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Research paper

Characterization of circulating steroid hormone profiles in the bottlenose dolphin (*Tursiops truncatus*) by liquid chromatography–tandem mass spectrometry (LC–MS/MS)

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ABSTRACT

Systemic steroid hormone measurements are often used in the assessment of reproductive, developmental, and stress physiology in vertebrates. In protected wildlife, such as the common bottlenose dolphin (*Tursiops truncatus*), these measures can provide critical information about health and fitness to aid in effective conservation and management. Circulating steroid hormone concentrations are typically measured by immunoassays, which have imperfect specificity and are limited to the measurement of a single hormone per assay. Here we demonstrate that reverse phase solid phase extraction (SPE) coupled to liquid chromatography tandem mass spectrometry (LC–MS/MS) allows for the simultaneous, precise (< 15% relative standard deviation), and accurate (between 70% and 120% recovery of spiked quantities) measurement of at least seven steroid hormones in dolphin plasma. These seven steroid hormones include three hormones that have been measured previously in bottlenose dolphin blood (progesterone, testosterone, and cortisol) and three hormones which have never been quantified in dolphin blood (17-hydroxyprogesterone, androstenedione, cortisone, and corticosterone). While 17β-estradiol was not detected endogenously, we were able to accurately and precisely measure spiked quantities estradiol. Measures from plasma were more precise (i.e., lower RSD) than serum, and thus we recommend plasma as the preferred matrix for this analytical method. In order to facilitate comparison of current and future plasma-based studies to previous serum-based studies, we characterized the relationships between hormone measurements in matched plasma and serum, and found that measurements across matrices are significantly and positively correlated. Lastly, to demonstrate potential applications of this method, we examined how steroid hormone profiles vary by pregnancy, sexual maturity, and stress status – pregnancy was associated with elevated progesterone, adult males had higher testosterone, and capture stress was associated with elevated corticosteroids. Overall, we conclude that this method will enable investigators to more thoroughly and efficiently evaluate steroid hormone homeostasis in bottlenose dolphins compared to immunoassay methods. These methods can potentially be applied to the assessment of sexual maturity/seasonality, pregnancy status, and stress in free-ranging bottlenose dolphins as well as those maintained under human care, and potentially other marine mammals.

1. Introduction

Monitoring steroid hormones in marine mammals can provide valuable health and fitness information to support effective conservation. Circulating steroid hormone concentrations have been used to assess pregnancy status, sexual maturity/cyclicity/seasonality, and the

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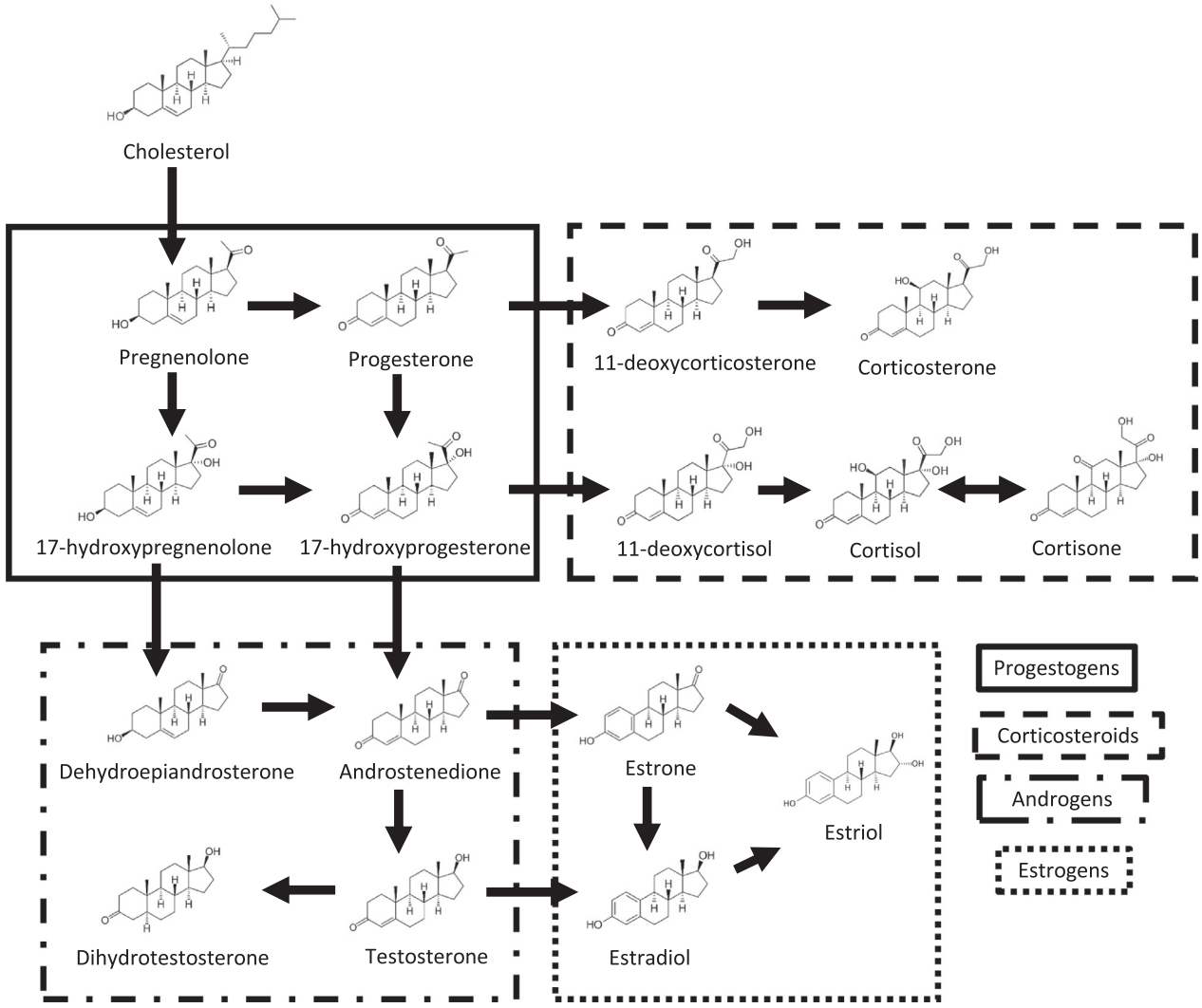

Fig. 1. Steroidogenesis pathway inclusive of the 16 hormones we screened using the described LC–MS/MS assays. Hormones are grouped by class per the key. Arrows indicate metabolic relationships.

stress response in bottlenose dolphins (Bergfelt et al., 2011; Cornell et al., 1987; Fair et al., 2014; Harrison and Ridgway, 1971; Houser et al., 2011; Kirby and Ridgway, 1984; O'Brien and Robeck, 2012; Ortiz and Worthy, 2000; Robeck et al., 1994; Sawyer-Steffan et al., 1983; Schroeder and Keller, 1989; St. Aubin et al., 1996; Steinman et al., 2016; Thomson and Geraci, 1986; Yoshioka et al., 1986). Systemic endocrine assessments in vertebrates are commonly performed with blood matrices (serum or plasma) because circulating hormone concentrations reflect systemic homeostasis (i.e. the status of the equilibrium between hormone secretion, storage, transport, and clearance). Here we aim to validate a liquid chromatography tandem mass spectrometry (LC–MS/MS) steroid hormone assay, which has several advantages over immunoassay-based methods, in bottlenose dolphin blood matrices.

Steroid hormones are categorized into four classes based on structure and function (progestogens, androgens, estrogens, and corticosteroids), all of which are derived from cholesterol and exist within a common metabolic pathway (Fig. 1) (Miller, 1988; Norris and Carr, 2013). Progestogens are typically associated with pregnancy and the luteal phase of the estrous cycle, but are also precursors to androgens and corticosteroids (Miller, 1988; Norris and Carr, 2013). Androgens are commonly referred to as “male sex hormones” because they are observed at high concentrations in males and regulate the expression of masculine traits, though they, are also important for female physiology (Miller, 1988; Norris and Carr, 2013). Estrogens are commonly called “female sex hormones” for analogous reasons, and, similarly, are important in male physiology (Miller, 1988; Norris and Carr, 2013). Corticosteroids are involved in mediating the stress response, and are further divided into glucocorticoids, which regulate energy homeostasis by impacting peripheral glucose utilization, gluconeogenesis via protein and lipid catabolism, and glycogenesis, and mineralocorticoids, which regulate ionic (Na^+/K^+) homeostasis (Miller, 1988; Norris and Carr, 2013). For reasons detailed below, investigators commonly measure only a single hormone within the specific class(es) of interest. In bottlenose dolphins, these are typically progesterone (progestogen), testosterone (androgen), cortisol (glucocorticoid), aldosterone (mineralocorticoid), and estradiol (estrogen).

Previously, circulating steroid hormones in bottlenose dolphins have been measured by immunoassays (enzyme immunoassay (EIA) or radioimmunoassay (RIA)), which utilize antibodies to detect hormones (Bergfelt et al., 2011; Cornell et al., 1987; Fair et al., 2014; Houser et al., 2011; Kirby and Ridgway, 1984; O'Brien and Robeck, 2012; Ortiz and Worthy, 2000; Sawyer-Steffan et al., 1983; Schroeder and Keller, 1989; St. Aubin et al., 1996; Steinman et al., 2016; Thomson and Geraci, 1986; Yoshioka et al., 1986). Immunoassays are indirect-detection methods, meaning the detected endpoints (i.e., radioactivity, color change, or light production) are secondary signals generated by the binding of the target hormone to cognate antibodies. While these

methods provide excellent sensitivity, immunoassay specificity is imperfect due to the potential for antibodies to cross-react with non-target analytes. Because steroid hormones are chemically/structurally similar (Fig. 1), antibodies cannot distinguish between steroids with absolute specificity. Therefore, presence of non-target, cross-reactive hormones in a sample may lead to artificial enhancement of the target signal in an immunoasssay. Furthermore, due to the use of indirect-detection, immunoassays are limited to the analysis of a single analyte per assay. In order to comprehensively assess endocrine status in an individual, investigators must perform independent immunoassays for each hormone. For this reason, studies reliant on immunoassays tend to be highly targeted, typically quantifying only one hormone or a small suite of hormones. While utilitarian, this approach provides only a partial assessment of steroid hormone homeostasis and disregards potentially interesting inter- and intra-class relationships.

Boggs et al. recently demonstrated the feasibility of using a solid phase extraction (SPE) to LC–MS/MS method to simultaneously quantify multi-class steroid hormones in human blood matrices and bottlenose dolphin blubber (Boggs et al., 2016; Boggs et al., 2017). Unlike immunoassays, mass spectrometry provides a direct-detection method, meaning the hormone (not a reaction with the hormone) is measured, thereby increasing specificity. In LC–MS/MS, the monitoring of compound-specific transitions (i.e., fragmentation patterns) at compound-specific retention time ensures specificity. Additionally, multiple hormones can be measured in a single assay. This method allows investigators to perform a more thorough analysis of steroid hormone homeostasis with greater efficiency and specificity than is attainable with current immunoassays. This manuscript describes the validation of these methods for use in bottlenose dolphin blood matrices, and the application to dolphin plasma for the investigation of demographic differences in steroid hormone profiles.

2. Materials and methods

2.1. Animals and samples

2.1.1. Individual plasma samples

Blood was collected from free-ranging bottlenose dolphins from Barataria Bay, Louisiana, USA in September 2017. Methods for the temporary capture and blood collection have been previously described (Schwacke et al., 2013; Smith et al., 2017; Wells et al., 2005). Plasma was produced by centrifugation of whole blood collected in sodium-heparin vacutainers to prevent coagulation. Plasma was immediately frozen. Aliquots (5 mL) were frozen and shipped in nitrogen dry shippers to the National Institute of Standards and Technology (NIST) Environmental Specimen Bank (ESB) at Hollings Marine Laboratory (Charleston, SC) where they were stored at −80 °C until analysis.

2.1.2. Pooled samples

Blood samples were collected from adult bottlenose dolphins maintained at the U.S. Navy Marine Mammal Program (Space and Naval Warfare Systems Center Pacific, San Diego, CA) via the arteriovenous plexus of the ventral fluke on various dates in October and November 2012. All samples were collected under trained, voluntary participation of the dolphins to reduce handling-induced stress. Plasma was produced as described above. Serum was produced by centrifugation of whole blood that was allowed to clot for 45 min. Samples from each date were pooled by matrix and sex. Pools were frozen in approximately 5 mL aliquots at −80 °C, shipped frozen on dry ice to Hollings Marine Laboratory (Charleston, SC), and stored at −80 °C until analysis.

2.1.3. Individual-matched serum and plasma

Blood was collected from free-ranging bottlenose dolphins from three sites in the southeastern United States during capture-release health assessments, including: Barataria Bay, Louisiana (June 2014); Sarasota Bay, Florida (May 2013, 2015, and 2016); and Brunswick, Georgia (September 2015). This sample set includes pregnant (or suspected pregnant) and non-pregnant females (n = 4 and 5, respectively), subadult and adult males (n = 6 and 5, respectively), and samples collected at two different time points during collection, time point 1 (T1; collected as soon as possible following restraint) and time point 6 (T6; collected at the end of sampling, immediately preceding release of the animal) (n = 17 and 3, respectively). Pregnancy was diagnosed by ultrasound. Age was determined either through lifelong observation (i.e. known birth date) or through examination of growth layer patterns in teeth using methods that have been described previously (Hohn et al., 1989; McFee et al., 2010). Age class was defined by age (individuals ≥ 10 years old were classified as adults) or length (individuals ≥ 240 cm total length classified as adult), in the absence of age data. Serum and plasma were produced from whole blood as described above for pooled samples. Aliquots (1–2 mL) were frozen and shipped in nitrogen dry shippers to the National Institute of Standards and Technology (NIST) Environmental Specimen Bank (ESB) at Hollings Marine Laboratory (Charleston, SC) where they were stored at −80 °C until analysis.

2.2. Calibration and internal standards

Calibration and isotopically-labeled internal standards were acquired from various manufacturers (Table 1). Calibration (Cal) and internal standard (IS) stock solutions were gravimetrically prepared from neat standards, and mixture solutions were prepared from these stock solutions and diluted in methanol, with the concentration of each compound calculated gravimetrically (ng compound/g mixture) (Tables S1–4).

2.3. Reverse phase solid-phase extraction

Steroid hormones were extracted via a method originally developed for human blood matrices (validated with NIST Standard Reference Material 971, Hormones in Human Serum) described in Boggs et al. (2016). Briefly, 100 µL or 150 µL of IS mixture was added to clean borosilicate culture tubes, and was dried under nitrogen gas (N_2) at 100 kPa to 130 kPa in a water bath at 40 °C to prevent potential precipitation of blood proteins by the methanol associated with the IS. Approximately 2 mL of serum or plasma (thawed at room temperature for approximately 30 min to 40 min), or 0.5 mL to 1.0 mL of calibration standard was added. The masses of IS and sample matrix (serum, plasma, or calibration standard) were tracked gravimetrically. IS-only blanks were also included, but received no additional matrix. Sodium acetate buffer (4 mL, 0.01 mol/L, pH 5) was added to each tube, vortexed briefly, and incubated at room temperature for 1 h to facilitate liberation of hormones bound to circulating proteins (Tai and Welch, 2004). During sample incubation, Supelclean LC-18, 6 mL capacity, 1 g bed weight solid-phase extraction cartridges (Sigma-Aldrich; St. Louis, MO) were arranged on a vacuum SPE manifold and conditioned sequentially with 5 mL of methanol, 5 mL of MilliQ water, and 1 mL of sodium acetate buffer (0.01 mol/L, pH 5). After incubation, the sample/buffer mixture was loaded onto the conditioned SPE cartridges. A vacuum (−33.3 kPa) was applied as necessary to facilitate the flow of sample through the column. Cartridges were washed with 12 mL of MilliQ water followed by 5 mL of 80:20 MilliQ water:acetonitrile (volume fraction). A vacuum was applied to ensure removal of all wash solution. Samples were eluted into clean borosilicate culture tubes with 2.5 mL of methanol. Eluent was dried under N_2 at 100 kPa to 130 kPa in a water bath at 40 °C, reconstituted in 200 µL of methanol, and transferred to amber autosampler vials with 250 µL glass inserts.

2.4. Dansyl chloride derivatization for measurement of estrogens

Dansyl chloride derivatization was performed using methods

Table 1
Calibration and internal standard compound manufacturer, purity information, and monitored transitions.

Common Name	Manufacturer	Stated Purity	Precursor Ion (*m/z*)	Quantitative Fragment (*m/z*)	Qualitative Fragment (*m/z*)
Progesterone	Sigma-Aldrich	≥99%	314.7	109.2	97.2
17-hydroxyprogesterone	Sigma-Aldrich	≥95%	331.0	97.2	109.2
Androstenedione	Steraloids	≥98%	287.1	97.2	109.2
Testosterone	Sigma-Aldrich	≥98%	288.9	109.2	97.1
Estrone	Sigma-Aldrich	≥99%	504.5[a]	171.3[a]	440.1[a]
Estradiol	Sigma-Aldrich	≥98%	506.2[a]	170.9[a]	442.3[a]
Cortisol	Sigma-Aldrich	≥98%	363.2	121.3	267.3
Cortisone	Sigma-Aldrich	≥98%	361.1	163.3	121.3
11-deoxycortisol	Steraloids	99.1%	347.3	109.2	97.0
Corticosterone	Sigma-Aldrich	≥98.5%	347.3	135.0	121.0
11-deoxycorticosterone	Steraloids	≥98%	331.1	97.1	109.2
Progesterone-$^{13}C_3$	Cambridge Isotopes	98%	318.3	100.0	112.1
17-hydroxyprogesterone-$^{13}C_3$	Cerilliant	99.99%	334.1	112.1	112.2
Androstenedione-$^{13}C_3$	Cerilliant	99.99%	290.2	100.3	112.2
Testosterone-$^{13}C_3$	Cerilliant	99.99%	292.1	112.0	100.0
Estradiol-$^{13}C_3$	Cerilliant	99.99%	509.4[a]	170.9[a]	NA[b]
Cortisol-d_4	Cerilliant	99.99%	367.3	121.2	271.5
Cortisone-$^{13}C_3$	Sigma-Aldrich	98%	364.2	166.5	124.1

[a] These are the dansyl chloride-derivatized *m/z* values.
[b] No suitable secondary fragment was identified for estradiol-$^{13}C_3$.

modified from Nelson et al. (2004). A 50 µL aliquot of the final 200 µL SPE extract) in methanol was transferred to borosilicate tubes containing 200 µL of acetone and 500 µL of sodium bicarbonate buffer (0.1 mol/L, pH 10.5) and was vortexed for 1 min. Dansyl chloride solution (500 µL of a 1 mg/mL; Sigma-Aldrich; St. Louis, MO) in acetone was added, and vortexed for 1 min. This mixture was incubated for 3 min in a heat block at 60 °C, and then dried under N_2 at 100 kPa to 130 kPa in a water bath at 40 °C. Dried samples were reconstituted in 2 mL of methanol, filtered by UniPrep 0.2 µm PTFE syringeless filter (Whatman Inc, Piscataway, NJ) to remove excess salts, and transferred into new borosilicate tubes. Filtered samples were dried under N_2 at 100 kPa to 130 kPa in a water bath at 40 °C, reconstituted in 50 µL of methanol, and transferred into a new amber autosampler vial with a 250 µL glass insert.

2.5. Instrumental methods

Instrumental methods used here have been described previously by Boggs et al. (2016). Three different chromatographic separations were performed: 1) biphenyl separation of underivatized steroids, 2) biphenyl separation of derivatized estrogens, and 3) C18 separation to improve detection of corticosteroids (Fig. 2). Boggs et al. discussed the value provided by using these three distinct separations in bottlenose dolphin blubber, reporting that the biphenyl separations provided the best quantitation for the gonadal steroids whereas C18 separation produced the best quantitation for the corticosteroids; and derivatization was necessary for estrogen quantitation (Boggs et al., 2017). Instrumental and compound parameters were consistent across methods. We used an Agilent (Santa Clara, CA) 1200 Series HPLC system with a binary pump and an autosampler linked to an AB Sciex (Framingham, MA) API 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer with the parameters outlined by Boggs et al. (2016). Separation of androgens, progestogens, and estrogens was conducted using a Restek (Bellefonte, PA) Ultra Biphenyl column (250 mm × 4.6 mm, 5 µm particle size) and a gradient of acetonitrile and methanol (both containing 0.1% formic acid) beginning at 80% methanol which was decreased to 65% methanol over 20 min, then decreased to 0% methanol over 1 min, held for 5 min, increased to 80% methanol over 0.1 min, and held for 9.9 min. Prior to the C18 separation for corticosteroid measurement, extracts were solvent exchanged into 50:50 methanol:water (volume fraction) by transferring 50 µL of extract into a clean borosilicate culture tube, drying under N_2 at 100 kPa to 130 kPa in a water bath at 40 °C, reconstituting in 50:50 methanol: water solution (volume fraction), and transferring to a new autosampler vial with glass insert. An Agilent Eclipse Plus C18 column (150 mm × 21 mm, 5.0 µm particle size), and a gradient of methanol and milliQ water (both with 0.1% acetic acid) was used to separate the corticosteroids beginning with 46% methanol and held for 10 min, increased to 82.5% methanol over 10 min, then increased to 83.3% methanol over 5 min. The column was then washed with 100% methanol for 5 min, and re-equilibrated to 46:54 methanol:water (volume fraction) for 10 min. Scheduled multiple reaction monitoring (sMRM) was used. Two transitions were monitored per compound in all separations – the transition with the largest signal was used for quantification, while the other was used for qualitative identity confirmation (Tables 1; S5).

2.6. Quantification

Chromatographic peaks for target compounds and internal standard compounds were integrated using Sciex Analyst software (Version 1.5; Framingham, MA). Target compound peak areas were divided by the peak area of the matched isotopically labeled internal standard (cortisol-d_4 was used for 11-dexoycortisol, corticosterone, and 11-deoxycorticosterone due to a lack of suitable commercially available isotopically labeled standards). These area ratios were interpolated on regressions calculated from extracted calibration comprised of at least three calibration standards, which bracketed the range of sample values (Tables S2–4). Standard curves were tailored to the range of values measured in the samples in each experiment, with the maximal point in the curve being as close to the top of the maximum measured value as possible while still exceeding. This was to ensure that curve parameters were not influenced by high inflection points in great excess of the maximum value measured. Linear or quadratic curves were used; the selection of one versus the other was based on inspection of curve shape at the tailored range. Observed reporting limits (RL_{obs}) are defined as the lowest calibration standard used in the calibration curve, while theoretical reporting limits (RL_{calc}) were calculated as three times the standard deviation of the extracted blank measurements plus the mean of the extracted blanks (Table 2), and the maximum of these two RL values was used as the censoring threshold, as has been done previously (Alava et al., 2011; Boggs et al., 2016; Boggs et al., 2017; Hoguet et al., 2013; Keller et al., 2012; Ragland et al., 2011; Stewart et al., 2011).

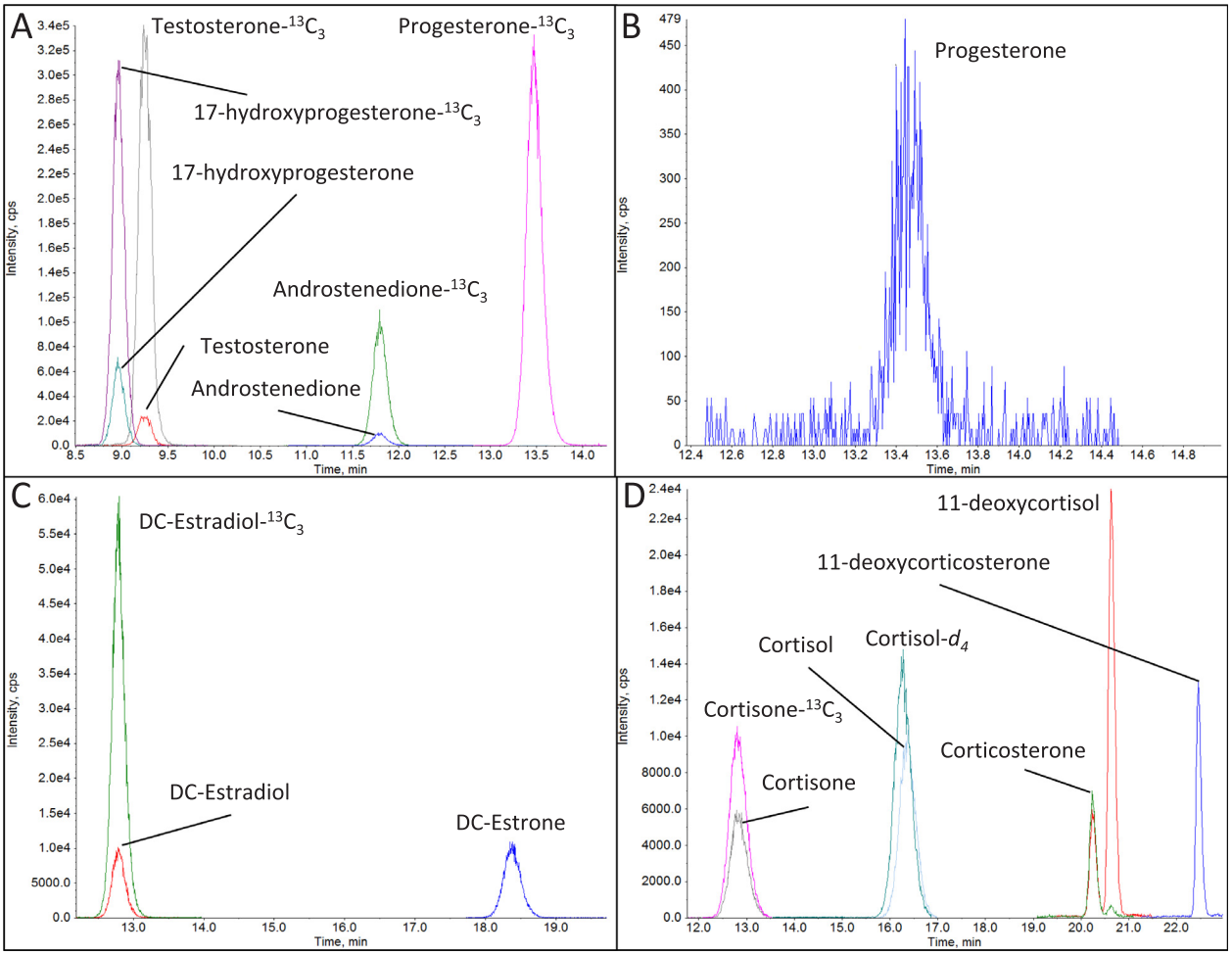

Fig. 2. Example chromatograms for each of the three separations utilized in this study. Intensity (y-axes) units are counts per second (cps). A) Biphenyl separation of progestogens and androgens without derivatization, B) progesterone chromatogram from biphenyl separation (not visible in A), C) biphenyl separation for dansyl chloride-derivatized estrogens, D) C18 separation of corticosteroids.

2.7. Accuracy and matrix interference assessment: spike retrieval

We performed a spike recovery experiment to assess method accuracy and precision in male and female plasma. The extraction method was slightly modified for this spike retrieval experiment. Following addition of IS, 400 µL of a calibration standard mixture was gravimetrically amended to ten tubes (n = 5 each for male and female plasma) to constitute the steroid spike (Table 3). Then both the IS and spike were dried, after which plasma was added (2 mL; n = 5 per sex) and extraction proceeded as described above. For each of the ten individual samples, an additional 2 mL aliquot was extracted without the additional spike to measure endogenous concentration for use in the

Table 2
Reporting limits (RL) (ng) by experiment. Observed RL (RL_{obs}) is determined by the lowest calibration standard used in the calculation of the standard curve. Calculated RL (RL_{calc}) calculated as three times the standard deviation of blank measurements plus mean of blank measurements.

	Accuracy Assessment		Precision Assessment		Matrix Assessment	
	RL_{obs}	RL_{calc}	RL_{obs}	RL_{calc}	RL_{obs}	RL_{calc}
Progesterone	4.79	3.06	NQ	NQ	0.116	0.270
17-Hydroxyprogesterone	0.115	0.0953	0.114	0.105	0.114	0.107
11-Deoxycorticosterone	0.104	–	NQ	NQ	NQ	NQ
Corticosterone	0.769	–	0.806	NA	0.447	1.66
11-Deoxycortisol	0.0781	0.0737	NQ	NQ	NQ	NQ
Cortisol	0.864	1.97	0.0903	NA	0.853	1.87
Cortisone	0.203	–	0.0836	NA	0.200	0.845
Testosterone	0.261	0.277	0.259	1.64	0.259	–
Androstenedione	0. 229	0.369	0.195	–	0.00971	0.0164
Estradiol	0.0343	0.378	NQ	NQ	NQ	NQ
Estrone	1.17	–	NQ	NQ	NQ	NQ

– = Negative value.
NQ = analyte not detected in experiment.
NA = could not be calculated.

Table 3
Mean spike mass by hormone compared to the maximum value of each measured in plasma of free-ranging animals.

Hormone	Mean Spike Mass (ng)	Maximum Value in Plasma (ng)
Progesterone	9.014	20.60
17-Hydroxyprogesterone	10.47	12.67
Androstenedione	1.069	3.907
Testosterone	11.41	57.25
Estrone	2.012	NA
Estradiol	3.562	NA
Cortisol	16.75	30.18
Cortisone	3.926	6.721
11-Deoxycortisol	3.393	NA
Corticosterone	8.776	16.24
11-Deoxycorticosterone	2.300	NA

calculation of percent recovery.

Eleven hormones, progesterone, 17-hydroxyprogesterone, androstenedione, testosterone, estradiol, estrone, 11-deoxycortisol, cortisol, cortisone, 11-deoxycorticosterone, and corticosterone, were included in this experiment. Method accuracy was determined by calculating percent recovery of each hormone per the following equation:

$$\%Recovery = \frac{RecoveredHormoneMass}{ExpectedHormoneMass} \times 100 = \frac{(a \times b)}{(c \times b) + (d \times e)} \times 100$$

where "a" is the measured hormone concentration (ng hormone/g sample), "b" is the sample mass (g), "c" is the endogenous hormone concentration measured in aliquot-matched unspiked samples (ng/g), "d" is the hormone concentration in the spike mixture (ng/g), and "e" is the spike mass (g). Relative standard deviations (RSDs) of percent recoveries were calculated by sex and matrix to assess method precision. A percent recovery between 70% and 120% with an RSD below 15% was considered comparable to existing techniques for accuracy and precision.

2.8. Precision assessment: comparison of endogenous steroid concentrations in plasma and serum pools

Due to a lack of sufficient serum from any single sampling date, serum pools from multiple sampling dates were thawed and re-pooled (by sex) to provide adequate volume of a homogenous pool for analysis (serum: n = 5 per sex, plasma: n = 4 per sex, calibration standard solution: n = 7, and blanks: n = 3). Upon addition of sodium acetate buffer to female serum samples, the serum coagulated preventing it from mixing with the buffer. Thus, after addition of buffer, these samples were sonicated for 1–2 min and vortexed for 30–60 s to try to disperse the clots. This sonicating-vortexing cycle was repeated one to two times until the clots appeared entirely dispersed or showed no improvement in dispersal. Any remaining solid debris was not transferred to the SPE column due to potential for clogging.

2.9. Matrix assessment: comparison of individual-matched plasma and serum

Individual-matched plasma and serum (n = 20), calibrants (n = 10), and blanks (n = 4) were extracted as described above. Serum coagulation occurred in six samples, and was remedied as before by repeated sonication and vortexing. To control for potential batch effects, two control materials (adult male plasma and pregnant female plasma) were produced by pooling individual samples. An aliquot of each was run once per day during sample processing; mean and RSDs for these replicates are reported in Table S6.

2.10. Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics 23 (IBM, North Castle, NY, USA). For all hypothesis tests, $\alpha = 0.05$. Pearson's (r) or Kendall's tau-b (τ_b) correlations were utilized for the matrix assessment experiment to examine the relationship between hormone measurements in matched serum and plasma. Kendall's tau-b was used for progesterone, 17-hydroxyprogesterone, testosterone, and androstenedione because these variables are left-censored (i.e., contain measurement values below RL), and, rather than substituting arbitrary values for measurements below the RL, we censored values below RL to zero and utilized this non-parametric test. Zero was utilized to ensure that the censored values were below the lowest true value, and that all values below RL would be tied in rank-based statistical tests. Cortisone and corticosterone measurements were not left censored, but neither raw nor $\log_{10}$ transformed values met the assumptions of Pearson's correlation, thus these relationships were also analyzed by Kendall's tau-b. For corticosterone, two extreme outliers were removed to improve clarity of graphs, but their inclusion did not influence the results of the statistical tests. Cortisol was not censored and met the assumptions of Pearson's correlation once $\log_{10}$ transformed, therefore the relationship between plasma and serum cortisol was assessed by Pearson's correlation. The relationships among plasma hormones were also analyzed by Kendall's tau-b.

Mann-Whitney U tests was used to assess how plasma progesterone varied by pregnancy status in females, and how testosterone, androstenedione, and 17-hydroxyprogestesrone varied by age class/sex (subadult and adult males). The relationships between elapsed time (i.e., time in minutes between onset of capture process [i.e., deployment of the net] to sample collection) and cortisol ($\log_{10}$ transformed), cortisone, and corticosterone measurements were assessed by Pearson's correlation and Kendall's tau-b, respectively.

3. Results

3.1. Accuracy assessment: spike retrieval

We performed a spike recovery experiment to examine method accuracy and precision in male and female plasma. The spike values were comparable in magnitude to the maximum values measured in plasma from free-ranging dolphins (Table 3). Eight of the eleven hormones met the criteria of acceptable accuracy (70%–120% recovery) and precision (< 15% RSD) in both sexes (Fig. 3). These were progesterone, 17-

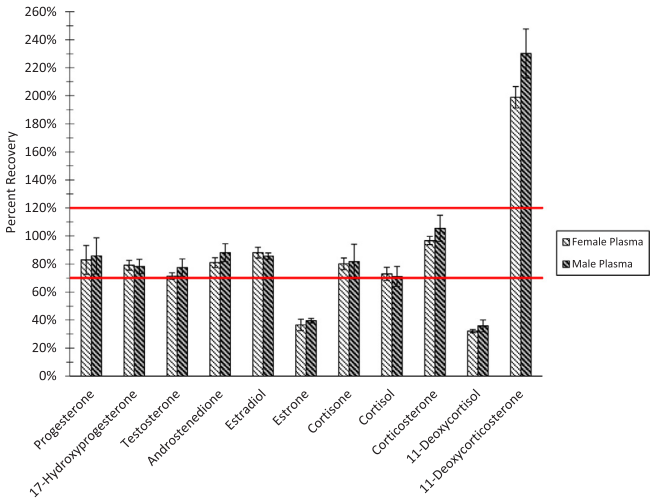

Fig. 3. Average percent recovery of each steroid hormone by sample matrix. Error bars indicate standard deviation, solid red lines indicate the upper and lower threshold values for acceptable recovery (between 70% and 120%).

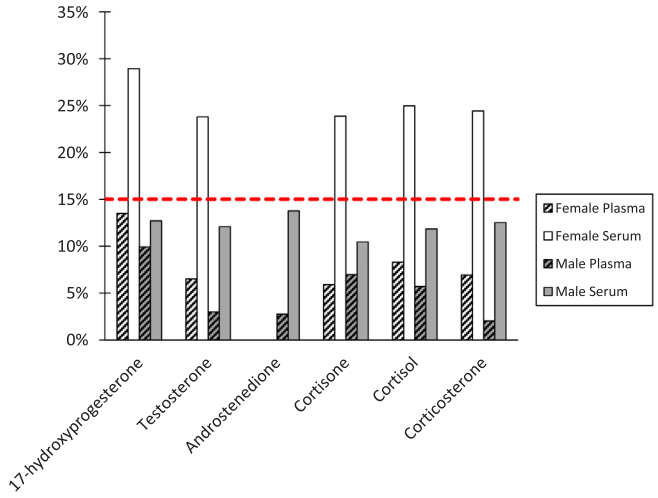

Fig. 4. Percent RSD of endogenous steroid hormone measurements made in quadruplicate or quintuplicate. Dashed red line indicates the threshold for acceptable precision (< 15% RSD).

hydroxyprogesterone, androstenedione, testosterone, estradiol, cortisol, cortisone, and corticosterone. Extraction efficiencies for estrone, 11-deoxycortisol, and 11-deoxycorticosterone did not meet the criteria for acceptance.

3.2. Precision assessment: comparison of endogenous steroid concentrations in plasma and serum pools

We assessed method precision by measuring endogenous hormone concentrations in pooled male and female plasma and serum in quadruplicate (plasma) or quintuplicate (serum), and calculating RSDs for each hormone within each matrix. As in the extraction efficiency experiment, an RSD less than 15% is considered acceptable precision. Endogenous progesterone concentrations were below the RL in all matrices. Androstenedione was only detectable and quantifiable in male matrices and exhibited RSD less than 15% in both serum and plasma (Fig. 4). 17-hydroxyprogesterone, testosterone, cortisone, cortisol, and corticosterone were detectable in both matrices from both sexes. 17-hydroxyprogesterone, testosterone, cortisone, cortisol, and corticosterone were below the 15% RSD threshold in female plasma, male plasma, and male serum, but not in female serum. RSDs for all quantifiable hormones were lower in plasma than in serum, regardless of sex.

3.3. Matrix assessment: comparison of individual matched plasma and serum

We assayed endogenous steroids in individual-matched serum and plasma from free-ranging bottlenose dolphins, and examined the relationships between hormone concentrations in each matrix. Hormone measurements in serum compared to plasma were significantly ($p < 0.05$) and positively correlated for all seven detectable hormones (17-hydroxyprogesterone [$\tau_b = 0.730$], progesterone [$\tau_b = 0.465$], testosterone [$\tau_b = 0.644$], androstenedione [$\tau_b = 0.674$], cortisol [$r = 0.822$], cortisone [$\tau_b = 0.758$], and corticosterone [$\tau_b = 0.569$]) (Fig. 5). Note that unlike in the previous experiments, which utilized pooled blood matrices, progesterone was quantifiable in several samples in this sample set.

We examined several hormone measurements by various sampling and demographic variables: age class (males only), pregnancy status (females only), and sample collection time. Plasma testosterone was significantly ($p < 0.05$) elevated in adult males compared to subadult males, while androstenedione and 17-hydroxyprogesterone were

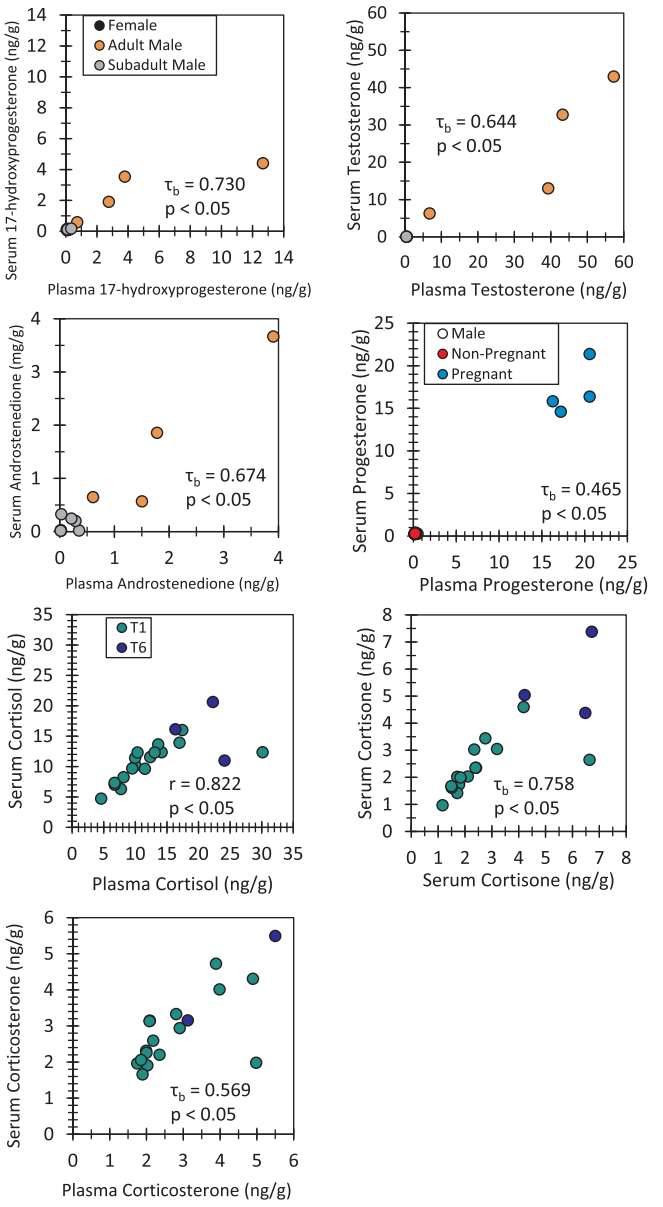

Fig. 5. Relationships between steroid hormone measurements in individual-matched plasma and serum.

elevated in adult males but not significantly so ($p = 0.052$ and 0.056, respectively) (Fig. 6A). Plasma progesterone was significantly ($p < 0.05$) elevated in pregnant females compared to non-pregnant females (Fig. 6B). Plasma cortisol and cortisone were significantly ($p < 0.05$) and positively ($r = 0.476$ and $\tau_b = 0.542$, respectively) correlated with elapsed time to sample collection, while plasma corticosterone was not ($\tau_b = 0.268$, $p = 0.127$) (Fig. 6C). Elapsed time ranged between 13 min and 47 min for T1 samples (median = 20 min) and 108 and 172 min for T6 samples (median = 116 min).

Several relationships between hormones within plasma were also assessed. Testosterone was significantly ($p < 0.05$) and positively correlated with androstenedione ($\tau_b = 0.557$) and 17-hydroxyprogesterone ($\tau_b = 0.360$), but androstenedione was not correlated with 17-hydroxyprogesterone ($\tau_b = 0.271$, $p = 0.121$) (Fig. 7). Testosterone and 17-hydroxyprogesterone were both positively correlated with cortisone ($\tau_b = 0.441$ and 0.362, respectively) (Fig. 7). Cortisol and cortisone were also positively correlated ($\tau_b = 0.705$) (Fig. 7). Corticosterone was significantly and positively correlated with plasma cortisol ($\tau_b = 0.556$), cortisone ($\tau_b = 0.621$), and 17-hydroxyprogesterone ($\tau_b = 0.477$) (Fig. 7)

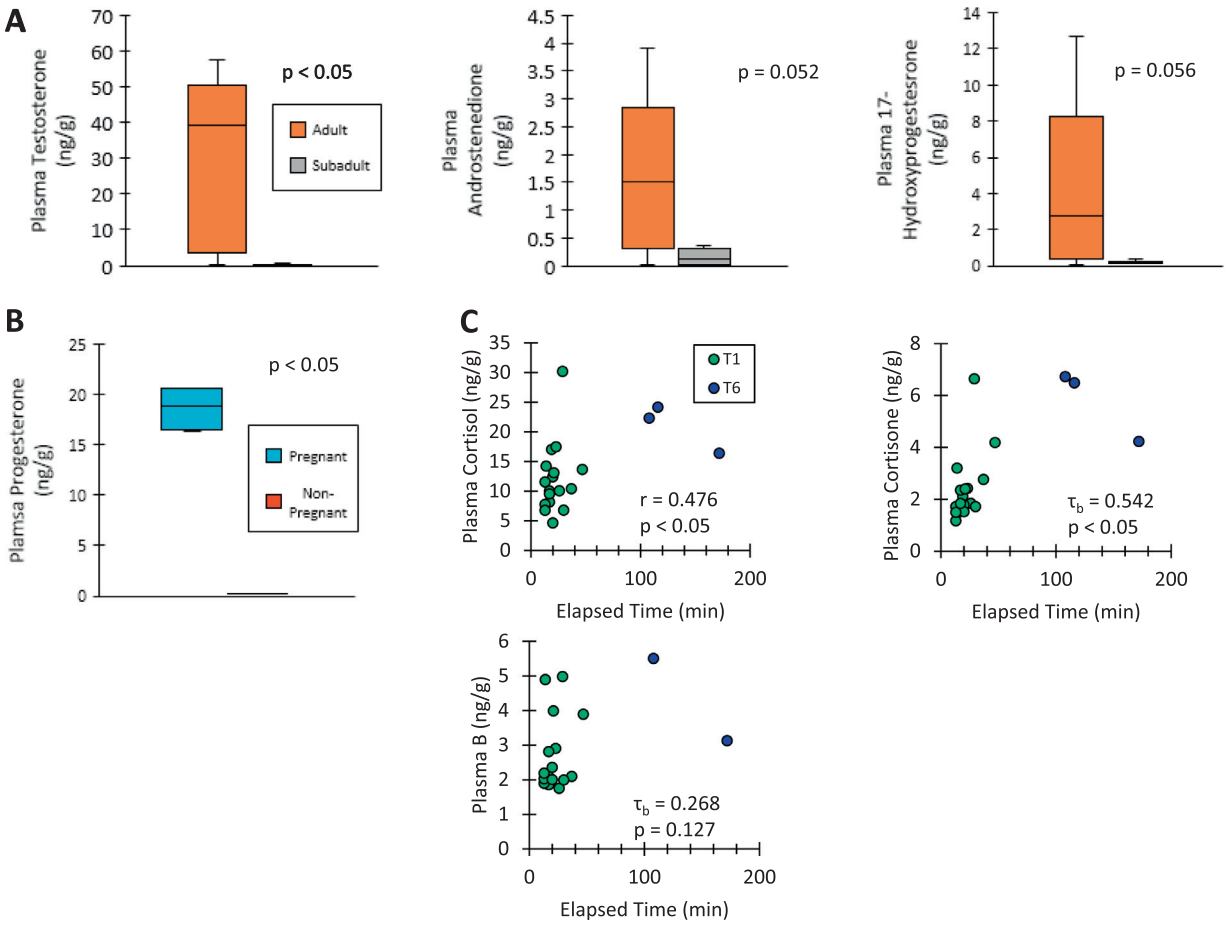

Fig. 6. Plasma steroid hormones by demographic and sampling variables. A) Plasma testosterone, androstenedione, and 17-hydroxyprogesterone concentrations by age class in males. B) Progesterone by pregnancy status in females. For A and B, box lower bound indicates the first quartile, the upper bound indicates the third quartile, and the horizontal line indicates the median. Whiskers are 1.5 times the interquartile range plus or minus the upper or lower bound, respectively. Values external to this range are included as individual points. C) Relationship between elapsed time to sample collection and plasma cortisol, cortisone, and corticosterone concentrations.

4. Discussion

The purpose of this study was to validate the use of SPE to LC–MS/MS methods to measure circulating steroid hormone profiles in bottlenose dolphins. Through the spike recovery experiment, we demonstrated that spiked quantities of eight of the eleven tested hormones (progesterone, 17-hydroxyprogesterone, testosterone, androstenedione, estradiol, cortisol, cortisone, and corticosterone) can be accurately and precisely extracted and quantified by SPE coupled to LC–MS/MS in bottlenose dolphin plasma. Thus, this method provides sufficient extraction efficiency and minimization of matrix effects to allow accurate steroid measurement. Dansyl chloride derivatization was required for the accurate measurement of estradiol, which exhibited poor percent recoveries in underivatized extracts. We did not use matched isotopically labeled internal standards for estrone, 11-deoxycortisol, and 11-deoxycorticosterone, which may be the reason they fail to meet the criteria for acceptance. Rather, we used alternative internal standard compounds to quantify these hormones by isotopic dilution; for estrone we used estradiol-$^{13}C_3$, and we tested both cortisol-d_4 and cortisone-$^{13}C_3$ for 11-deoxycortisol and 11-deoxycorticosterone, but these did not produce acceptable results. The difference in retention time in our chromatography between the target analytes (estrone = 18.7 min, 11-deoxycortisol = 20.5 min, 11-deoxycorticosterone = 22.3 min) and internal standards (estradiol-$^{13}C_3$ = 13.0 min, cortisone-$^{13}C_3$ = 12.4 min, and cortisol-d_4 = 16.0 min) indicates that estradiol-$^{13}C_3$ cortisone-$^{13}C_3$ and cortisol-d_4 are poor internal standards for these analytes. This experiment should be repeated with matched internal standards, if available. Without such standards, this method should not be used to measure estrone, 11-deoxycortisol, or 11-deoxycorticosterone in dolphin plasma. However, utility for qualitative assessments (i.e., absence/presence) is acceptable.

Having established that this method can accurately and precisely measure known quantities of several steroid hormones in plasma, we examined precision of endogenous hormone measurements in both plasma and serum. Six hormones were detected; these were 17-hydroxyprogesterone, testosterone, androstenedione, cortisol, cortisone, and corticosterone. For all six hormones, plasma exhibited lower RSDs than serum in both sexes. Measurements made in female serum were imprecise, exceeding the 15% RSD threshold by 8.9–14%, which may stem from the difficulties that arose during extraction (i.e. the coagulation issues discussed in Methods section). Because we were unable to fully disperse the clots and the remaining solid debris was not loaded onto the SPE columns, variable quantities of hormones could have been retained in the solid debris and thus left unextracted. This loss should be accounted for because the IS mixture was added before the buffer. Nonetheless, this could have introduced additional variation to female serum measurements, contributing to lower precision.

To our knowledge, this is the first time 17-hydroxyprogesterone, androstenedione, cortisone, and corticosterone have been measured in dolphin blood. Furthermore, because our chromatographic method enables us to do so, we qualitatively screened for endogenous concentrations of 10 other hormones, including pregnenolone, 17-

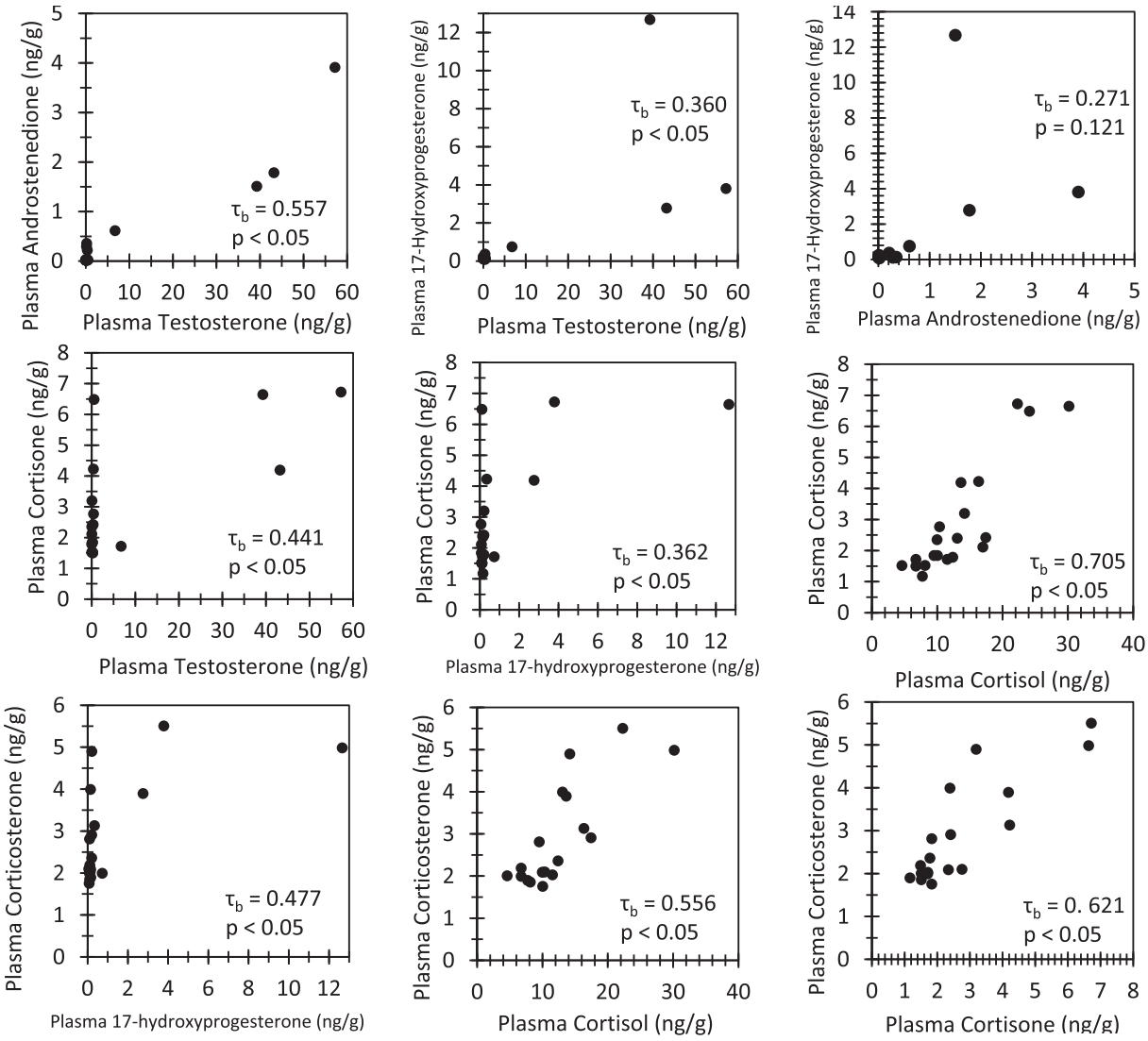

Fig. 7. Relationships among plasma hormones.

hydroxypregnonolone, progesterone, 11-deoxycorticosterone, 11-deoxycortisol, dehydroepiandrosterone, dihydrotestosterone, estrone, estradiol, and estriol. However, none of these eleven were detected in our pooled matrices. This is unsurprising for numerous reasons. Progesterone and estrogens have been measured in bottlenose dolphin blood in other studies, and circulating concentrations of these hormones depend on reproductive status (Bergfelt et al., 2011; Cornell et al., 1987; Kirby and Ridgway, 1984; O'Brien and Robeck, 2012; Sawyer-Steffan et al., 1983; Yoshioka et al., 1986). Progesterone should only be elevated in female individuals that are pregnant or in the luteal phase of the estrous cycle, while estrogens would be elevated in female individuals in the follicular phase of the estrous cycle, particularly immediately preceding ovulation (Bergfelt et al., 2011; Cornell et al., 1987; Kirby and Ridgway, 1984; O'Brien and Robeck, 2012; Robeck et al., 2005; Sawyer-Steffan et al., 1983; Yoshioka et al., 1986). None of the females in this portion of the study were pregnant. Bottlenose dolphins have been shown to exhibit somewhat seasonal patterns in reproductive activity, with the reproductively active season typically falling between spring and early fall (Kirby and Ridgway, 1984; Sawyer-Steffan et al., 1983; Urian et al., 1996; Yoshioka et al., 1986). Therefore, because sampling occurred in mid-fall, it is unlikely that any of the females sampled to produce the blood matrix pools were actively cycling. Furthermore, because these are pooled samples, even if an individual had elevated progesterone/estrogens, these hormones may be diluted to below RL by pooling with other, non-cycling animals. Thus, low progesterone and estrogen concentrations are to be expected in these blood matrix pools. Through our first (method accuracy) experiment, we demonstrated that we are able to accurately and precisely measure spiked quantities of progesterone and estradiol. Thus, this method could potentially be used for quantification of these hormones. Overall, this method provides improvement over traditional methods (i.e. immunoassays) by allowing for the simultaneous measurement of at least six (and potentially eight) steroid hormones at endogenous concentrations (Bergfelt et al., 2011; Cornell et al., 1987; Fair et al., 2014; Houser et al., 2011; Kirby and Ridgway, 1984; O'Brien and Robeck, 2012; Ortiz and Worthy, 2000; Sawyer-Steffan et al., 1983; Schroeder and Keller, 1989; St. Aubin et al., 1996; Steinman et al., 2016; Thomson and Geraci, 1986; Yoshioka et al., 1986). This will allow investigators to more thoroughly assess steroid hormone homeostasis and characterize relationships between hormones within and among steroid hormone classes in bottlenose dolphins.

Plasma is the preferred matrix for future applications of this method because plasma measurements exhibit better precision than serum measurements. Furthermore, plasma is unaffected by the coagulation issue observed in serum that we described above, making plasma much easier to process. Additionally, upon thawing serum pools, we found that a significant portion (roughly 20%–50%) of the volume of serum was coagulated before the addition of buffer. This coagulation

precluded mixing of the aliquot and caused difficulty when transferring serum into the culture tube. Again, this makes processing more difficult and potentially introduces additional variation. Therefore, due to concerns over precision and feasibility, plasma is better suited to this method. Because measurements in female serum all had RSDs greater than the 15% threshold, future applications of this method to female blood should use plasma. Some investigators might be hesitant to use plasma due to the use of anti-coagulant additives (sodium-heparin in this case) in the production of plasma, which introduces the potential for plasma-specific interferences. However, if plasma-specific interferences were problematic, they would have been evident in the method accuracy experiment. Plasma measurements were within the acceptable percent recovery range, meaning if plasma-specific interferences were present, they did not significantly impact method accuracy.

It is important to note that we did not compare hormone concentrations across matrices from our pooled samples because each matrix was derived from a separate pool. In other words, the blood used to produce the serum pools was collected on different dates than that for the plasma pools – roughly two weeks apart – which also means the pools may have been comprised of samples collected from different animals. Considering that hormone concentrations could vary temporally and by individual, comparing hormone concentrations across matrices with these pools would be inappropriate. Since several previous studies of bottlenose dolphin endocrinology have used serum while we, instead, recommend using plasma, it is important that we characterize the relationship between hormone measurements in both matrices.

Thus, we used individual-matched plasma and serum samples from free-ranging bottlenose dolphins to assess and compare between serum and plasma hormone measurements. We found that measurements were significantly and positively correlated across matrices. For cortisol and cortisone, these relationships seemed the strongest at low-to-mid plasma concentrations, while high plasma values were not well matched in serum. This could potentially be due to loss of hormone associated with coagulation during processing and/or extraction. Nonetheless, we have demonstrated that serum and plasma hormone values, as measured by SPE to LC–MS/MS, are in good agreement, providing assurance that measurements made in plasma are sufficiently comparable to serum. Future experiments with larger samples sizes and wider ranges of endogenous values could potentially yield quantitative models that can be used to predict plasma hormone concentrations from serum measurements (or vice versa).

This sample set included pregnant and non-pregnant females, subadult and adult males, and samples collected at different time points for specific individuals at both endpoints of the sampling process (T1 and T6). Progesterone secretion increases during pregnancy; thus we would anticipate elevated progesterone in pregnant compared to non-pregnant females, and this is indeed the case (Kirby and Ridgway, 1984; Sawyer-Steffan et al., 1983). Importantly, the inclusion of pregnant animals allowed us to detect and quantify endogenous progesterone, whereas it was undetectable in pooled samples. However, because we only measured progesterone once per sample, we cannot assess precision of these measurements. Testosterone is a marker of sexual maturity in male bottlenose dolphins (Harrison and Ridgway, 1971; Schroeder and Keller, 1989). Therefore, we anticipated that adult males would exhibit elevated concentrations of testosterone and its precursors compared to subadult males. Our results partially support this hypothesis – adult males have higher plasma testosterone concentrations compared to subadult males, while androstenedione and 17-hydroxyprogesterone are not significantly elevated. However, the relationships between age class and androstenedione and 17-hydroxyprogesterone in males are nearly significant ($p = 0.052$ and 0.056, respectively), indicating further investigation is warranted. While androstenedione and 17-hydroxyprogesterone are not elevated in adult males, the positive correlations between testosterone and these precursors suggest that production of androstenedione and 17-hydroxyprogesterone increases to support elevated testosterone secretion. This conclusion is somewhat subverted by the fact that androstenedione and 17-hydroxyprogesterone are not significantly correlated. The strength of each of these conclusions is limited by low sample size; future studies should target to sample more adult males to better assess these relationships.

Capture and handling stimulates the hypothalamic-pituitary-adrenal axis, leading to elevated secretion of cortisol in bottlenose dolphins (St. Aubin et al., 1996; Thomson and Geraci, 1986). Thus, we anticipated that cortisol would be positively correlated with elapsed time to sample collection. We included T1 and T6 samples to widen the range of elapsed time in our sample set. Furthermore, cortisone and cortisol are metabolically linked (Fig. 1); therefore, we anticipated that cortisone would exhibit a similar relationship with elapsed time. Corticosterone is also a glucocorticoid and may be a minor product of the dolphin adrenal gland, therefore we examined the relationship between corticosterone and elapsed time also. Cortisol and cortisone were positively correlated with elapsed time, and there was a strong positive relationship between plasma cortisol and cortisone values, supporting our hypotheses. Corticosterone was not significantly related to elapsed time but was positively correlated plasma cortisol and cortisone. Interestingly, cortisone was also positively correlated with testosterone and 17-hydroxyprogesterone, and corticosterone was positively correlated with 17-hydroxyprogesterone, which could potentially arise from direct gonadal-adrenal axis crosstalk (i.e., testosterone regulating corticosteroid metabolism, or vice versa), which has been observed in other species (Rabin et al., 1988; Rivier and Rivest, 1991; Whirledge and Cidlowski, 2010). Alternatively, since testosterone was only elevated in adult males, this may be a spurious relationship resulting from age and/or sex-specific changes in corticosteroid metabolism unrelated to testosterone and 17-hydroxyprogesterone. As before, these conclusions are limited by small sample sizes and left censoring. These relationships are currently being investigated in additional studies with larger sample sizes. We performed these tests and report these limited conclusions simply to demonstrate several potential applications of this method.

As in the previous experiments, endogenous estrogens were not detected in any matched serum/plasma samples. In this experiment, our lowest calibration standard, with estradiol concentration of 45.3 pg/g, had a distinct estradiol peak, indicating we should be able to detect concentrations at this level. In bottlenose dolphins, baseline circulating estradiol concentrations have been measured at less than 50 pg/mL (approximately 48.8 pg/g, calculated from density of human serum), while concentrations during the preovulatory surge at the end of the follicular phase of the estrous cycle tend to fall between 50 pg/mL and 100 pg/mL (approximately 97.7 pg/g) (Robeck et al., 2005; Sniegoski and Moody, 2002; Yoshioka et al., 1986). Thus, we conclude that this method likely has the capacity to detect and quantify estradiol surge values, but potentially cannot be used to measure baseline values as currently defined within the literature. Granted, these baseline and surge values were established by immunoassay in serum, meaning they could potentially be different in plasma and in measurements made by LC–MS/MS, given the limitations of immunoassays discussed above and considering potential differences in serum and plasma.

Overall, we have demonstrated that use of an SPE to LC–MS/MS method allows for the simultaneous measurement of multi-class steroid hormones in bottlenose dolphin blood matrices, including not only hormones that have been measured previously by immunoassay (progesterone, testosterone, estradiol, and cortisol) but four hormones that, to our knowledge, have not been reported for dolphin blood (17-hydroxyprogesterone, androstenedione, cortisone, and corticosterone). Immunoassay methods typically used for endocrine assessment in dolphins are limited to the measurement of a single hormone per assay and are hindered by antibody cross-reactivity. Therefore, this SPE to LC–MS/MS method allows for more thorough assessment of steroid hormone homeostasis in bottlenose dolphins with improved efficiency

and specificity.

5. Compliance with ethical standards

Sample collections at the U.S. Navy Marine Mammal Program were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the Biosciences Division, Space and Naval Warfare Systems Center Pacific and the Navy Bureau of Medicine and Surgery, and followed all applicable U.S. Department of Defense guidelines for the care and use of laboratory animals. Sarasota Bay sampling was performed under National Marine Fisheries Service (NFMS) Scientific Research Permit No. 15543 and annually renewed IACUC approvals through Mote Marine Laboratory. Barataria Bay and Brunswick sampling was conducted under NMFS permit No. 932-1905/MA-009526 with protocols reviewed and approved by National Oceanic and Atmospheric Administration IACUC.

Conflict of interest

The authors declare that they have no conflict of interest in the publication of this manuscript. Commercial equipment, instruments, or materials are identified to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology nor the National Oceanographic and Atmospheric Administration, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2018.04.003.

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