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1 Remote Blubber Sampling Paired with Liquid Chromatography Tandem Mass Spectrometry for
2 Steroidal Endocrinology in Free-Ranging Bottlenose Dolphins (*Tursiops truncatus)*

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Abbreviations:
ACE: Ashepoo, Combahee, Edisto River
CAWS: Charleston Area Waterways
ECDFs: Empirical Cumulative Distribution Functions
DMSO: Dimethyl Sulfoxide
LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry
LOD: Limit of Detection
PCA: Principal Components Analysis
POPs: Persistent Organic Pollutants
RL: Reporting Limit
RSD: Relative Standard Deviation
SRM: Standard Reference Material

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27 **Abstract**

28 Liquid chromatography tandem mass spectrometry allows for the measurement of steroid
29 hormone suites in the blubber of marine mammals. By combining this technology with
30 minimally invasive techniques such as remote biopsy, endocrine profiles can be assessed,
31 allowing for studies of hormonal profile variation over time. In this study, we explored
32 associations among different steroidogenic pathways and seasonal differences in blubber
33 hormone profiles of free-ranging common bottlenose dolphins along the coast of South Carolina,
34 USA. Male dolphins experience a peak in testosterone, androstenedione, progesterone, and 17-
35 hydroxyprogesterone in the spring, likely related to an upregulation of the androgen
36 steroidogenic pathway during mating season. We also observed increased cortisol
37 concentrations during summer compared to winter. Among females, there was an increase in
38 androstenedione with elevated progesterone concentrations indicative of pregnancy, highlighting
39 another potential endocrine marker for pregnancy in free-ranging dolphins. This work
40 emphasizes the importance of selecting the appropriate season for studies on endocrine status to
41 effectively uncover physiological variation or disruption in free-ranging cetaceans.

42 **Keywords**

43 Remote biopsy, hormones, cetacean, blubber, reproductive steroids, stress

44

1. Introduction

Monitoring steroid hormones is an effective method for determining reproductive status and can contribute to the assessment of health status in free-ranging marine mammals (De Mello and De Oliveira, 2016; Kellar et al., 2015; Kellar et al., 2006; Lane et al., 2015; Schwacke et al., 2014; Steinman et al., 2016). Steroid hormones circulate throughout the body in the blood and regulate sexual maturation, reproduction, and stress response. However, collecting blood samples from free-ranging individuals to monitor stress hormones (corticosteroids) in cetaceans is difficult as these species, are highly mobile and require time consuming methods for safe restraint to obtain a blood sample (Schwacke et al., 2009), which in turn provides time for cortisol concentrations to rise in the blood due to the acute stress event. Studies involving temporary capture of some cetacean species have been conducted; however, these are limited to a few small cetacean species and are generally constrained to shallow, nearshore waters (Schwacke et al., 2014; Wells et al., 2004). Capture and handling of the subject animal can also be problematic for the assessment of steroid hormones, particularly corticosteroids, as the time to collection can stress the animals and alter the endocrine profile (Champagne et al., 2017; Kellar et al., 2015). Therefore, identifying an endocrinologically-relevant matrix that can be collected remotely would be highly beneficial for monitoring steroid hormones and improve cross-study comparison.

Remote biopsy sampling of cetaceans is proven to be a safe and efficient methodology to collect blubber from free-ranging individuals (Gorgone et al., 2008; Kiszka et al., 2010; Weller et al., 1997), and blubber is an endocrinologically relevant tissue and has been used to examine male sexual maturity (testosterone), pregnancy (progesterone), and stress in managed individuals or collected post-mortem (cortisol) (Champagne et al., 2017; Champagne et al., 2018; Kellar et

68 al., 2015; Kellar et al., 2009; Kellar et al., 2006; Krutzen et al., 2002). However, because the
69 animal cannot be handled using this method, collection of additional demographic data (size,
70 age, sexual maturity) is not possible in untracked populations and requires a separate analysis for
71 sex determination. Additionally, the technique has been shown to be biased towards sampling of
72 male dolphins (Quérouil et al., 2009). Despite these shortcomings, this sampling technique
73 requires fewer resources than capture-release studies, allowing for increased sample size from
74 less sampling effort, and provides rapid sampling, which limits the changes in stress hormones
75 due to collection processes.

76 The recent application of liquid chromatography tandem mass spectrometry (LC-
77 MS/MS) methods to measure steroids in blubber provides substantial advantages over the
78 traditional steroid quantification methods, i.e. immunoassays (Boggs et al., 2017; Mary et al.,
79 2017). One major advantage of LC-MS/MS over immunoassay techniques is that multiple
80 hormones are determined simultaneously. Although the main criticism of LC-MS/MS is that
81 immunoassays can attain superior sensitivity, the method developed by Boggs et al. (2017) has
82 demonstrated limits of detection in the pg/g range, making it sufficient for most analyses
83 attempting to detect physiological differences. If greater sensitivity for differences in low
84 concentration hormones is necessary, immunoassays could provide a benefit. None-the-less, by
85 analyzing suites of steroid hormones, collecting broader information on reproductive and stress
86 physiology is possible from a single analysis which can then better inform follow up analyses.
87 While pilot data from Boggs et al. (Boggs et al., 2017) demonstrated that steroid hormones are
88 quantifiable by LC-MS/MS in remote biopsies from common bottlenose dolphins, further
89 analysis is required to determine the biological relevance of these hormones at ambient
90 concentrations in a free-ranging population.

91 Understanding seasonal hormone variation in free-ranging populations is critical for
92 assessing changes in hormone concentrations induced by reproductive events, endocrine
93 disruption, or chronic stressors. Many species of cetaceans experience seasonal fluctuations in
94 reproductive hormone concentrations (Kellar et al., 2009; Kirby, 1984; Schroeder and Keller,
95 1989; Yoshioka et al., 1986) that, when not considered during a study design, could confound the
96 interpretation of other exogenous influences on hormones at the population level. Although
97 dolphin reproductive hormones are known to fluctuate with season, little is known about
98 seasonal fluctuations in stress hormones. Annual fluctuations in serum cortisol have been
99 described in managed orcas (*Orcinus orca*) and common bottlenose dolphins (Orlov et al., 1988;
100 Suzuki et al., 2003) which both displayed elevated circulating cortisol in the winter or spring
101 months. However, these fluctuations have not been described in free-ranging common
102 bottlenose dolphins nor has it been found using blubber biopsies. Elevated seasonal
103 concentrations of cortisol, if they do exist among free-ranging populations, can be a confounding
104 factor when attempting to assess potential disturbances such as acoustic noise, contaminants, or
105 other anthropogenic activities.

106 Here we describe the analysis of steroid hormone profiles in remote blubber biopsies
107 from common bottlenose dolphin (*Tursiops truncatus*) in the confluence of the Ashepoo,
108 Combahee, and Edisto Rivers (hereafter referred to as the ACE Basin) and waters in the
109 Charleston area of South Carolina, USA (Charleston Area Waterways System hereafter referred
110 to as CAWS), from October 2011 to August 2012. These populations represent some of the
111 northernmost resident estuarine stocks in the United States, and are hypothesized to display
112 greater seasonal variation in steroid hormones, if such patterns exist. The ACE Basin contains a
113 National Estuarine Research Reserve that is considered to have relatively low anthropogenic

114 activity. Additionally, the ACE Basin and CAWS have no statistical difference in concentrations
115 of persistent organic pollutants (POPs) in the male populations (Neely et al., 2018). Blubber
116 POP concentrations were comparable and intermediate to other southeastern populations of
117 common bottlenose dolphins and are not found in concentrations of high concern (Balmer et al.,
118 2015b; Kucklick et al., 2011). Therefore, these populations were selected to establish seasonal
119 baseline ranges for blubber steroid hormone concentrations. These data constitute the first report
120 on seasonal variation of baseline multi-steroid hormone profiles from blubber of free-ranging
121 common bottlenose dolphins.

122 ## 2. Materials and Methods

123 ### *2.1 Sample Collection*

124 Common bottlenose dolphins from inshore waters of the South Carolina, including the
125 ACE Basin and CAWS, were targeted for this study. The CAWS collectively includes the North
126 Edisto River, the Charleston Harbor, the Cooper, Ashley, and Wando Rivers, and the Stono
127 River estuary (Figure 1). Remote biopsies (n = 93) were collected from October 2011 to August
128 2012 using the methods described in Balmer et al. (2015a). Seasons were classified by
129 equinoxes and solstices for that collection year (Fall = October 1, 2011 – December 21, 2011,
130 Winter = December 22, 2011 – March 19, 2012, Spring = March 20, 2012 – June 19, 2012,
131 Summer = June 20, 2012 – August 31, 2012). For each season (winter, spring, summer, and fall)
132 the number of males sampled were 18, 14, 33, and 16 respectively and the number of females
133 were 7, 2, 3, and 0 respectively. Upon collection, skin was removed from the biopsy sample and
134 stored in 20 % dimethyl sulfoxide (DMSO) saturated with sodium chloride and were sent to the
135 National Oceanic and Atmospheric Administration National Marine Fisheries Service Southeast
136 Fisheries Science Center, Marine Mammal Molecular Genetics Laboratory for determination of

137 sex and population stock. Using the methods described by Rosel et al. (2003), X and Y
138 chromosomes were amplified using polymerase chain reaction in DNA extracted from the skin to
139 identify sex. Blubber samples were full depth (determined qualitatively by presence of
140 connective tissue, muscle, or gradation of vasculature) and maximally 10 mm X 25 mm deep and
141 0.8 g. The blubber was halved length-wise to produce two full depth samples approximately 0.4
142 g to 0.6 g in mass. These sections were placed in separate cryovials then flash frozen in a liquid
143 nitrogen vapor shipper within a mean time of 12 min after collection. One of the frozen blubber
144 subsections was allocated for hormone analysis and was transported to the National Institute of
145 Standards and Technology (NIST) biorepository, in Charleston, SC. There, samples were
146 archived at -80 °C until processing. The remaining subsection was archived for future analyses.
147 *2.2 Sample Preparation and Analysis*
148 Samples were homogenized and steroids extracted according to the methodology
149 described in Boggs et al. (2017). Briefly, an internal standard mixture (concentrations in
150 Supplemental Table 1) consisting of testosterone-$^{13}C_3$, androstenedione-$^{13}C_3$, 17-
151 hydroxyprogesterone-$^{13}C_3$, and cortisol-d_4 (Cerilliant; Round Rock, TX, USA; 99.99 % purity)
152 and progesterone-$^{13}C_3$ (Cambridge Isotopes; Tewksbury, MA, USA; 98 % purity) was
153 gravimetrically added to a garnet bead homogenization tube (Mo Bio, San Diego, CA, USA).
154 When exact matched internal standards were not available, the most structurally similar internal
155 standard was substituted (see Boggs et al. 2017 for more details on this method). Remote
156 biopsies were then minced in a glass beaker on dry ice, added to the homogenization tube, and
157 mass of the added blubber recorded. Samples were homogenized at 6500 rpm for 30 s four times
158 using a Precellys bead homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France).
159 Homogenates were extracted and cleaned using the Bond Elut QuEChERS EN Extraction kit and

the Bond Elut QuEChERS dispersive-SPE kit for Drug Residues in Meat (Agilent, Santa Clara, CA, USA). The extract was then filtered through a 0.22 µm cellulose acetate spin filter before being reduced to dryness under nitrogen and reconstituted in 200 µL of methanol.

The quality control sample used was NIST Standard Reference Material (SRM) 1945, Organics in Whale Blubber. While there is no matrix matched SRM currently with certified or reference hormone measurements, this material is a large sample of homogenous whale blubber and thus, could be used as quality control across batches. Means and relative standard deviations (RSDs) for SRM 1945 were calculated to track repeatability of the method across the different extraction days. Blanks were also collected on all extraction days to test for contamination.

A six point calibration curve and internal standard blanks were extracted identically to the samples. Calibrants were androstenedione, testosterone, 17-hydroxyprogesterone, progesterone, corticosterone, cortisone, and cortisol (Sigma Aldrich, St. Louis, MO; ≥ 98 % purity), 11-deoxycorticosterone, and 11-deoxycortisol (Steraloids, Newport, RI; ≥ 98 % purity). Calibration standard range of steroid masses (Supplemental Table 2) and internal standard mixture concentrations were calculated and tracked gravimetrically.

2.3 Instrumental method

An Agilent 1200 Series HPLC system coupled to an AB Sciex API4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer was used to monitor two product ions, one for quantitation and one for identity confirmation, using scheduled multiple reaction monitoring for each steroid and internal standard. Chromatography was conducted on a Restek (Bellefonte, PA, USA) Ultra Biphenyl column (250 mm x 4.6 mm, 5.0 µm particle size) with acetonitrile and methanol both containing 0.1 % formic acid (volume fraction) for reproductive hormones. A flow of 500 µL/min was used for a solvent gradient of 20 % acetonitrile increased to 45 % over

183 30 min., then increased to 80 % over 1 min and held for 4 min, then washed with 100 %
184 acetonitrile for 5 min and re-equilibrated at 20 % for 10 min. Corticosteroids were separated on
185 an Agilent (Santa Clara, CA, USA) Eclipse Plus C18 column (21 mm X 150 mm, 5.0 µm particle
186 size) with methanol and water both containing 0.1 % acetic acid (volume fraction). Column
187 conditions were as follows: flow rate of 250 µL/min, and isocratic method of 46 % methanol for
188 20 min, a wash of 100 % methanol for 13 min, and re-equilibrated for 10 min. Additional
189 information on compound and instrument parameters can be found in Boggs et al. (Boggs et al.,
190 2017).

191 *2.4 Quantitation*

192 Masses of each analyte were calculated using linear regression of calibration standards
193 that bracketed observed sample peak area ratios (area of the analyte divided by the area of the
194 appropriate internal standard; Supplemental Table 2). Concentrations were determined by
195 dividing the calculated mass of each analyte by the extracted sample mass (mass fraction).
196 Therefore, results are presented in ng of steroid per g of wet weight blubber. Steroid
197 concentrations were not normalized by lipid mass because cortisol has been shown to correlate
198 with percent lipid in the blubber (see Galligan et al. (2019) for further explanation). Limit of
199 detection (LOD) was determined as the mean plus three times the standard deviation of the batch
200 blanks (methanol with internal standard extracted identically to the samples) for each analyte.
201 Reporting limit (RL) of the method was defined by the lowest calibration standard in the
202 regression analysis or the LOD if it was higher than the lowest calibration standard. This is a
203 conservative method of defining the RL as discussed by Ragland et al. (2014).

204 *2.5 Statistical Analysis*

Values below the RL used the RL as a replacement value and were flagged. Statistical analyses and visualizations were generated using R (Team, 2013) (primarily packages “tidyverse” (Wickham, 2017) and “NADA” (Lee and Lee, 2017)). Concentration data were grouped by analyte, sex, and season of collection. Sample sets under these defined criteria with 100 % detection used standard distribution-based estimates of central tendency and spread as well as standard t-test/ analysis of variance (ANOVA) comparison tests with distribution-appropriate transformation if necessary. Statistical assessment in this traditional manner suffers from the presence of values below the RL. Replacement of values below the RL with an arbitrary value (e.g. zero, RL, RL/2, etc.) modifies the underlying distribution and skews measures of central tendency as well as confounding comparison tests (Helsel, 2012). Helsel’s approaches for central tendency estimates and significance testing as implemented in NADA were used for data sets with < 100 % detection frequency (Helsel, 2012). Briefly, for central tendency estimates, percent of samples above the group maximum RL was used where detection frequency was less than 20 %, sample sets where detection frequencies were 20 % to 50 % used robust regression on order statistics, and sample sets with detection frequencies 50 % to 99.9 % used the Kaplan-Meier method to estimate empirical cumulative distribution functions (ECDFs); measures of central tendency and spread were drawn from these statistics, as appropriate given the parameters of each group, minimizing the impacts of data below the RL. Due to sparsity and detectability, Helsel’s methods (Helsel, 2012) were used for seasonal and geographic comparison between sample sets. This approach 'flips' left-censored data (data below the RL) around an arbitrarily large constant, resulting in right-censored data suitable to survivorship analysis statistics, such as group wise rank order comparison tests across ECDFs. These tests - and others suited to different data set properties - are available and easily implemented using the NADA package in R.

228 Females were excluded from seasonal analysis due to the small number of samples. Hypothesis
229 testing between sample sets with < 100 % detection in all sample sets used NADA's "cendiff()"
230 comparison between ECDFs. Principal components analysis ("prcomp()") and package
231 "factoextra" (Kassambara and Mundt, 2017) were used for visual data exploration of
232 multivariate analyte pattern relationships. Correlations across hormones, and between hormones
233 and TEO, were assessed using a censored version of Kendall's tau as implemented in NADA
234 ("NADA::cenken()") and visualized as tile plots (across hormones) and annotated scatter plots
235 (hormones and TEO, using the Akritas-Theil-Sen slope estimate and the Turnbull intercept
236 estimate). One outlier was identified in the hormone/TEO data set and removed as a case study
237 and the hormone/TEO assessment repeated. Potential seasonal fluctuation of TEO between
238 spring and summer (the only two seasons for which data were available) was assessed by t-test
239 after meeting assumptions of normality and homoscedasticity. Significance levels ($\alpha = 0.05$) for
240 rejection of H_0 were consistent throughout; all tests were two-sided.

241 ## 3. Results and Discussion

242 *3.1 Quality Control*

243 SRM 1945 had good repeatability for the detectable sex steroids (RSD < 15 %; Table 1).
244 However, this material is from a stranded female pilot whale. Therefore, progesterone
245 concentrations were elevated, while testosterone was not detectable. The RSDs for SRM 1945
246 were comparable to limits for immunoassays (< 12 %) for all the corticosteroids except for 11-
247 deoxycorticosterone (RSD = 25 %). The high RSD (greater than 15 %) of 11-
248 deoxycorticosterone at concentrations comparable to the dolphin blubber biopsies indicates that
249 the quantification of this hormone is not acceptable for the low concentrations in this study.

250 *3.2 Reproductive Steroids*

251 Androstenedione, 17-hydroxyprogesterone, and progesterone were detected in males and
252 females, while testosterone was only quantifiable in males (Figure 2). Of the 12 females
253 sampled, only three (ID: TYP-111215-01, TYP-120314-02, and TYP-120814-06) had
254 quantifiable concentrations of progesterone. Using the blubber progesterone concentration limits
255 for pregnancy (100 ng/g) defined for other dolphin species (Kellar et al., 2013; Kellar et al.,
256 2006; Trego et al., 2013), TYP-120814-06 (summer; 135 ng/g) would be classified as pregnant.
257 TYP-111215-01 (winter; 77.7 ng/g) and TYP-120314-02 (spring; 11.9 ng/g) had elevated
258 progesterone concentrations which would define the individual as a non-pregnant mature female.
259 However, androstenedione was elevated in these two female dolphins exhibiting moderately
260 elevated progesterone signals. Androstenedione production increases in humans, horses, and
261 killer whales (*Orcinus orca*) during pregnancy (Castracane and Asch, 1995; Kuijper et al., 2013;
262 Legacki et al., 2016; Robeck et al., 2017) potentially as a pathway to increase estrone and estriol
263 production as well as via direct stimulation of luteal progesterone production in early pregnancy
264 (Begumhasan and Murphy, 1992; Carrizo et al., 1994). While demographic information to
265 determine the pregnancy status of female dolphins in this study were not available, it was
266 demonstrated that androstenedione can be quantified in remotely collected blubber of female
267 dolphins should it be found to be an important indicator of pregnancy.

268 Androgens were quantifiable in most male samples (testosterone = 90 %,
269 androstenedione = 95 %; Figure 2). Because remote biopsy sampling precludes determination of
270 reproductive maturity using parameters such as age or testis size, we cannot conclude whether
271 individuals were immature, quiescent, or senescent. However, demonstration of the
272 quantification of an additional androgen in remote biopsies provides an additional target to study
273 male maturity.

274 Among males, progestogens and androgens were correlated with each other (Figure 3). 275 A positive relationship between the two androgens is expected ($p < 0.001$, tau = 0.745) as 276 androstenedione is a precursor hormone to testosterone. There is also a positive correlation 277 between progesterone and 17-hydroxyprogesterone and both androgens (progesterone: 278 testosterone $p < 0.001$, tau = 0.371, progesterone: androstenedione < 0.001 tau = 0.354, 17- 279 hydroxyprogesterone: testosterone $p < 0.001$, tau = 0.704, and 17-hydroxyprogesterone: 280 androstenedione $p < 0.001$ tau = 0.640). Progesterone and 17-hydroxyprogesterone are also 281 precursors to the cortisol pathway, but the correlations between the androgens and progestogens 282 suggest that the testes, rather than the adrenal glands, are the source of circulating progesterone 283 and 17-hydroxyprogesterone in unstressed male common bottlenose dolphins. Thus, caution 284 should be taken in including progestogens in future analyses to assess stress response in remote 285 biopsied males during reproductive events.

286 *3.3 Corticosteroids*

287 Cortisone, 11-deoxycortisol, corticosterone, and cortisol were detected in both males and 288 females (Figure 2). Cortisol was quantified in 81 % of the male samples and 75 % of the female 289 samples. All but 7 samples measured for cortisol were below 2.0 ng/g (Figure 2) with a RL of 290 0.181 ng/g. The lowest comparable concentrations measured in the blubber from dolphins were 291 4 ng/g from bycatch dolphins that assumingly died quickly after an acute stress (Kellar et al., 292 2015) and 1.4 ng/g lipid from volunteered samples from managed dolphins at baseline stress 293 (Champagne et al., 2017). Though this value is in ng/g lipid and not directly comparable, if we 294 generously assume the lipid percentage to be even 75 % of the tissue by weight, this would yield 295 an estimate of 1.1 ng/g, a value comparable to measurements from this study. Three additional 296 corticosteroids were quantified. 11-Deoxycortisol was the next most frequently detected

297 hormone at 57 % and 75 % for male and female samples respectively. Corticosterone and
298 cortisone were quantified in ≤ 20 % of male and female sample sets. Therefore, it is reasonable
299 to conclude that remote biopsy to LC-MS/MS techniques can be used for the assessment of
300 baseline stress hormone concentrations.

301 Cortisone and cortisol concentrations were correlated ($p = 0.001$, $tau = 0.264$) suggesting
302 that either blood cortisone, like cortisol, is transferred to the blubber or that cortisol/cortisone
303 metabolism could occur in blubber from a living dolphin. In vitro enzymatic conversion of
304 cortisol and cortisone occurs in blubber of marine mammals (Galligan et al., 2018). However,
305 cortisone was only detectable in 15 % of the males sampled, and there was a minimum threshold
306 concentration of blubber cortisol of 1.6 ng/g before cortisone was detectable. If the statistical
307 analysis is limited to individuals where both cortisone and cortisol are quantifiable with this
308 method ($n = 12$), the p-value remains the same and the tau increases to 0.697. Therefore, the
309 relationship between cortisol and cortisone in the blubber potentially is stronger than the
310 detection limits of this method allow us to investigate and a future analysis using immunoassay
311 techniques or dolphins under higher stress conditions could elucidate this relationship.

312 Progesterone is a precursor to corticosteroid biosynthesis. Thus, as one would expect,
313 analysis of male corticosteroid pathways showed a relationship between progesterone and
314 cortisone, cortisol, and corticosterone ($p < 0.05$, $tau = 0.119, 0.182, 0.114$, respectively; Figure
315 3A). In stressed male cattle, circulating progesterone and corticosteroid concentrations are
316 correlated (Welsh and Johnson, 1981). However the relationship in this study was weaker than
317 the relationship between progesterone and the androgens, as would be expected in a dart biopsied
318 individual that has presumably not experienced a major stressor before sampling.

319 When the full steroid pathway in males was analyzed using principal components
320 analysis (PCA), reproductive steroids separated from corticosteroids (Figure 3B). As the
321 correlational data and vertebrate steroid hormone pathways suggest, progesterone is a pivotal
322 hormone relating to both the reproductive pathways and the stress pathways in males. Therefore,
323 as previously stated, progesterone should be investigated as a stress hormone, but caution should
324 be given during reproductive events that activate the androgen pathway and could confound
325 results.

326 *3.5 Seasonality*

327 All hormones were assessed for differences among the dolphins sampled in CAWS and
328 ACE. Hormones did not differ significantly in CAWS and ACE dolphins within any season.
329 Therefore, the dolphins sampled in these sites were combined and assessed as one regional
330 group. Insufficient sample sizes were collected for females to conduct seasonal analysis. Thus,
331 all seasonal data discussed are from male dolphins.

332 Seasonal differences were found in reproductive steroids among males (Figure 4). A
333 peak in all measured progestogens and androgens (17-hydroxyprogesterone, progesterone,
334 androstenedione, and testosterone; p-values < 0.001 for all hormones) occurred during spring.
335 Spring peaks in reproductive hormones likely coincides with mating seasons in this region
336 (McFee et al., 2014). There was also a nadir in testosterone and 17-hydroxyprogesterone during
337 fall, potentially indicating a seasonal period of reproductive quiescence for this population.

338 Progesterone, while normally considered a female hormone for its role in pregnancy, also
339 increased during the spring in males ($p < 0.001$). However, as discussed previously,
340 progesterone, through its conversion to 17-hydroxyprogesterone, can lead to androgen synthesis.
341 Additionally, progesterone and 17-hydroxyprogesterone were correlated among males in this

342 study ($p < 0.001$, tau = 0.403; Figure 3A) as well as with the two androgens. This suggests that
343 the significant increase in progesterone in males in spring could be a contributing pathway for
344 the synthesis of androgens for reproductive activities.

345 We were unable to categorize the remotely sampled dolphins into age-classes, therefore
346 the steroid measurements represent an unknown mix of sexually mature and immature males.
347 This likely contributed to the variance of androgen measures in the spring when sexually mature
348 males would be expected to have elevated androgen concentrations, while immature males
349 would not. Regardless of this disadvantage to remote biopsy techniques, a peak in androgens and
350 progestogens in the spring was detected and the relationship among the androgens and
351 progestogens was defined both through correlations and the PCA. Therefore, this technique can
352 be used to identify reproductive seasons in understudied populations. Additionally, three
353 hormones in addition to testosterone have been identified as possible biomarkers to investigate
354 male maturity through blubber dart biopsy. This study demonstrates the importance of selection
355 of the appropriate hormone targets as well as ideal season for analysis to increase the likelihood
356 of successful determination of male maturity of free-ranging dolphins.

357 Seasonal analysis of corticosteroids was conducted on the biopsies of males. Cortisol
358 concentrations were significantly elevated in the summer compared to winter ($p = 0.029$, Figure
359 4). This raises the question whether increase in cortisol could be due to the increased
360 temperatures during the summer compared to cold winter temperatures. Champagne et al. (2018)
361 observed that blubber cortisol concentrations are increased in relation to increasing ambient air
362 temperatures during out of water events, potentially due to increased perfusion of the blubber
363 due to dilation of the blood vessels to offload heat. However, if perfusion were the only driving
364 factor in this study, one would expect an increase in all of the hormones due to increased

365 temperatures during summer versus winter seasons as indicated by the water temperature data
366 from the ACE Basin National Estuarine Research Reserve System (the warmest water
367 temperatures during this study occurred July through August with the coldest water temperatures
368 through December and January; http://cdmo.baruch.sc.edu/aqs/). However, the relationship
369 between water temperature, perfusion, and blubber hormone concentrations is more complex. A
370 seasonal study on Indo pacific bottlenose dolphins (*Tursiops aduncus*) under human care, found
371 that the highest serum cortisol concentrations coincided with highest rectal temperatures, but
372 these occurred in the season with the coldest water temperatures, spring (Funasaka et al., 2011).
373 In other studies on cetaceans under human care, serum cortisol were highest in winter (male
374 orcas and common bottlenose dolphins) or spring (common bottlenose dolphins) (Orlov et al.,
375 1988; Suzuki et al., 2003). However, all of these studies were conducted on animals under
376 human care where water temperatures are often controlled independent of natural environmental
377 factors such as ambient air temperatures and photoperiods. These differences emphasize the
378 need to establish cortisol baselines using consistent collection methods on free-ranging
379 populations rather than applying results from populations under human care to free-ranging
380 populations.

381 Despite a statistically significant elevation of cortisol concentrations in the summer, these
382 data should be considered in a biological context. The differences in mean seasonal cortisol
383 concentrations are minor (less than 0.7 ng/g between winter and summer). The biological
384 significance of such a small difference may not be relevant. However, the relationship between
385 blood and blubber cortisol concentrations during chronic stress is unknown. Baseline
386 measurements of cortisol from dolphins managed under human care showed a five-fold increase
387 in blood cortisol (ng/mL) compared to blubber cortisol (ng/g) after oral administration of 60 mg

388 of cortisol every 6 h (Champagne et al., 2017). This suggests that blood cortisol concentrations
389 could be much greater than what is reflected in the blubber. However, the five-fold increase was
390 measured from a simulated stress event in managed animals and long term offloading of the
391 initial cortisol response was not studied. Therefore, the results should be applied with caution to
392 this study where the slow partitioning of cortisol during acute or chronic stressors might affect
393 the blubber concentrations of cortisol at the moment of sample collection. This study serves to
394 emphasize the importance of conducting field experiments to understand the baseline seasonal
395 physiology of free-ranging populations of marine mammals before disturbances can be detected.

396 **4. Conclusions**

397 This is the first study to analyze seasonal baseline concentrations of steroid hormones,
398 including reproductive and stress steroids, in the blubber of free-ranging dolphins where
399 momentary stress during sample collection would not have affected the measured hormone
400 concentrations. This allows for the characterization of baseline hormone values, with potential
401 differences likely reflecting seasonal environmental influences and/or exposure to other stressors
402 without the confounding effect of sampling-induced stress.

403 By coupling remote biopsies with the LC-MS/MS method, suites of reproductive steroid
404 hormones can be measured together from a single sample, providing steroid hormone profiles
405 without the need for stressful and costly capture and release procedures. LC-MS/MS method
406 precision was comparable to immunoassay methods (RSDs $<$ 12 %) for the eight hormones
407 reliably quantified in this study, making it a precise and efficient method for the investigation of
408 hormone pathways compared to running eight separate immunoassays.

409 Using the approach in this study, demographic reproductive profiles, reproductive health,
410 and stress could be defined using tracked populations and applied in a greater proportion of the

411 free-ranging populations with less stress to this protected species. While more data are needed
412 on female seasonality and reproductive outcomes, this information serves as a starting point to
413 explore progesterone and androstenedione concentrations in blubber as a technique for the
414 detection of pregnancy, thereby improving estimations of miscarriages and successful births.
415 Additionally, this study emphasizes the importance of selecting the appropriate season for the
416 desired investigation. The method clearly demonstrates a significant increase in blubber
417 androgens and other reproductive hormones in male common bottlenose dolphins during seasons
418 of increased reproductive activity, which could serve as useful targets for the investigation of
419 male maturity. Also, understanding seasonal variation in stress hormones is critical to
420 investigating potentially disturbed populations. With this information, scientists in the field can
421 better examine populations of common bottlenose dolphins that may be under chronic stress,
422 and, by doing so, can aid in the monitoring and conservation of this protected marine mammal
423 and potentially other species.

424 **Compliance with ethical standards**

425 All research protocols used were approved by a NOAA Institutional Animal Care and
426 Use Committee. Collections were conducted in concordance with ethical standard guidelines
427 provided by the Office of Protected Resources, Marine Mammal Health and Stranding Response
428 Program and Animal Welfare Act and under the NOAA authorization 109(h) of the Marine
429 Mammal Protection Act. The authors declare that they have no competing interests in the
430 publication of this manuscript. Commercial equipment, instruments, or materials are identified
431 to specify adequately the experimental procedure. Such identification does not imply
432 recommendation or endorsement by NIST nor NOAA, nor does it imply that the materials or

433 equipment identified are necessarily the best available for the purpose. All samples were
434 collected under Marine Mammal Protection Act Permit No. 779-1633.

435 **Data accessibility**

436 The datasets supporting this article are publicly available from NIST (MIDAS record ID 1961) at
437 https://doi.org/10.18434/T4/1503309.

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Tables

Table 1: Statistics on steroid hormone concentrations for replications (n = 6) of Standard Reference Material 1945, Organics in Whale Blubber.

Hormone	Mean (ng/g blubber)	Standard Deviation	RSD
Cortisone	5.82	0.21	3.6 %
11-Deoxycortisol	4.18	0.40	10 %
Corticosterone	2.46	0.28	11 %
Cortisol	8.56	0.85	10 %
11-Deoxycorticosterone*	2.43	0.61	25 %
17-Hydroxyprogesterone	3.37	0.18	5.4 %
Androstenedione	0.441	0.06	13 %
Testosterone§	< RL		
Progesterone	206	31	15 %

Notes: < RL is below the reporting limit of this method; RSD = relative standard deviation; * = not measured in dolphin samples; δ = not detected in 1945 because it is a female whale

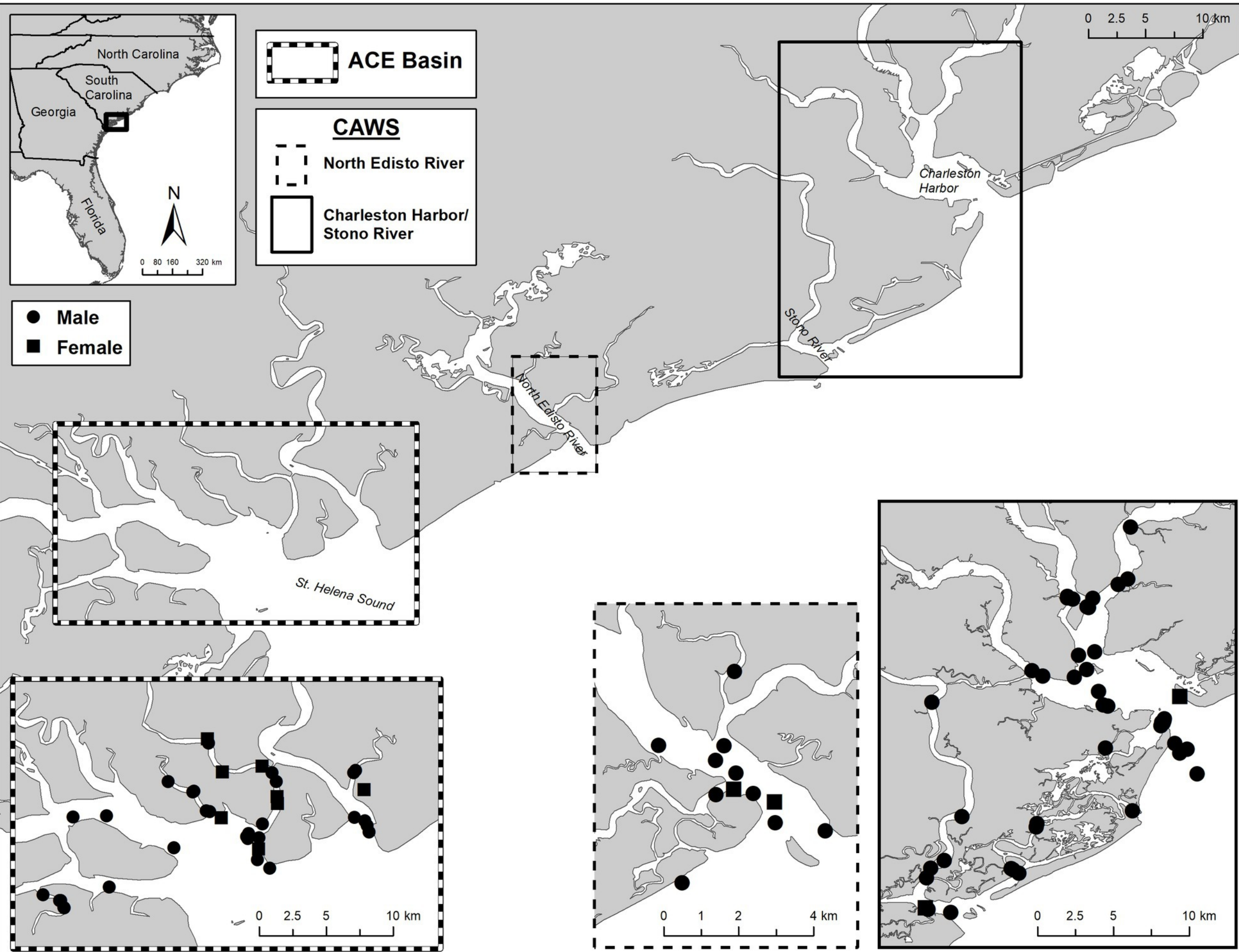
Figure Captions

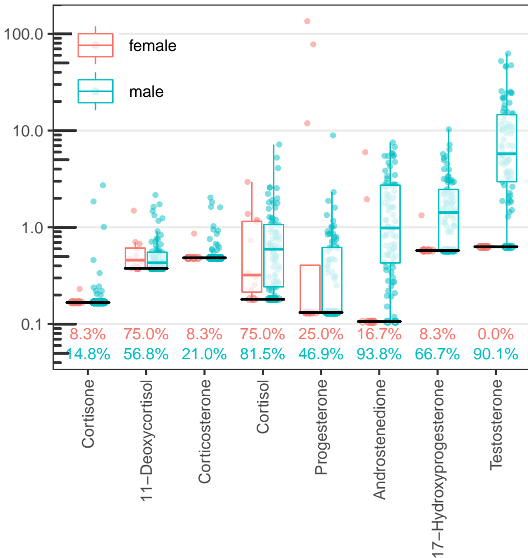
Figure 1: Map of the Ashepoo, Combahee, Edisto Basin (ACE) and the Charleston Area Waterways System (CAWS) where common bottlenose dolphin remote blubber biopsies were collected. Each dot represents the GPS locations at which an individual was biopsied.

Figure 2: Boxplots of blubber hormone concentrations from all common bottlenose dolphins sampled in this study on the Ashepoo, Combahee, Edisto Basin and surrounding Charleston-area waterways in South Carolina. Boxplots represent the median with quartiles. The lower solid black bar represents the reporting limit for this method. Detection frequencies for each hormone are listed by sex above the x-axis.

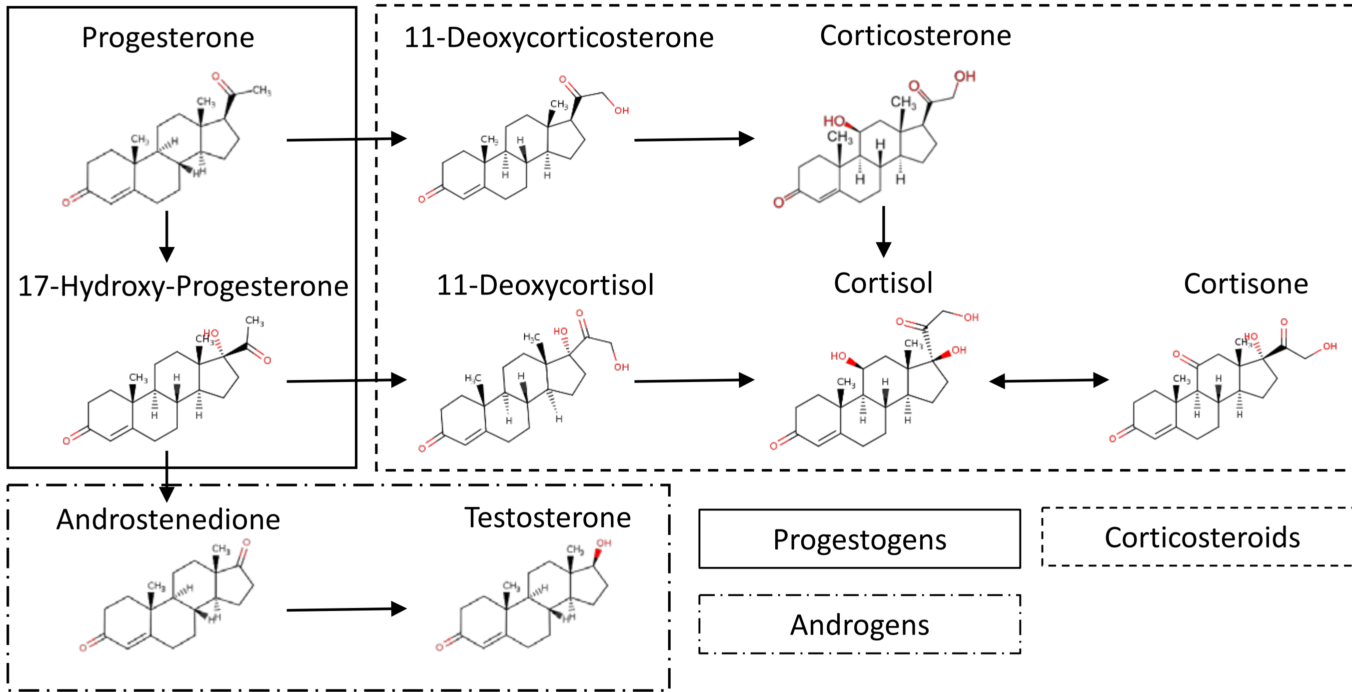
Figure 3: Relationships among hormones in blubber from common bottlenose dolphins. Panel A: The steroid synthesis pathway and classification for hormones measured in this study. Panel B: Correlations heat map using Kendall's Tau. Intensity approximates tau. Statistical significance ($p < 0.05$) is represented by (+) for a positive tau and (-) for a negative tau. Spaces are white where no information is available. Sample sizes were 81 for males and 12 for females. Panel C: Principal components analysis of quantified blubber hormones for male common bottlenose dolphins.

606 Figure 4: Comparison of seasonal blubber hormone concentrations in male common bottlenose
607 dolphins. Sample sizes for each season were as follows: Winter n = 18, Spring n = 14, Summer n
608 = 33, Fall n = 16. Significant differences assessed by post-hoc analysis are indicated by different
609 letters.

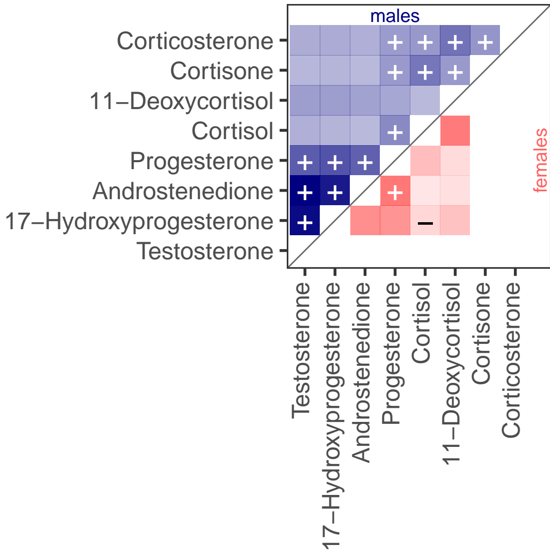

North Carolina
South Carolina
Georgia
Florida
N
0 80 160 320 km
ACE Basin
CAWS
North Edisto River
Charleston Harbor/
Stono River
Male
Female
0 2.5 5 10 km
Charleston Harbor
Stono River
North Edisto River
St. Helena Sound
0 2.5 5 10 km
0 1 2 4 km
0 2.5 5 10 km



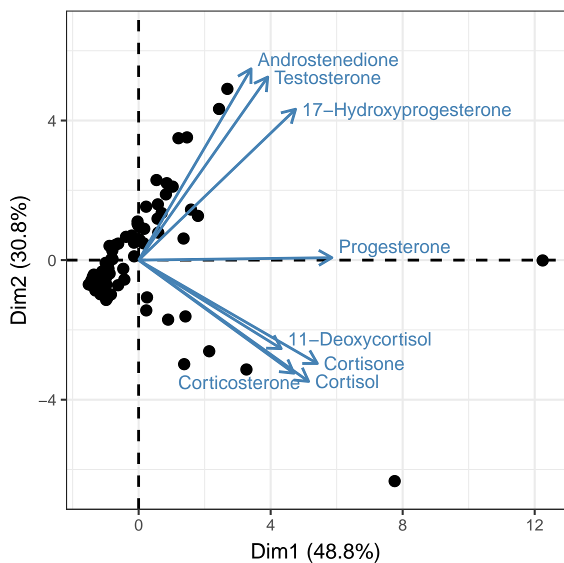
Concentration (ng/g blubber)
100.0
10.0
1.0
0.1
female
male
8.3%
14.8%
75.0%
56.8%
8.3%
21.0%
75.0%
81.5%
25.0%
46.9%
16.7%
93.8%
8.3%
66.7%
0.0%
90.1%
Cortisone
11−Deoxycortisol
Corticosterone
Cortisol
Progesterone
Androstenedione
17−Hydroxyprogesterone
Testosterone

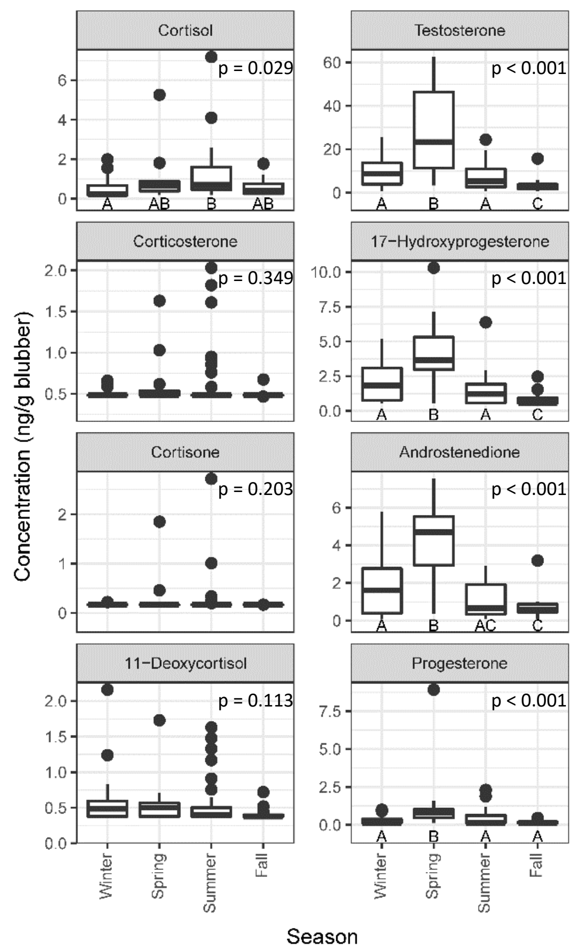


Progesterone
11-Deoxycorticosterone
Corticosterone
17-Hydroxy-Progesterone
11-Deoxycortisol
Cortisol
Cortisone
Androstenedione
Testosterone
Progestogens
Corticosteroids
Androgens



males
females
Corticosterone
Cortisone
11−Deoxycortisol
Cortisol
Progesterone
Androstenedione
17−Hydroxyprogesterone
Testosterone


Androstenedione
Testosterone
17−Hydroxyprogesterone
Progesterone
11−Deoxycortisol
Cortisone
Corticosterone
Cortisol
4
0
−4
Dim2 (30.8%)
0
4
8
12
Dim1 (48.8%)



Cortisol
p = 0.029
A
AB
B
AB
Testosterone
p < 0.001
A
B
A
C
Corticosterone
p = 0.349
17−Hydroxyprogesterone
p < 0.001
A
B
A
C
Cortisone
p = 0.203
Androstenedione
p < 0.001
A
B
AC
C
11−Deoxycortisol
p = 0.113
Progesterone
p < 0.001
A
B
A
A
Concentration (ng/g blubber)
Winter
Spring
Summer
Fall
Season