehabilitating female manatee sampled repeatedly during early pregnancy, serum progesterone concentrations peaked at 4.8 ng/mL in Month 2 of gestation, and declined to 0.9 ng/mL by gestational

Table 2
Serum progesterone (P4) concentrations for pregnancy diagnosis throughout gestation in the manatee

P4 cutoff (ng/mL)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Positive likelihood ratio
≥0.1	100	0	33.3	–[b]	1.0
≥0.4[a]	**93.3**	**86.7**	**77.8**	**96.3**	**7.0**
≥0.5	73.3	96.7	91.7	87.9	22.0
≥1.0	46.7	100	100	78.9	–[c]

[a] ROC analysis indicated that assay sensitivity and specificity as well as PPV and NPV were optimized when 0.4 ng/ml was selected as the threshold for pregnancy diagnosis.
[b] There was no negative predictive value at ≥0.1 ng/mL because there can be no negative results at this threshold, which encompasses all progesterone results.
[c] The positive likelihood ratio was calculated as sensitivity/(1 − specificity), which results in a denominator of zero at ≥1.0 ng/mL.

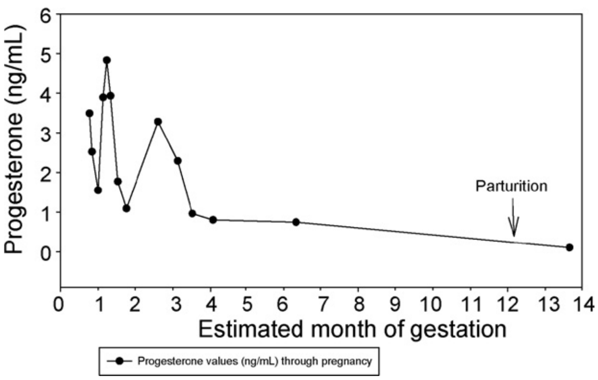

Fig. 2. Serum progesterone profile in a single manatee of known gestation. Blood sampling was routine until the gestational Month 4, after which only one additional sample was collected prior to parturition (arrow). Concentrations were 1.2–4.8 ng/mL (gestational Months 1–3), approximately 1.0 ng/mL (Months 4 and 6), and 0.1 ng/mL 50 d after parturition.

Table 3
Serum progesterone concentrations throughout gestation in manatees, determined from study females

Gestational month	Average progesterone ± S.D. (ng/mL)	Number of manatees	Sample size
1	1.7	1	1
2	3.6 ± 1.3	1	4
3	4.7 ± 1.2	3	3
4	1.7	1	1
5	0.8	1	1
6	1.0	1	1
7	0.8 ± 0.1	4	4
8	1.3 ± 0.5	2	2
9	No data	–	–
10	0.4 ± 0.06	3	3
11	0.3 ± 0.07	1	2
12	No data	–	–

–, no available samples.

Month 7 (Fig. 2). For pregnant, free-ranging females, estimation or confirmation of a parturition date allowed a blood sample and its associated progesterone concentration to be linked to the stage of gestation (Table 3). Based upon available data, early gestation (Months 1–4) was characterized by progesterone concentrations ranging from approximately 1.7–4.7 ng/mL. During mid-gestation (Months 5–8), progesterone concentrations were near 1.0 ng/mL, and in Months 10 and 11 (a portion of late gestation), concentrations between 0.3 and 0.5 ng/mL were measured.

3.4. Abortion detection

Progesterone data were available for three female manatees that aborted a fetus while being treated for injuries (#1 and #2 = entanglement; #3 = watercraft strike) at rehabilitation facilities (Table 4). All females

Table 4
Abortion-associated serum progesterone (P4) (ng/mL) in late-pregnant female manatees

Sampling time frame	Female #1	Female #2	Female #3
At rescue	0.1	0.3	0.1
Within 2 wk after rescue	0.1	0.4	0.1
Fetal length at abortion (cm)	109	115	124
Post-abortion	0.1 ($n = 1$)	–	0.1 ($n = 4$)
Normal late pregnancy	0.3–0.4	0.3–0.4	0.3–0.4

–, no available data.

Table 5
Mean (±S.D.) serum progesterone concentrations by season for non-pregnant and pregnant Florida manatees

Season	Non-pregnant (ng/mL) (*n*)	Pregnant (ng/mL) (*n*)
Winter	0.1 ± 0.0 (3)	0.7 ± 0.2 (4)
Spring	0.1 (1)	0.6 ± 0.3 (2)
Summer	0.1 ± 0.09 (5)	0.6 ± 0.3 (4)
Autumn	0.2 ± 0.1 (12)	3.9 ± 1.8 (4)

were estimated to be in late pregnancy (the final 4 mo of gestation) at the time of their abortion, based on fetal length. Females #1 and #3 displayed baseline serum progesterone concentrations of 0.1 ng/mL prior to abortion.

3.5. Seasonal variation

Mean progesterone concentrations in pregnant females were 6–20 times greater than those of non-pregnant females for a given season (Table 5). The highest mean progesterone concentration observed was associated with pregnant females in autumn (3.9 ± 1.8 ng/mL), which was much greater than concentrations observed in pregnant or non-pregnant females during any other season. There was no evidence of seasonal variation in progesterone concentration for non-pregnant females within the sample population.

3.6. Progesterone concentrations by gender and age class

Progesterone concentrations in pregnant adult females (median, 0.8 ng/mL) were significantly higher than concentrations in female and male calves and subadults, non-pregnant adult females, and adult males (Table 6).

4. Discussion

4.1. Assay analytical and diagnostic validation

Serum progesterone concentration is a reliable diagnostic tool for detection of pregnancies in species where there are clear differences between progesterone concentrations in diestrus and pregnancy [9,15–18]. Pregnancy in many domestic species is characterized by serum progesterone concentrations much greater than those observed in manatees [18]. Therefore, validation of a highly sensitive assay was desired for the manatee. The IMMULITE system, which was developed for human use and validated for several species, including small carnivores (J. Verstegen, unpublished), was validated for manatees. Any commercially available progesterone assay must be validated for use in manatees prior to routine use, and assays with a more sensitive LLOD (≤0.1 ng/mL) are most useful.

The progesterone assay was analytically validated, with acceptable coefficients of variation <25% [35], adjusted R^2 values that yielded linear results for accuracy [36], and a correlation coefficient ≥0.975 for methods comparison [37] (Table 1). For methods comparison, the three results that fell outside of the region of total allowable error occurred below the threshold for pregnancy diagnosis and close to the assay's LLOD, where the total allowable error margin is smallest. This error was not clinically significant.

In order to create a sample population representative of the free-ranging population, as required for diagnostic validation, 33.3% of samples originated from pregnant females, which corresponds to the suspected prevalence of pregnancy in the free-ranging manatee population [10]. Based on ROC analysis, sensitivity and specificity were both optimized at 0.4 ng/mL, identifying this as the appropriate threshold for diagnosing manatee pregnancy. These values were

Table 6
Serum progesterone concentrations in manatees by gender and age class

Gender and age categories	Mean ± S.D. (ng/mL) (*n*)	Median (ng/mL)	Min–max (ng/mL)
Female calf	0.3 ± 0.3 (7)[a]	0.1	0.1–0.9
Female subadult	0.1 ± 0.1 (17)[a]	0.1	0.1–0.7
Non-pregnant female adult	0.2 ± 0.2 (30)[a]	0.1	0.1–0.9
Pregnant female adult	1.2 ± 1.3 (15)[b]	0.8	0.2–5.3
Male calf	0.1 ± 0.1 (2)[a]	0.1	0.1–0.2
Male subadult	0.2 ± 0.2 (16)[a]	0.1	0.1–0.7
Male adult	0.2 ± 0.1 (27)[a]	0.1	0.1–0.6

The assay lower limit of detection (LLOD) was 0.1 ng/mL.
[b] Pregnant female adults had significantly higher progesterone concentrations than any other age class of females or males ([a]).

diagnostic until the final 2 mo of gestation, when progesterone concentrations may decrease below 0.4 ng/mL. At this time, females are visibly pregnant, making progesterone analysis unnecessary to diagnose pregnancy.

Previous attempts to measure progesterone in manatees have involved fecal samples and a limited number of plasma (assay LLOD = 0.08 ng/mL) and serum samples [11,12]. It was found that plasma concentrations were lower than those recovered from fecal samples and there was no correlation between matching fecal and plasma samples [12]. The long gut transit time in manatees and the associated degradation and metabolism of progesterone result in imprecise and inaccurate fecal hormone concentrations, which complicate the accurate medical assessment of pregnancy using fecal hormones.

4.2. Effects of freeze–thaw

Freeze–thaw cycles did not significantly affect progesterone assay results, which is consistent with previously findings [38]. Although some changes were observed throughout the subsequent trials, no clear pattern existed and the differences among the means were not statistically significant.

4.3. Normal gestational changes

Though manatee progesterone concentrations overall are lower than those of some other species [18,39], particularly during early pregnancy, manatees can have a fivefold or greater increase in serum progesterone during the first 4 mo of pregnancy. We hypothesized that serum progesterone concentrations would decline in the latter stages of pregnancy, and such a decline was observed; concentrations peaked during early pregnancy (1.7–4.7 ng/mL), and became progressively lower during mid (~1 ng/mL) and late (0.3–0.5 ng/mL) gestation. Progesterone concentration varies during gestation in other species, such as the mare, where serum progesterone declines to 1–2 ng/mL by 180–200 d of the 340 d pregnancy, as a result of conversion to feto-placental 5α-reduced progestins, which appear in the circulation by approximately 70 d of gestation [39]. In African and Asian elephants, progesterone concentrations were low throughout gestation (0.07–1.3 ng/mL) because progesterone metabolites serve as progestins and have progesterone-like activity [19,40,41]. Lower progesterone concentrations observed closer to term in the manatee should not affect the diagnostic utility of this assay, which is most needed to detect pregnancy before visual indicators appear after Month 6 of the 12-mo gestation. Until diestrus progesterone concentrations can be fully documented in the manatee, serial sampling of progesterone is suggested to confirm pregnancy, as has been recommended for elephants [20]. If a progesterone result ≥0.4 ng/mL is measured from a female manatee, a second sample should be submitted for analysis 2 wk after the initial sample was collected (based on fecal estimates of manatee estrous cycle duration [12] and gut transit time [14]). If this second sample also measures a progesterone concentration ≥0.4 ng/mL, the female can be considered pregnant. If the second sample results in a progesterone concentration <0.4 ng/mL, then the original sample likely represented diestrus within that female. This serial sampling technique does not apply to long-term captive females who cannot become pregnant due to the absence of male contact.

4.4. Abortion detection

Progesterone concentrations at or near baseline (0.1 ng/mL) appeared to be indicative of abortion in manatees previously diagnosed as being pregnant, which confirmed our hypothesis that abortion would be associated with a decline in serum progesterone concentration to basal values. In the pregnant mare, abortion was associated with a rapid decline in progestins or progestin concentrations <2 ng/mL for more than 3–4 d [42]. This threshold is lower in manatees, as evidenced by baseline progesterone concentrations (0.1 ng/mL) observed at least 2 wk prior to abortion in two late pregnancy females. Because progesterone concentrations associated with a healthy pregnancy in the manatee also declined to 0.3–0.4 ng/mL in the final months of gestation, diagnosing an impending abortion may be more difficult during this time than at an earlier stage of gestation, when progesterone concentrations are higher. For clinicians who are rehabilitating pregnant manatees, with such traumatic injuries as watercraft strikes and entanglements, progesterone should be evaluated regularly due to the effects of chronic stress on pregnancy and the possibility of abortion [43,44]. Additionally, in cases where pregnancy is suspected or confirmed, treatment strategies for rehabilitating females should be modified to prevent crushing of the fetus and abortion, by avoiding situations and activities (e.g. removal from the water and lateral rolling for wound treatment or examination) that may result in additional pressure to the abdomen or either lateral aspect of the body.

4.5. Seasonal variation

Manatees are believed to be diffusely seasonal breeders [10]. The breeding season for the Florida manatee has been proposed to range from March through September [10,32,45], based on the presence of follicles and corpora lutea in the ovaries of female carcasses [32], maximum perinatal carcass recovery [46], and the occurrence of mating herds [10,45]. Patterns in male sperm production also imply decreased likelihood of successful mating during the winter months [47].

In this study, the maximum progesterone concentrations during pregnancy were observed in autumn (September 23–December 21), which corresponded to mating and implantation between early summer and autumn (based on peak progesterone concentrations observed during early pregnancy in the current study). Descriptive summary statistics were presented without statistical analysis of seasonal variation due to the small sample size ($n = 1–4$) available for most seasons. The observed autumnal progesterone peak for pregnant females was later than expected, compared to previous studies of manatee mating and behavior [10,32,45], and may be due to this low sample size. The deviation from the expected summer progesterone peak was caused in part by two females in late pregnancy (with low progesterone concentrations) in the summer population. Because the mating and birthing seasons overlap [32] and because serum progesterone concentrations in the manatee declined throughout gestation, seasonal values were affected by the presence of females at various gestational stages, with variable progesterone concentrations.

As hypothesized, the highest progesterone concentrations were measured in pregnant females, during all seasons of the year. We also hypothesized that seasonal differences in progesterone concentration would be apparent in the non-pregnant population, with higher concentrations associated with episodes of diestrus during the breeding season. However, no appreciable seasonal differences existed in the non-pregnant population, for which the mean progesterone concentrations were at baseline (0.1 ng/mL) for all seasons except autumn, where the mean was 0.2 ± 0.1 ng/mL. We inferred that the non-pregnant sample population contained only a small number of diestrus females, which eliminated the possibility of viewing seasonal progesterone differences within the non-pregnant population.

4.6. Progesterone concentrations by gender and age class

Pregnant adult females had the highest progesterone concentrations in the manatee population, as expected. These findings concur with a previous study of progesterone concentrations in manatee [12].

4.7. Future studies

A separate study, currently being prepared for publication (K. Tripp, unpublished), investigated progesterone metabolites in the peripheral circulation of the manatee. Additional studies of manatee progesterone and pregnancy should monitor concentrations of this hormone throughout gestation, which would require sample collection from well-trained, healthy, captive or rehabilitated females, to minimize stress associated with handling. Analysis of serum progesterone throughout non-pregnant estrous cycles is also required to determine the duration and range of progesterone concentrations that characterize non-pregnant diestrus. Such an investigation would require the use of captive females housed with males to help guarantee normal reproductive cyclicity, as there has been some evidence to suggest abnormal progesterone concentrations in isolated females (K. Tripp, unpublished) and female elephants who have undergone numerous infertile estrous cycles have been found to have reproductive pathology [31].

4.8. Conclusion

The IMMULITE 1000®'s progesterone assay was determined to be a valid and highly sensitive and specific tool for diagnosing pregnancy in the manatee, particularly during early and mid-gestation. Progesterone analyses following one and two freeze–thaw cycles did not significantly affect assay results. Changes in serum progesterone concentrations throughout manatee gestation were identified, with the highest concentrations occurring during early pregnancy. The progesterone concentration associated with abortion in two late pregnancy females was also identified (0.1 ng/mL). The highest progesterone concentrations in pregnant females were observed in autumn and pregnant adult females exhibited the highest progesterone concentrations in the manatee population. This assay will allow manatee biologists and veterinarians to monitor reproductive health in individual free-ranging and captive females and will be beneficial in managing captive breeding of sirenians.

Acknowledgements

Samples were collected under United States Fish and Wildlife Service (USFWS) Permit Nos. MA773494-8 (FWC-FWRI) and MA791721 (USGS Sirenia Project) and Mexico samples were collected under permits NUM/SGPA/DGVS/03144 and 04513 from the Dirección General de Vida Silvestre (wildlife ministry) and transported to the USA under CITES export permit MX24463 (2005) and import permit US808447/9. Serum was analyzed at the University of Florida under USFWS permit # MA067116-0 and with University of Florida Institutional Animal Care and Use Committee approval No. D956.

We gratefully acknowledge Melanie Pate and Jim Burrow for their technical expertise in the laboratory. We also thank the capture teams from Ecosur Chetumal, the USGS Sirenia Project, and the FWC Fish and Wildlife Research Institute as well as the veterinary staff of the Lowry Park Zoo, Miami Seaquarium, and Sea World of Florida. Finally, we thank Dr. Greg Bossart, Janeth Padilla, Susan Butler, Ken Arrison, Margie Barlas, and Kari Higgs, whose assistance facilitated this research.

References

[1] Daels PF, Albrecht BA, Mohammed HO. Equine chorionic gonadotropin regulates luteal steroidogenesis in pregnant mares. Biol Reprod 1998;59:1062–8.

[2] Kirkpatrick J, Lasley BL, Shideler SE, Roser JF, Turner Jr JW. Non-instrumented immunoassay field tests for pregnancy detection in free-roaming feral horses. J Wildl Manage 1993;57: 168–73.

[3] McNeilly AS, Martin RD, Hodges JK, Smuts GL. Blood concentrations of gonadotrophins, prolactin and gonadal steroids in males and in non-pregnant and pregnant female African elephants (*Loxodonta africana*). J Reprod Fertil 1983;67:113–20.

[4] Hess DL, Schmidt AM, Schmidt MJ. Reproductive cycle of the Asian elephant (*Elephas maximus*) in captivity. Biol Reprod 1983;28:767–73.

[5] Niemuller C, Gray C, Cummings E, Liptrap RM. Plasma concentrations of immunoreactive relaxin activity and progesterone in the pregnant Asian elephant (*Elephas maximus*). Anim Reprod Sci 1998;53:119–31.

[6] Kellogg ME, Burkett S, Dennis TR, Stone G, Gray BA, McGuire PM, et al. Chromosome painting in the manatee supports Afrotheria and Paenungulata. BMC Evol Biol 2007;7:6.

[7] Pardini AT, O'Brien PCM, Fu B, Bonde RK, Elder FFB, Ferguson-Smith MA, et al. Chromosome painting among Proboscidea, Hyracoidea and Sirenia: support for Paenungulata (Afrotheria, Mammalia) but not Tethytheria. Proc Roy Soc B: Biol Sci 2007;274:1333–40.

[8] Boyd JS, Omran SN, Ayliffe TR. Evaluation of real time B-mode ultrasound scanning for detecting early pregnancy in cows. Vet Rec 1990;127:350–2.

[9] Engeland IV, Ropstad E, Andresen Ø, Eik LO. Pregnancy diagnosis in dairy goats using progesterone assay kits and oestrous observation. Anim Reprod Sci 1997;47:237–43.

[10] Rathbun GB, Reid JP, Bonde RK, Powell JA. Reproduction in free-ranging Florida manatees. In: O'Shea TJ, Ackerman BB, Percival HF, editors. Population biology of the Florida manatee, information and technology report 1. Washington, DC: U.S. Department of the Interior, National Biological Service; 1995. p. 135–56.

[11] Francis-Floyd R, White JR, Chen CL, Cardeilhac PT, Cichra CE. Serum progesterone and estradiol concentrations in captive manatees *Trichechus manatus*. J Aquat Anim Health 1991;3: 70–3.

[12] Larkin IL. Reproductive endocrinology of the Florida manatee (*Trichechus manatus latirostris*): estrous cycles, seasonal patterns and behavior. Ph.D. dissertation. Gainesville, FL: University of Florida; 2000. p. 40, 44, 48, 55, 56, 78, 103.

[13] Palme R. Measuring fecal steroids: guidelines for practical application. Ann NY Acad Sci 2005;1046:75–80.

[14] Larkin IL, Gross TS, Reep RL. Use of faecal testosterone concentrations to monitor male Florida manatee (*Trichechus manatus latirostris*) reproductive status. Aquat Mamm 2005;31:52–61.

[15] Karen A, Beckers J-F, Sulon J, de Sousa NM, Szabados K, Reczigel J, et al. Early pregnancy diagnosis in sheep by progesterone and pregnancy-associated glycoprotein tests. Theriogenology 2003;59:1941–8.

[16] Raggi LA, Ferrando G, Parraguez VH, MacNiven V, Urquieta B. Plasma progesterone in alpaca (*Lama pacos*) during pregnancy, parturition and early postpartum. Anim Reprod Sci 1999;54: 245–9.

[17] Dionysius DA. Pregnancy diagnosis in dairy goats and cows using progesterone assay kits. Aust Vet J 1991;68:14–6.

[18] Chadio S, Xylouri E, Kalogiannis D, Michalopoulou E, Evagelatos S, Menegatos I. Early pregnancy diagnosis in swine by direct radioimmunoassay for progesterone in blood spotted on filter paper. Anim Reprod Sci 2002;69: 65–72.

[19] Brown JL, Lehnhardt J. Serum and urinary hormones during pregnancy and the peri- and postpartum period in an Asian elephant (*Elephas maximus*). Zoo Biol 1995;14:555–64.

[20] Brown JL. Reproductive endocrine monitoring of elephants: an essential tool for assisting captive management. Zoo Biol 2000;19:347–67.

[21] Weigle BL, Wright IE, Ross M, Flamm R. Movements of radio-tagged manatees in Tampa Bay and along Florida's west coast, 1991–1996. Technical report. St. Petersburg, FL: Florida Marine Research Institute; 2001, p. 3.

[22] Deutsch CJ, Edwards HH, Barlas ME. Florida manatee foraging behavior around a winter warm-water refuge. Final report to the Wildlife Foundation of Florida. St. Petersburg, FL: FWC Fish and Wildlife Research Institute; 2006. 42 pp.

[23] Slone DH, Stith BM, Reid JP. Manatee use within Everglades National Park: data summary and management implications. Abstract only. In: Greater Everglades ecosystem restoration conference. 2006. p. 210.

[24] Morales-Vela B, Olivera-Gómez D, Reynolds III JE, Rathbun GB. Distribution and habitat use by manatees (*Trichechus manatus manatus*) in Belize and Chetumal Bay, Mexico. Biol Conserv 2000;95:67–75.

[25] Reid JP. Cooperative manatee research in Puerto Rico. Endangered Species Bull 2006;31:18–9.

[26] Marmontel M. Age determination and population biology of the Florida manatee, *Trichechus manatus latirostris*. Ph.D. dissertation. Gainesville, FL: University of Florida; 1993. p. 144, 172, 185.
[27] Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 1993;39:561–77.
[28] Munsterjhelm C, Valros A, Heinonen M, Hälli O, Peltoniemi OAT. Welfare index and reproductive performance in the sow. Reprod Domest Anim 2006;41:494–500.
[29] Harr K, Harvey J, Bonde R, Murphy D, Lowe M, Menchaca M, et al. Comparison of methods used to diagnose generalized inflammatory disease in manatees (*Trichechus manatus latirostris*). J Zoo Wildl Med 2006;37:151–9.
[30] Chrousos GP, Torpy DJ, Gold PW. Interactions between the hypothalamic–pituitary–adrenal axis and the female reproductive system: clinical implications. Ann Intern Med 1998;129: 229–40.
[31] Agnew D, Munson L, Ramsay E. Cystic endometrial hyperplasia in elephants. Vet Pathol 2004;41:179–83.
[32] Marmontel M. Age and reproduction in female Florida manatees. In: O'Shea TJ, Ackerman BB, Percival HF, editors. Population biology of the Florida manatee, information and technology report 1. Washington, DC: U.S. Department of the Interior, National Biological Service; 1995. p. 98–119.
[33] Cornbleet PJ, Gochman N. Incorrect least-squares regression coefficients in method-comparison analysis. Clin Chem 1979;25:432–8.
[34] Chinchilli VM, Miller WG. Evaluating test methods by estimating total error. Clin Chem 1994;40:464–71.
[35] Royal College of Pathologists of Australasia. Appendix A: programs, analytes and allowable limits of performance. In: Analytical quality requirements in chemical pathology quality assurance programs information handbook. Adelaide, Australia: Australasian Association of Clinical Biochemists; 2001 . p. 27.
[36] Westgard JO, Carey RN, Wold S. Criteria for judging precision and accuracy in method development and evaluation. Clin Chem 1974;20:825–33.
[37] Stöckl D, Dewitte K, Thienpont LM. Validity of linear regression in method comparison studies: is it limited by the statistical model or the quality of the analytical input data? Clin Chem 1998;44:2340–6.
[38] Comstock GW, Burke AE, Norkus EP, Gordon GB, Hoffman SC, Helzlsouer KJ. Effects of repeated freeze–thaw cycles on concentrations of cholesterol, micronutrients, and hormones in human plasma and serum. Clin Chem 2001;47:139–42.
[39] Allen WR. Luteal deficiency and embryo mortality in the mare. Reprod Domest Anim 2001;36:121–31.
[40] de Villiers DJ, Skinner JD, Hall-Martin AJ. Circulating progesterone concentrations and ovarian functional anatomy in the African elephant (*Loxodonta africana*). J Reprod Fertil 1989;86: 195–201.
[41] Hodges JK, Heistermann M, Beard A, van Aarde RJ. Concentrations of progesterone and the 5α-reduced progestins, 5α-pregnane-3,20-dione and 3α-hydroxy-5α-pregnan-20-one, in luteal tissue and circulating blood and their relationship to luteal function in the African elephant, *Loxodonta africana*. Biol Reprod 1997;56:640–6.
[42] LeBlanc MM. Equine perinatology: what we know and what we need to know. Anim Reprod Sci 1996;42:189–96.
[43] Turner AI, Hemsworth PH, Tilbrook AJ. Susceptibility of reproduction in female pigs to impairment by stress or elevation of cortisol. Domest Anim Endocrinol 2005;29:398–410.
[44] Joachim RA, Hildebrandt M, Oder J, Klapp BF, Arck PC. Murine stress-triggered abortion is mediated by increase of CD8+ TNF-α+ decidual cells via substance P. Am J Reprod Immunol 2001;45:303–9.
[45] O'Shea TJ, Hartley WC. Reproduction and early-age survival of manatees at Blue Spring, upper St. Johns River, Florida. In: O'Shea TJ, Ackerman BB, Percival HF, editors. Population biology of the Florida manatee, information and technology report 1. Washington, DC: U.S. Department of the Interior, National Biological Service; 1995. p. 157–70.
[46] Ackerman BB, Wright SD, Bonde RK, Odell DK, Banowetz DJ. Trends and patterns in mortality of manatees in Florida, 1974–1991. In: O'Shea TJ, Ackerman BB, Percival HF, editors. Population biology of the Florida manatee, information and technology report 1. Washington, DC: U.S. Department of the Interior, National Biological Service; 1995. p. 223–58.
[47] Hernandez P, Reynolds III JE, Marsh H, Marmontel M. Age and seasonality in spermatogenesis of Florida manatees. In: O'Shea TJ, Ackerman BB, Percival HF, editors. Population biology of the Florida manatee, information and technology report 1. Washington, DC: U.S. Department of the Interior, National Biological Service; 1995. p. 84–95.