Title: Blubber steroid hormone profiles as indicators of physiological state in free-ranging common bottlenose dolphins (*Tursiops truncatus*)

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Abstract

Blubber has been proposed as a possible alternative to blood in the assessment of endocrine physiology in marine mammals because it can be collected via remote biopsy, which removes some of the confounding variables and logistical constraints associated with blood collection. To date, few studies have directly assessed the relationships between circulating versus blubber steroid hormone profiles in marine mammals, and these studies have been limited to a small subset of steroid hormones, which collectively limit the current utility of blubber steroid hormone measurements. In this study, we used liquid-chromatography tandem-mass spectrometry (LC-MS/MS) to screen for 16 steroid hormones in matched blood and blubber samples from free-ranging common bottlenose dolphins (*Tursiops truncatus)*. Seven steroid hormones were detected and quantified, including two progestogens, two androgens, and three corticosteroids. Using principal components analysis (PCA), we explored relationships between hormones in both matrices and three physiological states: sexual maturity in males, pregnancy, and acute stress response. Plasma and blubber testosterone and its precursors, 17-hydroxyprogesterone and androstenedione, loaded to the first principal component (PC1), and PC1 scores were higher in mature males. Plasma and blubber progesterone loaded to PC2, and pregnant/probable pregnant females had significantly higher PC2 scores. Pregnant females also had higher PC1 scores than other females, suggesting differences in androgen profiles between these groups. There was disagreement between plasma and blubber corticosteroid profiles, as indicated by their loading to different PCs; plasma corticosteroids loaded to PC3 and blubber corticosteroids to PC4. PC3 scores were significantly predicted by elapsed time to blood collection (i.e., time between initiating the capture process and blood collection), while elapsed time to blubber collection significantly predicted PC4 scores, indicating that corticosteroid profiles shift in both tissues during acute stress. Corticosteroid profiles were not related to demographic group, site-month, body mass index, water temperature, or time spent outside of the water on the processing boat. Overall, these results demonstrate that blubber steroid hormone profiles reflect changes in endocrine function that occur over broad temporal scales.

1. Introduction

Blood is the most common matrix used for endocrine assessments in vertebrates, but collecting blood from free-ranging wildlife typically requires capture and restraint, which is a stressful event and inherently induces shifts in circulating hormone measurements, particularly of stress hormones. As such, it is difficult to measure endocrinological baselines in wildlife, including marine mammals, using blood and current sampling techniques. Furthermore, in free-ranging cetaceans, collection of blood is a labor-intensive and expensive process (Balmer et al., 2014). Using remotely collected sample matrices for marine mammal endocrine assessments could minimize stress to the animals, allow for the measurement of endocrinological baselines, reduce sampling costs for researchers, and increase the number of animals that can be feasibly sampled. Several such matrices have been used, including: feces (Champagne et al., 2018; Wasser et al., 2017), respiratory vapor (Hunt et al., 2014a), baleen (Hunt et al., 2014b), earwax (Trumble et al., 2013), and blubber. Blubber, a form of subcutaneous adipose tissue in marine mammals, can be collected via remote biopsy (Noren and Mocklin, 2012), and contains measurable concentrations of numerous steroid hormones (Boggs et al., 2017; Champagne et al., 2017; Kellar et al., 2015; Kellar et al., 2009; Kellar et al., 2006; Kershaw and Hall, 2016; Kershaw et al., 2017; Mansour et al., 2002; Pallin et al., 2018a; Pallin et al., 2018b; Pérez et al., 2011; Trego et al., 2013; Vu et al., 2015). Thus, blubber could potentially serve as an alternative to blood in marine mammal endocrine assessments involving lipophilic hormones, such as steroids.

Blubber steroid hormone measurements vary with physiological states in cetaceans, including stress (Kellar et al., 2015), sexual maturity (Inoue et al., 2018; Kellar et al., 2009), and pregnancy (Kellar et al., 2006; Mansour et al., 2002; Pérez et al., 2011; Trego et al., 2013). In general, blubber steroid hormone profiles qualitatively reflect circulating profiles. In adult male cetaceans, both circulating and blubber testosterone (T) values increase during the breeding season (Harrison and Ridgway, 1971; Kellar et al., 2009; Schroeder and Keller, 1989; Vu et al., 2015). Similarly, progesterone (P_4) is elevated in the blubber and blood of pregnant cetaceans (Kellar et al., 2006; Kirby and Ridgway, 1984; Mansour et al., 2002; Pallin et al., 2018a; Pallin et al., 2018b; Pérez et al., 2011; Sawyer-Steffan et al., 1983; Trego et al., 2013). Stressor-induced activation of the hypothalamo-pituitary-adrenal axis leads to elevated cortisol (F) concentrations in both blood and blubber in cetaceans (Champagne et al., 2018; Houser

et al., 2011; Kellar et al., 2015; Kershaw and Hall, 2016; Schroeder and Keller, 1989; St. Aubin et al., 1996; Thomson and Geraci, 1986). Blubber cortisol levels were also linked to body condition in harbor porpoises (*Phocoena phocoena*) (Kershaw et al., 2017). However, few studies have explicitly characterized the relationships between circulating and blubber hormone concentrations (Champagne et al., 2017; Champagne et al., 2018; Kellar et al., 2013).

Endocrine glands secrete hormones into blood, which then delivers hormones to peripheral tissues, including hormone target tissues, sites of peripheral hormone metabolism, and blubber. Thus, when changes in central endocrine function occur, circulating hormone values will change rapidly and these changes can be detected nearly instantaneously, while changes in hormone concentrations in peripheral tissue must lag behind changes in circulating concentrations and central endocrine function. Such a relationship was observed in domesticated pigs, in which peak adipose P_4 concentrations exhibited a one-to-two day lag behind peak plasma P_4 concentrations and returned to baseline concentrations more gradually than plasma concentrations (Hillbrand and Elsaesser, 1983). Thus, it is likely that blubber integrates circulating hormones over some period of time, and blubber hormone concentrations reflect an average circulating value over that period. Blubber hormone profiles are likely also influenced by in situ metabolism of steroid hormones—as demonstrated by Galligan et al. (2018b)— blubber perfusion rates, and perhaps other factors (e.g., lipid composition, concentration of steroid binding proteins in blood, etc.), which are also dynamic.

We currently have a poor understanding of the temporal relationships between circulating and blubber steroid hormone concentrations. In common bottlenose dolphins under human care, circulating F values were elevated 15 min following exposure to acute stress, remained high during the 120 min of the stress exposure, and then had returned to baseline within one hour post exposure (Champagne et al., 2018). Blubber was sampled at 0 min, 60 min, and 120 min into the stress exposure, and blubber F values had significantly increased at both 60 min and 120 min, though the magnitude of increase was lower compared to blood (Champagne et al., 2018). Notably, blubber was not sampled prior to 60 min, thus it is unclear how soon after the initiation of exposure that a change in blubber F could be detected. Furthermore, Champagne et al. (2018) did not sample blubber after cessation of exposure, meaning we do not know how long blubber F values are elevated after an acute change in circulating F concentration.

Therefore, while an increase in circulating F would indicate stressor exposure within the past several minutes-to-hours, elevated blubber F would indicate stressor exposure at some currently undetermined point(s) in the past, meaning that blubber cannot necessarily be used interchangeably with blood for assessment of acute stress. Conversely, blubber may be interchangeable with blood when measuring pregnancy-related shifts in P_4 physiology. In bowhead whales (*Balaena mysticetus*), during pregnancy when P_4 secretion increases and remains high for a prolonged period, circulating and blubber P_4 values are strongly correlated with one another (Kellar et al., 2013). This is likely because the persistent increase in P_4 secretion that occurs during pregnancy results in relatively stable circulating P_4 concentrations over a long period allowing sufficient time for blubber P_4 values to equilibrate with blood P_4. Taken together, this evidence suggests that blubber steroid measurements likely reflect changes in systemic endocrine function over a broader temporal scale, which has implications for how we interpret blubber hormone values in relation to physiological states.

Importantly, the blubber steroid hormone literature has primarily focused on F, T, and P_4 (Champagne et al., 2017; Kellar et al., 2015; Kellar et al., 2013; Kellar et al., 2009; Kellar et al., 2006; Mansour et al., 2002; Pérez et al., 2011; Trego et al., 2013), and no studies to date have directly studied the relationship between T concentrations in blood and blubber. Furthermore, several additional steroid hormones – 17-hydroxyprogesterone ($17OHP_4$), 11-deoxycorticosterone (DOC), corticosterone (B), 11-deoxycortisol (S), cortisone (E), and androstenedione (AE) – have recently been measured in free-ranging common bottlenose dolphin blubber and blood (Boggs et al., 2019; Boggs et al., 2017; Galligan et al., 2019; Galligan et al., 2018a). Galligan et al. (2018a) and Boggs et al. (2019) explored relationships between these hormones and various physiological states in blood and blubber, respectively. Both noted positive correlations between several of the hormones in the Δ_4 androgen biosynthesis pathway (specifically, $17OHP_4$, AE, and T) (Figure 1), and increases in AE during pregnancy. Herein, we build from these studies and conduct a comprehensive assessment of the relationships between physiological state and steroid hormone profiles in dolphin blood and blubber.

In this study we used individual-matched blood and blubber samples to assess the relationships between hormones in both matrices in the context of various physiological states, including sexual maturity, pregnancy, and acute stress response.

We hypothesized that generally hormone profiles would be comparable across the two sample matrices. Furthermore, we predicted that the hormones in the Δ_4-androgen biosynthesis pathway (Figure 1) would be elevated in adult males because sexual maturity is marked by an increase in T secretion and is detectable in both matrices (Harrison and Ridgway, 1971; Kellar et al., 2009; Schroeder and Keller, 1989). Progestogens should be elevated in pregnant females, as has been observed previously (Kellar et al., 2006; Kirby and Ridgway, 1984; Mansour et al., 2002; Pérez et al., 2011; Sawyer-Steffan et al., 1983; Trego et al., 2013), and in sexually mature females (Inoue et al., 2018). We suspected that androgens would also be elevated in both tissues in pregnant females as reported in bottlenose dolphins and killer whales (Boggs et al., 2019; Galligan et al., 2018a; Robeck et al., 2017; Steinman et al., 2016). Additionally, corticosteroids should be elevated during pregnancy (Valenzuela-Molina et al., 2018). Finally, the hormones in the glucocorticoid pathway should be positively correlated with elapsed time to sample collection because capture and handling stress induces cortisol secretion (Kellar et al., 2015; Kellar et al., 2013; St. Aubin et al., 1996; Thomson and Geraci, 1986) and impacted by body condition (Kershaw et al., 2017). This comprehensive comparison of blood-blubber hormone profiles will improve our ability to use remotely collected blubber biopsies to study endocrine function in free-ranging marine mammals.

2. Materials and Methods

2.1 Animals, Field Data Collection, and Sample Collection

Matched blubber and blood samples were collected from free-ranging common bottlenose dolphins (n = 77) from three locations in the southeastern United States during late spring and late summer/early fall (Barataria Bay, LA [June 2013, 2014; n = 34]; Brunswick, GA [September 2015; n = 16]; and Sarasota Bay, FL [May 2013-2016; n = 27]). Methods for the temporary capture, restraint, sampling, and release have been previously described (Schwacke et al., 2014; Smith et al., 2017; Wells et al., 2005). Briefly, a seine net was deployed encircling a dolphin or group of dolphins, and if necessary, the radius of the space enclosed by the net was reduced to force the dolphin to become entangled or enable handlers to safely restrain the dolphin without entanglement. When a dolphin entangled itself in the net, handlers would immediately restrain the animal and disentangle it. The time at which the capture net was deployed

was recorded, and is considered the start of the capture process. The times at which blood and blubber samples were collected were also recorded, and time elapsed between capture initiation (i.e., net deployment) and sample collection was calculated. Blood and blubber were not collected concurrently; blood was always collected first then blubber was collected second. On average, the interval between these collections was 87.9 min (standard deviation = 31.6 min; minimum = 9.00 min, median = 80.0 min, maximum = 185 min). Animals were transferred to a boat to measure body mass, and time of transfer was recorded. The blubber biopsy was generally collected on the boat (five animals were returned to the water prior to biopsy collection). Six individuals did not have body mass measured. Water temperature at time of sampling was also recorded for all but four animals. Sarasota Bay sampling was performed under National Marine Fisheries Service (NMFS) Scientific Research Permit No. 15543 and annually renewed IACUC approvals through Mote Marine Laboratory. Barataria Bay and Brunswick sampling were conducted under NMFS permit no. 932-1905/MA-009526 with protocols reviewed and approved by National Oceanic and Atmospheric Administration IACUC.

This sample set includes individuals from different demographic groups defined by different physiological states, including subadult and adult males and pregnant, probable pregnant, and non-pregnant females (adult and subadult) (Table 1). Males were sampled from Brunswick (n = 13) and Sarasota (n=13). Pregnant females were largely sampled from Barataria Bay (n = 14), with only one pregnant individual sampled from Brunswick. All probable pregnant females were sampled from Barataria (n= 4). Non-pregnant females were sampled from Barataria (n = 16), Sarasota (n = 14), and Brunswick (n = 2). Age was determined either through lifelong observation (i.e., known birth date) or, when possible, through examination of growth layer patterns in teeth using methods that have been described previously (Hohn et al., 1989; McFee et al., 2010); age was not determined in all individuals. Age classification was dictated by age, if known (individuals ≥ 10 years old were classified as adults; n = 47), or total length if age was not known (individuals with total length ≥ 240 cm were classified adults; n = 30). Pregnancy status was diagnosed by ultrasound examination of the uterus and ovaries and preliminary assessment of circulating P_4 by immunoassay (Schwacke et al., 2014; Smith et al., 2013; Smith et al., 2017; Wells et al., 2014). Females with a corpus luteum present on either ovary, serum progesterone concentrations greater than 5 ng mL^{-1}, and presence of a fetus and uterine fluid were classified as pregnant, per Smith et al. (2017).

225 Those with a corpus luteum present and serum P_4 > 5 ng mL^{-1} but without a fetus
226 observed were classified as probable pregnant. Body mass index (BMI) was calculated
227 from body length and weight measurements per Hart et al. (2013); no individuals we
228 classified as having low BMI (below the lower 95th percentile threshold).
229 Full-depth blubber samples were collected by surgical or punch biopsy
230 (Schwacke et al., 2014). Blood was collected from the vasculature of the ventral fluke
231 into sodium heparin vacutainers, and plasma was produced by centrifugation at site of
232 capture. After removal of the skin, blubber (average blubber biopsy mass = 0.400 g ±
233 0.168) and plasma (in 1 mL to 5 mL aliquots) were immediately frozen in a liquid
234 nitrogen dry shipper at approximately -150 ^{o}C, and shipped to the National Institute of
235 Standards and Technology (NIST) Environmental Specimen Bank at Hollings Marine
236 Laboratory (Charleston, SC, USA), where they were stored at -80 ^{o}C until analysis.
237 **2.2 Calibration and Internal Standards**
238 Calibration and isotopically-labeled internal standard manufacturer and purity
239 information are reported in Supplemental Table 1. Calibration (cal) and internal standard
240 (IS) mixture solutions were diluted in methanol, with the concentration of each
241 compound in the final mixture calculated gravimetrically (ng compound g^{-1} mixture).
242 Average mass of each IS compound amended to tubes is reported in Supplemental
243 Table 2.
244 **2.3 Hormone Extraction**
245 Blubber hormone extraction was completed using methods described by Boggs
246 et al. (2017) with a kit (Agilent, Santa Clara, CA, USA) that utilizes a salting-out assisted
247 liquid:liquid extraction (SALLE) to dispersive solid phase extraction (SPE) process (kits:
248 Agilent Bond Elut QuEChERS EN Extraction kit, p/n 5982-5650, and Agilent Bond Elut
249 QuEChERS dispersive-SPE kit for Drug Residues in Meat, 15 mL, p/n 5982-4956)
250 (Boggs et al., 2017; Fu and Zhai, 2010). Plasma hormones were extracted by reverse
251 phase solid phase extraction (SPE) via methods described by Galligan et al. (2018a).
252 For both matrices, process blanks containing only IS were extracted alongside samples
253 and cals.
254 **2.5 Instrumental Methods and Quantitation**
255 Chromatographic separation and quantification of steroids in both blubber and
256 plasma extracts proceeded according to methods described by Galligan et al. (2018a),
257 using an Agilent 1200 Series HPLC system with a binary pump and an autosampler

linked to an AB Sciex (Framingham, MA, USA) API 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer. This method allows for quantification of the following steroid hormones: pregnenolone, 17-hydroxypregnenolone, P_4, $17OHP_4$, AE, T, dihydrotestosterone, dehydroepiandrosterone, S, F, E, DOC, B, estradiol, estrone, and estriol (Figure 1). Two transitions were monitored per compound, and the transition with the larger signal was used for quantification. We used Sciex Analyst software (version 1.5) to integrate peaks. Steroid concentration was determined by interpolating analyte area ratios (analyte area:IS area) on a standard curve comprised of calibration standards which fully encompassed the range of sample values (Supplemental Tables 3 and 4). Observed reporting limits (RL_{obs}) were defined as the lowest calibration standard used in the calibration curve; calculated reporting limits (RL_{calc}) were defined as three times the standard deviation of the mean of process blank measurements plus the mean of the process blanks (Supplemental Table 5). The larger of the two RL values was used as the censoring threshold in statistical analyses. Censoring determination was based on raw area rather than calculated hormone value, but RLs are reported as hormone values for clarity. See section 2.6 “Statistics” for further details about censoring methods.

2.6 Statistics

Statistical analyses were performed with IBM SPSS Statistics 24 (IBM, North Castle, NY, USA) and R (version 3.6.1) with RStudio (R Core Team, 2018; RStudio Team, 2016). For all hypothesis tests, $\alpha = 0.05$. A principal components analysis (PCA) was performed to examine relationships between hormones in both matrices. A uniform distribution was assumed between 0 and RL, and hormone values below RL were substituted with a random value within this range. Data were $\log_{10}$ transformed, mean centered, and unit scaled. Suitability for PCA was confirmed by ensuring all variables had Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy > 0.5; that the KMO measure of adequacy for the entire data set was > 0.5; and that Bartlett’s test of sphericity was significant ($p < 0.05$) (Dziuban and Shirkey, 1974). Factors with eigenvalues > 1.0 were extracted. Varimax rotation was utilized to simplify interpretation. Two samples were lost during extraction (one plasma sample from a non-pregnant female and one blubber sample from a pregnant female), and were thus excluded from analysis (Supplemental Table 6).

290 We assessed relationships between PC scores and various demographic,
291 morphometric, and sampling variables via stepwise linear modeling in which we
292 removed variables based on p-value until all remaining variables were significant at α =
293 0.05. For PC1 and PC2, we included demographic group, site-month, and their
294 interaction as potential predictor variables. For PC3, we included elapsed time to blood
295 collection, BMI, demographic group, and site-month as potential predictor variables. Due
296 to a violation of assumptions, PC3 scores were rank transformed prior to analysis. For
297 PC4, we included elapsed time to blubber collection, water temperature, time spent on
298 boat, BMI, site-month, and demographic group as potential predictor variables.
299 Individuals that were brought onto the boat but then returned to the water prior to
300 blubber biopsy collection were excluded from the models including "time spent on boat"
301 as a variable because it would be impossible to interpret the effect of "time spent on
302 boat" in these animals. Pairwise comparisons via t-test, where appropriate, were
303 performed with Benjamini-Hochberg correction. We also assessed relationships between
304 age, length, and weight and PC1 scores (males) and PC2 scores (non-pregnant
305 females) via Pearson correlation or Kendall's tau correlation.
306
307 **3. Results**
308 Six hormones were detected and quantified in both blubber and plasma: P_4,
309 $17OHP_4$, AE, T, F, and E (Tables 1 and 2). S was detected and quantified in blubber
310 only (Tables 1 and 2). We did not detect pregnenolone, 17-hydroxypregnenolone, DOC,
311 B, dehydroepiandrosterone, dihydrotestosterone, estradiol, estrone, or estriol in any
312 samples. Hormone concentrations for each individual are reported in Supplemental
313 Table 6.
314 In the PCA, four components with eigenvalues > 1 were extracted explaining
315 27.2 %, 21.8 %, 12.8 %, and 9.19 % of the variance respectively (71.0 % cumulatively),
316 and simple structure was obtained by Varimax rotation (Table 3; Figure 2A and B).
317 Plasma and blubber T, AE, and $17OHP_4$ loaded (i.e., absolute value of loading > 0.4)
318 positively to PC1; plasma and blubber P_4 loaded positively to PC2; plasma F and E
319 loaded positively to PC3; and blubber S, F, and E loaded positively to PC4 (Table 3;
320 Figure 2A and B).
321 PC1 scores were significantly predicted (linear model, $R^2 = 0.799$, $F_{9,65} = 28.8$, p
322 < 0.001) by demographic group ($F_4 = 55.8$, $p < 0.001$), site-month ($F_2 = 10.9$, $p < 0.001$),

and their interaction ($F_3 = 4.66$, $p = 0.005$). Based on the interaction plot (Figure 2C), the site-month factor was important for males, particularly adult males, and not females. Therefore, we considered adult and subadult males collected from different sites-months to be distinct groups when performing pairwise comparisons. We found that adult males, regardless of site-month, exhibited higher scores than all other groups per pairwise t-test with Benjamini-Hochberg correction (Figure 2D). Among males, Sarasota (May) individuals exhibited higher PC1 scores than Brunswick (September) within both age classes (Figure 2D). Among females (inclusive of all sites-months), pregnant individuals had higher PC1 scores than non-pregnant and probable pregnant individuals per pairwise t-test with Benjamini-Hochberg correction (Figure 2D). Relationships between females and subadult males depended on site-month; Sarasota (May) subadult males had PC1 scores comparable to pregnant females and higher than non-pregnant and probable pregnant females, while Brunswick (September) subadult males exhibited PC1 scores comparable to non-pregnant and probable pregnant females and lower than pregnant females per pairwise t-test with Benjamini-Hochberg correction (Figure 2D). For PC2 scores, demographic group was the only significant predictor (linear model, $R^2 = 0.793$, $F_{4,70} = 66.9$, $p < 0.001$), with pregnant and probable pregnant females having higher scores than all other groups per pairwise t-test with Benjamini-Hochberg correction (Figure 2E).

Elapsed time to blood collection was the only significant predictor of PC3 scores (rank-transformed linear model, $R^2 = 0.256$, $F_{1,74} = 25.4$, $p < 0.001$) Similarly, elapsed time to blubber collection was the only significant predictor of PC4 scores (linear model, $R^2 = 0.267$, $F_{1,73} = 26.6$, $p < 0.001$) (Figure 3B).

Since three of four hormones in the Δ_4 androgen pathway (Figure 1)—specifically $17OHP_4$, AE, and T—loaded to PC1, we assessed PC1 scores by age and age-related morphometric variables (i.e., body length and weight) in males and non-pregnant females to further examine variation in androgen profiles by sexual maturity. Within males, we stratified this analysis by site-month due to the interaction between demographic group and site-month (Figure 2C). In males from both sites-months, PC1 scores were significantly and positively correlated with age (Sarasota [May]: Kendall tau correlation, $\tau = 0.623$, $z = 2.94$, $p = 0.003$; Brunswick [September]: Pearson correlation, $r = 0.833$, $t_5 = 3.37$, $p = 0.020$), body length (Sarasota [May]: Kendall tau correlation, $\tau = 0.632$, $z = 3.00$, $p = 0.003$; Brunswick [September]: Pearson correlation, $r = 0.722$, $t_{11} =$

3.46, p = 0.005), and body weight (Sarasota [May]: Kendall tau correlation, $\tau = 0.667$, T = 65, $p < 0.001$; Brunswick [September]: Pearson correlation, $r = 0.873$, $t_6 = 4.39$, p = 0.004) (Figure 5).

There were no significant relationships between PC2 scores and age (Kendall tau, $\tau = 0.0522$, $z = 0.339$, $p = 0.735$), length (Pearson correlation, $r = 0.210$, $t_{28} = 1.14$, p = 0.266), or weight (Pearson correlation, $r = 0.295$, $t_{25} = 1.54$, p = 0.136) in non-pregnant females (not shown).

4. Discussion

The goal of this study was to explore the relationships between blood and blubber steroid hormone profiles in common bottlenose dolphins, and thereby, provide information to subsequently improve our ability to use remotely collected blubber biopsies to assess endocrine status in marine mammals. We accomplished this using LC-MS/MS to measure a broad suite of steroid hormones in matched plasma and blubber samples from free-ranging common bottlenose dolphins. We performed a PCA to explore relationships among hormones, examine the relationships between hormone profiles in the two matrices, and study hormone profiles associated with three important physiological states: sexual maturity, pregnancy, and acute stress response.

The PCA allowed us to assess the relationships among hormones both within and across each matrix and thereby, provided information about the agreement between hormone profiles in each tissue. We also used the PCA to assess how hormone profiles (collectively in both tissues) vary by physiological state. In general, the variable loading patterns suggest that androgen and progestogen profiles were similar across matrices, and that most of the hormones in the Δ_4 androgen pathway are associated with one another. Conversely, corticosteroid profiles were poorly correlated across matrices, as evidenced by the fact that plasma and blubber corticosteroids loaded to separate PCs.

The range of F values reported here (blubber: 0.0596 ng g^{-1} to 21.0 ng g^{-1}; plasma: 1.67 ng g^{-1} to 30.2 ng g^{-1}) are comparable to previous studies in bottlenose dolphins and other cetaceans (blubber: approximately 1 ng g^{-1} to 70 ng g^{-1}; plasma: approximately 3.5 ng g^{-1} to 60 ng g^{-1}, assuming density of plasma is approximately 1.025 g mL^{-1}) (Champagne et al., 2018; Kellar et al., 2015). Capture and handling induces the secretion of corticosteroids in cetaceans (St. Aubin et al., 1996; Thomson and Geraci, 1986). We found that only elapsed time to blood collection was a significant predictor of

PC3 (plasma corticosteroids) scores. Thus, corticosteroid secretion increased during capture, handling, and sample collection, as would be expected during the acute stress response, while demographic group, BMI, and site-month had no impact on circulating corticosteroid profiles.

Since circulating corticosteroid concentrations were increasing during the capture, handling, and sampling process, we expected elapsed time to blubber collection to predict PC4 scores (blubber corticosteroids) as greater elapsed time would allow more time for corticosteroid secretion and for circulating corticosteroids to become incorporated into blubber. As expected, elapsed time to blubber collection was a significant predictor of PC4 score. It is also important to note that there was temporal mismatch between sample collection times for each tissue. Plasma was collected first (ranging from 6 min to 66 min post capture onset) and blubber was collected second (53 min to 215 min post capture onset). This mismatch likely allowed more time for equilibration between the matrices than if the two samples had been collected simultaneously, but the interval between blood and blubber collection varied between individuals, meaning individuals likely differed in their levels of blood-blubber equilibration at the time of blubber collection, which could have contributed to the lack of association between plasma and blubber corticosteroid profiles. Additionally, we cannot determine the rate at which corticosteroid profiles in blubber changed because we only have single timepoint measurements, as opposed to repeated measures, and our earliest sample occurred at 53 min, which is comparable to when Champagne et al. (2018) began assessing changes in blubber F in common bottlenose dolphins under human care exposed to acute stress. We conclude that corticosteroid concentrations increased in both matrices due to capture stress. Concurrently sampling blood and blubber repeatedly over a prolonged period of time would better clarify these temporal relationships. Developing novel, remote sampling devices that collect both blubber and blood simultaneously would greatly enhance our understanding of differences in corticosteroid profiles between these two matrices (e.g., an animal borne blood sampling device in development for pinnipeds (Takei et al., 2016)).

Corticosteroids are important regulators of energy metabolism. Therefore, we hypothesized that body condition (BMI) may be related to corticosteroid profiles in blood and blubber, as seen in harbor porpoises (Kershaw et al., 2017). However, we found no such relationship, potentially because no individuals in this study had low BMI (below the

lower 95th percentile threshold) (Hart et al., 2013). Future studies with broader ranges of BMI should further assess this relationship.

It is likely that the rate of perfusion also influences corticosteroid delivery to blubber (i.e., more blood flow to blubber would increase hormone delivery). Thus, factors influencing perfusion immediately prior to and during capture, handling, and sampling would also influence blubber corticosteroid profiles and, thus, PC4 scores. Water temperature and time spent on the boat prior to blubber collection could affect blubber perfusion since cetaceans modulate blood flow to the blubber and skin to adjust heat flux with the environment for thermoregulation. We did not observe an effect of water temperature or time spent on the boat on PC4 scores, potentially due to the fact that the dolphins were constantly sponged with water to cool their skin and keep it wet during out-of-water processing. This could potentially indicate that there was minimal influence of sampling procedure/temperature on perfusion and/or that changes in perfusion during sampling do not appreciably impact blubber hormone profiles. To explicitly elucidate these relationships, future studies will need to directly measure changes in perfusion in relation to circulating and blubber hormone profiles.

Some of the mismatch between blood and blubber corticosteroid profiles may arise from the fact that common bottlenose dolphin blubber has the capacity to metabolize corticosteroids (Galligan et al., 2018b); i.e., after corticosteroids are delivered to blubber via blood, they are metabolized by blubber, which could cause blubber corticosteroid profiles to shift away from circulating profiles. While the rates of metabolism are poorly defined, we would expect the influence of in situ metabolism to increase over time as this would allow greater quantities of hormone to be metabolized, leading to wider divergence between blood and blubber profiles in animals with greater interval between blood and blubber collection. Future research should seek to better characterize the metabolism of corticosteroids—and potentially other steroids—in blubber and examine how such metabolism may impact the relationships between blood and blubber steroid hormone profiles.

T secretion is elevated during breeding season in sexually mature male cetaceans; this leads to a seasonal increase in circulating and blubber T (Harrison and Ridgway, 1971; Kellar et al., 2009; Schroeder and Keller, 1989). Therefore, since sampling occurred between late spring and late summer, when breeding is likely occurring in these populations (McFee et al., 2014; Urian et al., 1996), we anticipated

that adult males would exhibit elevated T concentrations in both plasma and blubber. Furthermore, based on Galligan et al. (2018a) and Boggs et al. (2019), we expected that the upstream hormones in the Δ_4 androgen pathway – P_4, $17OHP_4$, and AE – would also be elevated in both matrices because increased production of these hormones is required to support increased T production. As a result, these hormones should be positively correlated in both matrices and exhibit maturity-dependent differences. Our results largely support these hypotheses. Blubber and plasma T, AE, and $17OHP_4$ loaded positively to PC1, indicating a positive association between these variables. Furthermore, PC1 scores were significantly higher in adult males, regardless of site-month, compared to all other groups, and PC1 scores were positively correlated with age, length, and weight in males from both sites-months, which indicates that the combined variance in T, AE, and $17OHP_4$ was related to maturity in males. The effect of site-month on PC1 scores within males may indicate that androgen profiles vary among populations or between months within the breeding season. One might also expect to observe elevated P_4 concentrations to support $17OHP_4$ production, but P_4 was not detected in any male blubber and was rarely detected in male plasma. This is likely due in part to the abnormally high RL for blubber P_4 in this study, which may have been due to use of a higher IS concentration for P_4 than Boggs et al. (2017) (18.9 ng vs. 5.26 ng), who achieved a lower detection limit for blubber P_4 (0.246 ng) which was comparable to immunoassay techniques (Inoue et al., 2018; Kellar et al., 2006; Mansour et al., 2002; Pallin et al., 2018a; Pallin et al., 2018b; Pérez et al., 2011; Trego et al., 2013). Nonetheless, our high RL for blubber P_4 impedes our ability to assess relationships between blubber P_4 and other hormones, especially in males and non-pregnant females, and thus is a key limitation to this study.

P_4 secretion increases during pregnancy in cetaceans. This increase can be observed in both plasma and blubber (Kellar et al., 2006; Kirby and Ridgway, 1984; Mansour et al., 2002; Pérez et al., 2011; Sawyer-Steffan et al., 1983; Trego et al., 2013). As expected, plasma and blubber P_4 loaded to the same principal component (PC2) in the PCA, and pregnant/probable pregnant females had significantly higher PC2 scores compared to non-pregnant females and other demographic groups. We expected that pregnant females would have higher corticosteroid levels, based on previous work in humpback whales (*Megaptera novaeangliae*) (Valenzuela-Molina et al., 2018), but found no such difference. Androgens, including T and AE, are also elevated during pregnancy

488 in bottlenose dolphins and killer whales (*Orcinus orca*) (Boggs et al., 2019; Galligan et
489 al., 2018a; Robeck et al., 2017; Steinman et al., 2016) , potentially to support ovarian
490 secretion of P_4 (Carrizo et al., 1994; Telleri´a et al., 1995; Waddell et al., 1992).
491 Therefore, it is unsurprising that pregnant females also had higher PC1 scores
492 compared to non-pregnant females.
493 Importantly, probable pregnant female PC1 scores were significantly lower than
494 pregnant, but not non-pregnant, females. Probable pregnant females are either newly
495 pregnant or in the luteal phase of their estrous cycle (Smith et al., 2017). Thus, this
496 finding suggests that blood and blubber androgen measurements could potentially be
497 used to differentiate pregnant females from early pregnant/luteal phase females without
498 the need for ultrasound tests. This could prove useful in assessing reproductive
499 dynamics and dysfunction in free-ranging cetacean populations. However, with low
500 sample size of probable pregnant females (n = 4), our conclusion here is limited and
501 should be the subject of further investigation. Additionally, it should be noted that all the
502 pregnant females in this study were in the first trimester of pregnancy when sampled.
503 Endocrine profiles likely change throughout pregnancy (Robeck et al., 2017; Robeck et
504 al., 2016; Steinman et al., 2016); therefore, future studies should examine the
505 relationships between circulating and blubber hormone profiles throughout pregnancy.
506 P_4 has been used to classify sexual maturity in female cetaceans. According to a
507 review of cetacean endocrinology, females in many cetacean species exhibit an
508 increase in circulating P_4 at the onset of sexual maturity, with sexually immature
509 individuals exhibiting concentrations < 1 ng/mL of P_4 (reviewed: Atkinson and Yoshioka,
510 2007). However, this threshold is misleading because P_4 concentrations will vary
511 significantly by pregnancy status and during the estrous cycle. In reality, circulating P_4
512 concentrations only rise above this threshold when a female is pregnant or in the luteal
513 phase of the estrous cycle (Kirby and Ridgway, 1984; Sawyer-Steffan et al., 1983). As
514 such, a circulating P_4 concentration < 1 ng/mL cannot be considered a marker of sexual
515 immaturity, but simply an indication that the female has not recently ovulated and/or is
516 not pregnant. Nonetheless, blubber P_4 concentration was a useful marker of maturity in
517 minke whales (*Balaenoptera acutorostrata*) (Inoue et al., 2018). We found no evidence
518 to suggest that PC2 scores are significantly related to age, length, or weight in non-
519 pregnant bottlenose dolphins. Inoue et al. (2018) used enzyme immunoassays to
520 measure P_4 and achieved lower a detection limit (0.2 ng g^{-1} vs. 5.62 ng g^{-1}), which likely

improved their ability to use P_4 to differentiate between mature and immature females. Had we achieved a lower limit of detection for blubber P_4 we would likely have been better able to address this question. However, over half of the mature non-pregnant minke whales in Inoue et al. (2018) had blubber P_4 levels within our range of detection; thus, if such a relationship existed in our study, we should have detected it despite our high RL. This suggests that there is either a difference between species and/or a seasonal component to the relationship between blubber P_4 and female maturity (notably, Inoue et al. (2018) only sampled in December, January, and February). Pallin et al. (2018b) found that most of the non-pregnant humpback whales in their study exhibited blubber P_4 concentrations that were indistinguishable from immature females, further supporting this conclusion. Thus, relationships between female maturity and steroid hormone profiles in cetaceans require further investigation.

Taken together, our results support our overall hypothesis that blubber is well-suited for assessing changes in endocrine function over relatively broad temporal scales but cannot currently be used to study instantaneous endocrine status. Our findings demonstrate that blubber hormone profiles can be used to study shifts in steroid hormone profiles associated with important physiological states, including sexual maturity, pregnancy status, and acute stress. In the future, with further assessment of these relationships using these techniques, it is possible that blubber could be used to classify an individual's sex, maturity, and reproductive status without having to perform physical/ultrasound exams or assessing genetic sex or morphometrics. This is particularly important because demographic, morphometric, and health data often cannot currently be collected when using remote sampling techniques. Overall, this study advances our understanding of cetacean endocrinology and improves our ability to assess cetacean reproductive, developmental, and stress physiology with remotely collected samples. Additionally, this study may provide important insights into the use of adipose tissue to assess endocrine physiology in other species and/or the use of other alternative matrices in marine mammals.

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564

565 **6. Conflicts of Interest**

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

Table 1. Sample size and hormone detection frequency by demographic group and sample matrix.

		Male		Female		
	RL (ng)	**Adult** n = 11	**Subadult** n = 15	**Non-Pregnant** n = 32	**Pregnant** n = 15	**Probable Pregnant** n = 4
Blubber Hormone						
17-hydroxyprogesterone ($17OHP_4$)	0.535	63.6 %	0.00 %	0.00 %	42.9 %	0.00 %
Testosterone (T)	0.260	63.6 %	0.00 %	0.00 %	0.00 %	0.00 %
Androstenedione (AE)	0.206	72.7 %	6.67 %	0.00 %	28.6 %	0.00 %
Progesterone (P_4)	5.62	0.00 %	0.00 %	0.00 %	100 %	100 %
Cortisone (E)	0.0838	90.9 %	93.3 %	90.6 %	64.3 %	100 %
Cortisol (F)	0.856	81.8 %	66.6 %	71.9 %	21.4 %	100 %
11-deoxycorticosterone (S)	0.0779	27.3 %	13.3 %	34.4 %	0.00 %	50.0 %
Plasma Hormone						
17-hydroxyprogesterone ($17OHP_4$)	0.114	90.9 %	33.3 %	38.7 %	73.3 %	50.0 %
Testosterone (T)	0.259	90.9 %	33.3 %	16.1 %	13.3 %	25.0 %
Androstenedione (AE)	0.0545	100 %	73.3 %	3.23 %	73.3 %	0.00 %
Progesterone (P_4)	0.459	0.00 %	0.00 %	6.45 %	100 %	100 %
Cortisone (E)	0.200	90.9 %	100 %	96.8 %	100 %	100 %
Cortisol (F)	0.853	90.9 %	100 %	96.8 %	100 %	100 %
11-deoxycorticosterone (S)	NQ	0.00 %	0.00 %	0.00 %	0.00 %	0.00 %

RL = reporting limit, NQ = not quantified
Non-pregnant female plasma and pregnant female blubber excludes one sample each which were lost during processing (Supplemental Table 6)

Table 2. Minimum, median, and maximum detected values of hormones by demographic group and sample matrix. Shaded cells indicate hormones that were not detected in specific matrices within groups.

		Blubber (ng g^{-1} wet weight blubber)							**Plasma** (ng g^{-1} wet weight plasma)					
		17OHP$_4$	**T**	**AE**	**P$_4$**	**E**	**F**	**S**	**17OHP$_4$**	**T**	**AE**	**P$_4$**	**E**	**F**
Adult Male	Min.	1.59	1.08	3.45	ND	0.492	0.0178	0.0383	0.245	2.04	0.315	ND	1.71	6.76
	Med.	4.02	4.24	10.3	ND	0.803	2.96	0.173	1.57	17.7	1.02	ND	3.23	12.8
	Max.	13.7	17.4	63.3	ND	2.31	14.6	0.780	12.7	56.9	2.86	ND	6.64	30.2
Subadult Male	Min.	ND	ND	3.48	ND	0.349	0.0596	0.159	0.115	0.395	0.0571	ND	1.23	4.62
	Med.	ND	ND	3.48	ND	0.709	3.13	0.247	0.129	0.571	0.136	ND	1.84	9.53
	Max.	ND	ND	3.48	ND	1.23	8.69	0.334	0.138	0.732	0.449	ND	3.20	17.4
Non-Pregnant Female	Min.	ND	ND	ND	ND	0.184	0.0940	0.0779	0.143	0.191	0.0352	0.402	0.886	2.89
	Med.	ND	ND	ND	ND	1.02	4.54	0.227	0.180	0.342	0.0352	1.71	2.18	10.6
	Max.	ND	ND	ND	ND	2.63	21.0	1.00	0.287	0.526	0.0352	3.02	4.19	17.5
Pregnant Female	Min.	2.05	ND	3.92	23.4	0.295	0.723	ND	0.265	0.203	0.0582	4.25	0.651	1.67
	Med.	2.12	ND	5.49	95.8	0.486	0.873	ND	0.371	0.237	0.223	13.8	1.06	7.63
	Max.	2.91	ND	8.88	174	0.900	6.13	ND	1.19	0.272	0.732	20.6	2.25	13.9
Probable Pregnant Female	Min.	ND	ND	ND	29.4	0.598	0.154	0.0791	0.213	0.213	ND	14.3	0.977	5.74
	Med.	ND	ND	ND	90.2	0.688	1.28	0.116	0.255	0.213	ND	16.8	1.62	9.85
	Max.	ND	ND	ND	146	0.859	5.00	0.154	0.297	0.213	ND	20.6	1.78	12.4

ND = not detected

Table 3. PCA rotated component matrices. Bolded values highlight variable loading with an absolute value greater than 0.4.

	PC1	PC2	PC3	PC4
% Variance	27.24	21.75	12.83	9.191
Blubber Hormone				
17-hydroxyprogesterone ($17OHP_4$)	**0.667**	0.304	0.130	0.034
Testosterone (T)	**0.699**	-0.156	-0.127	0.124
Androstenedione (AE)	**0.804**	-0.002	-0.122	0.001
Progesterone (P_4)	0.082	**0.912**	-0.061	-0.079
Cortisone (E)	0.002	-0.352	0.201	**0.751**
Cortisol (F)	-0.053	-0.140	-0.024	**0.772**
11-deoxycorticosterone (S)	0.157	0.176	0.183	**0.602**
Plasma Hormone				
17-hydroxyprogesterone ($17OHP_4$)	**0.781**	0.194	0.017	0.068
Testosterone (T)	**0.768**	-0.275	0.064	0.106
Androstenedione (AE)	**0.825**	0.025	0.114	-0.174
Progesterone (P_4)	-0.073	**0.900**	-0.132	-0.123
Cortisone (E)	0.087	-0.170	**0.943**	0.170
Cortisol (F)	-0.082	-0.036	**0.970**	0.121

Figure 1. Steroidogenesis pathway. Boxed pathway indicates the Δ_4 androgen pathway. Parentheticals indicate hormone abbreviation.

Figure 2. (A and B) Principal components analysis score and loading plots inclusive of both plasma and blubber hormone measurements from all bottlenose dolphin blubber and blood samples; markers indicate individuals (color and shape indicate demographic group and site per the key) and arrows indicate magnitude and direction of variable loading (the prefix "b_" indicates blubber hormone variable, while the prefix "p_" indicates plasma hormone variable). (**C**) Interaction plot for PC1 scores (i.e., mean PC1 scores by demographic group and site-month); error bars indicate standard deviation. (**D and E**) Differences in PC1 and PC2 scores, respectively, by demographic group and site-month; horizontal lines indicate demographic groups in which all sites-months were combined into a single group for pairwise comparison; groups with different letter headings are significantly different per pairwise t-test with Benjamini-Hochberg correction ($\alpha = 0.05$); numbers below boxes indicate sample size. AM = adult male, SM = subadult male, NPF = non-pregnant female, PF = pregnant female, PPF = probable pregnant female.

Figure 3. (A) Relationship between PC3 score (circulating corticosteroid profile) and elapsed time to blood collection; markers indicate individuals (color and shape indicate demographic group and site per the key); **(B)** Relationship between PC4 score (blubber corticosteroid profile) and elapsed time to blubber collection; markers indicate individuals (color and shape indicate demographic group and site-month per the key). Demographic group and site-month are only shown for visualization, neither of these variables are included in the final models for PC3 or PC4 scores. AM = adult male, SM = subadult male, NPF = non-pregnant female, PF = pregnant female, PPF = probable pregnant.

Figure 4. Relationship between age (**A**), body length (**B**), and body weight (**C**) and PC1 scores (androgen profile) in male bottlenose dolphins stratified by site-month (non-pregnant females shown with reduced size markers for comparison); markers indicate individuals (color and shape indicate demographic group and site-month per the key). AM = adult male, SM = subadult male, NPF = non-pregnant female.

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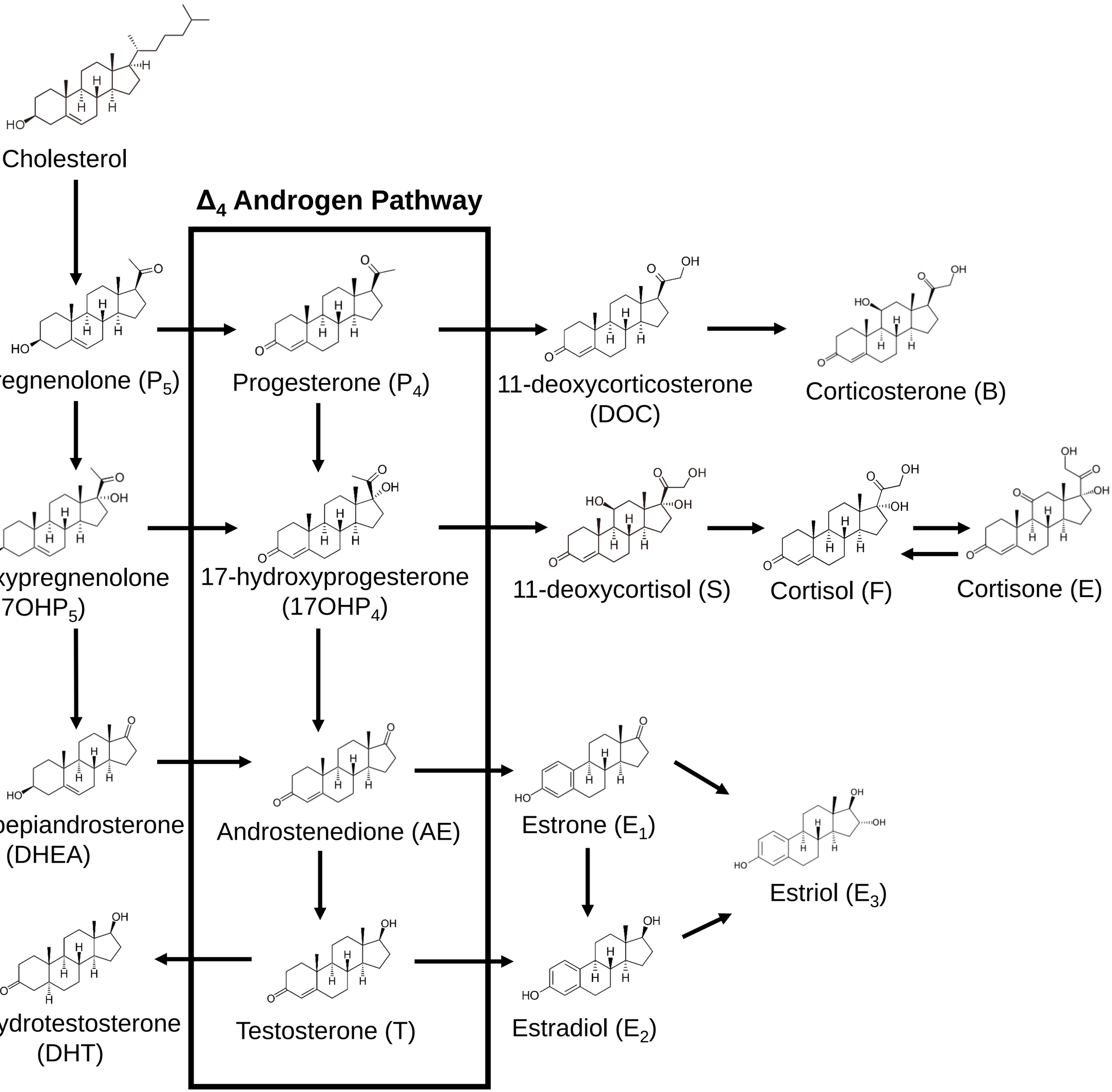
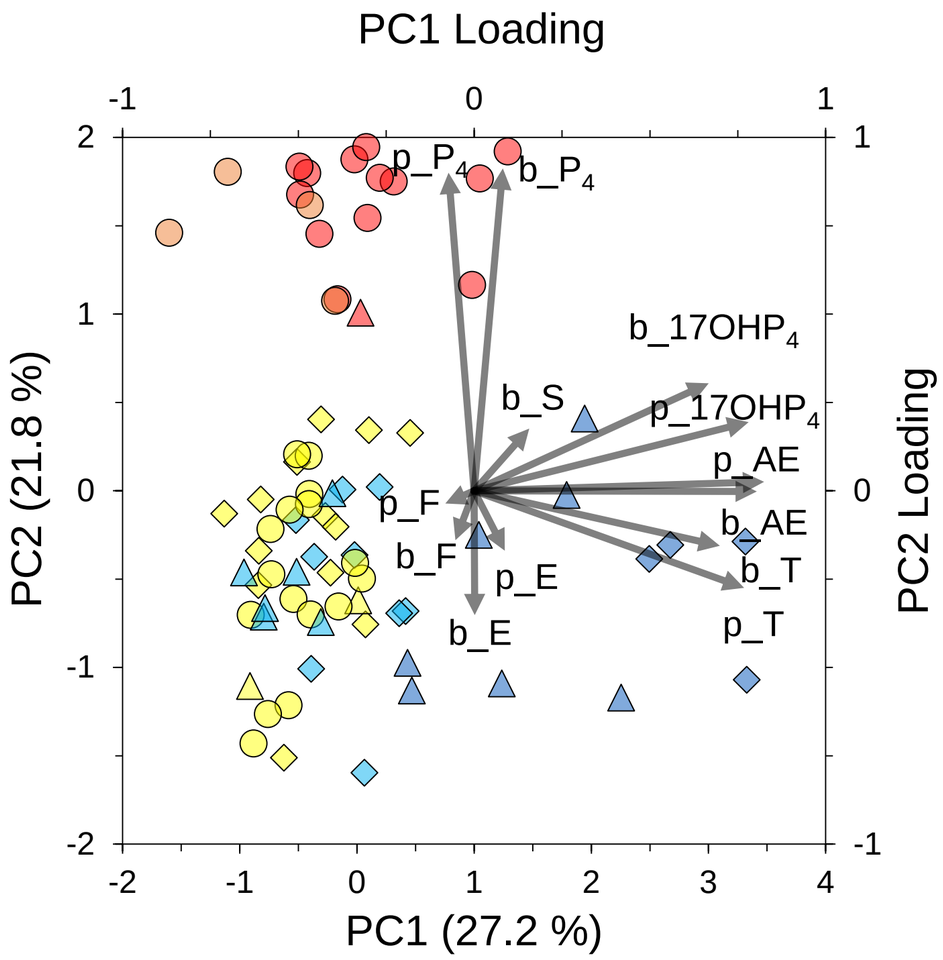
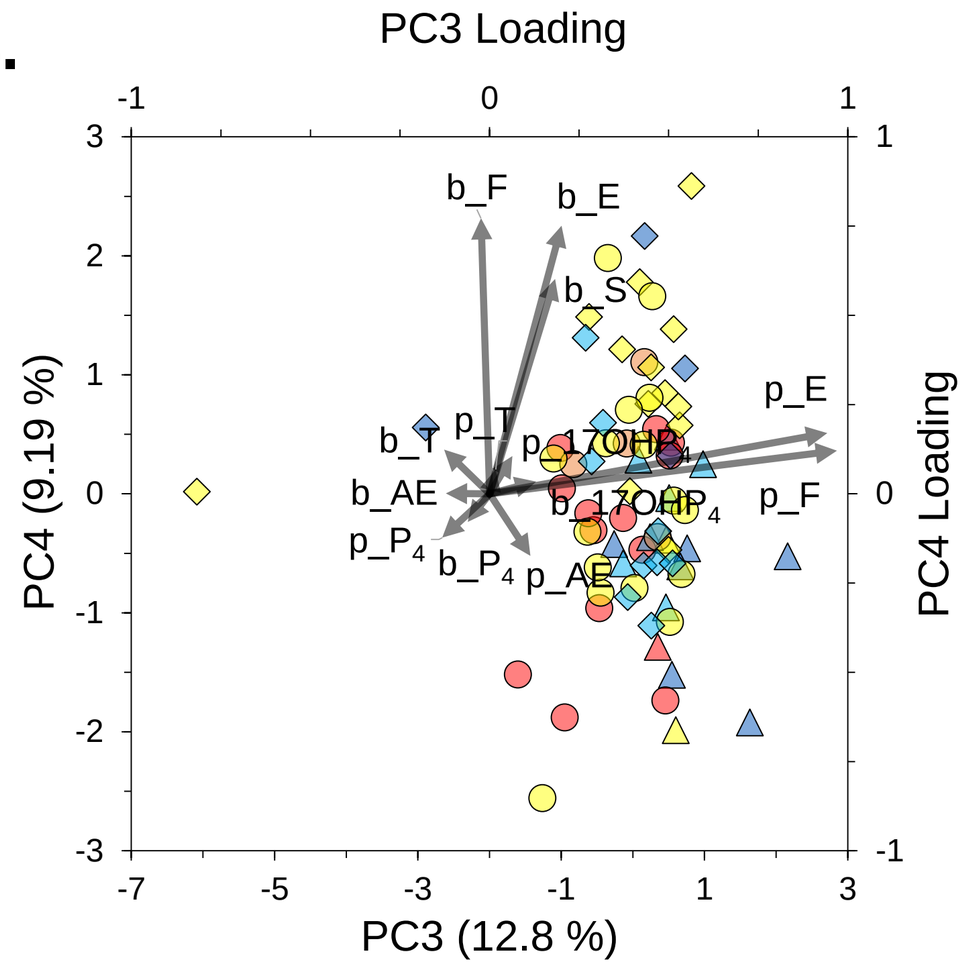
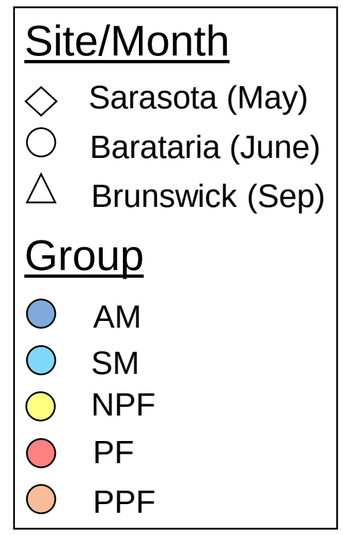

Cholesterol
Δ4 Androgen Pathway
Pregnenolone (P5)
Progesterone (P4)
11-deoxycorticosterone (DOC)
Corticosterone (B)
17-hydroxypregnenolone (17OHP5)
17-hydroxyprogesterone (17OHP4)
11-deoxycortisol (S)
Cortisol (F)
Cortisone (E)
Dehydroepiandrosterone (DHEA)
Androstenedione (AE)
Estrone (E1)
Estriol (E3)
Dihydrotestosterone (DHT)
Testosterone (T)
Estradiol (E2)

A.

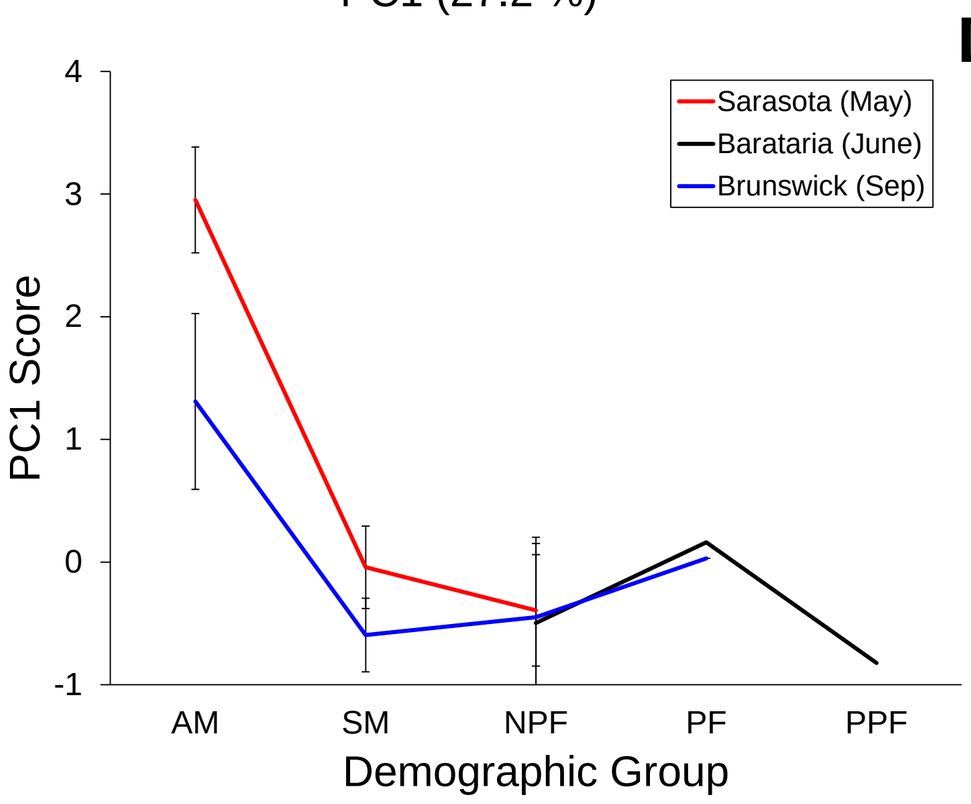

PC1 Loading
p_P4
b_P4
b_17OHP4
b_S
p_17OHP4
p_AE
p_F
b_AE
b_F
p_E
b_T
b_E
p_T
PC2 (21.8 %)
PC2 Loading
PC1 (27.2 %)

B.

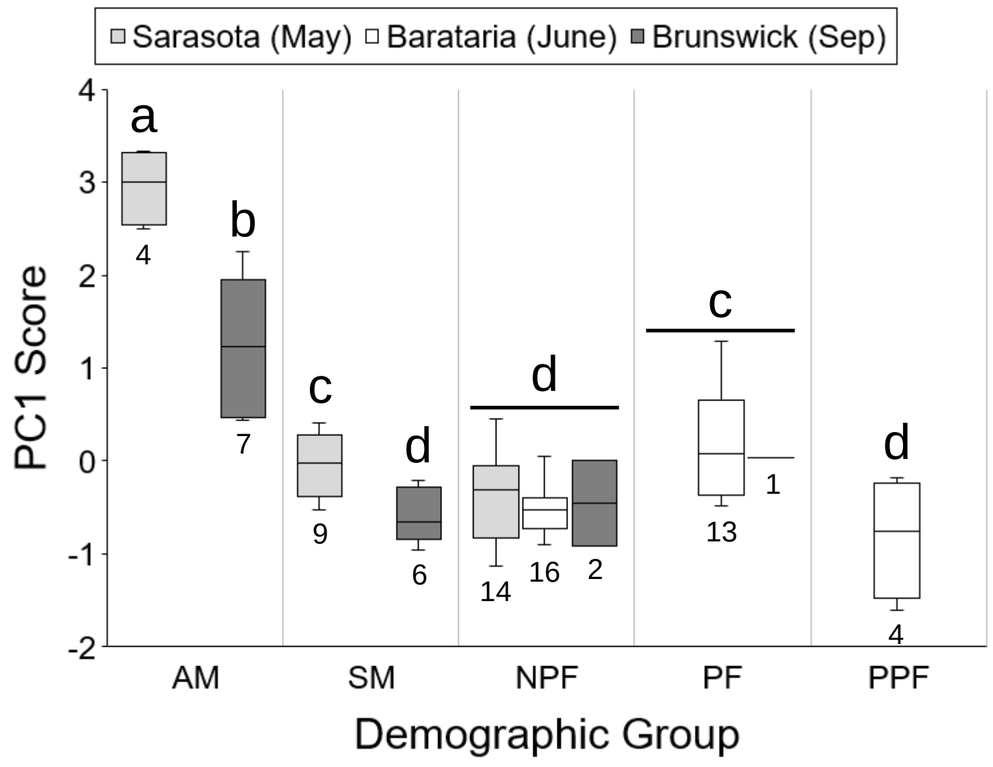

PC3 Loading
b_F
b_E
b_S
p_E
p_T
b_T
p_17OHP4
b_AE
p_F
b_17OHP4
p_P4
b_P4
p_AE
PC4 (9.19 %)
PC4 Loading
PC3 (12.8 %)


Site/Month
Sarasota (May)
Barataria (June)
Brunswick (Sep)
Group
AM
SM
NPF
PF
PPF

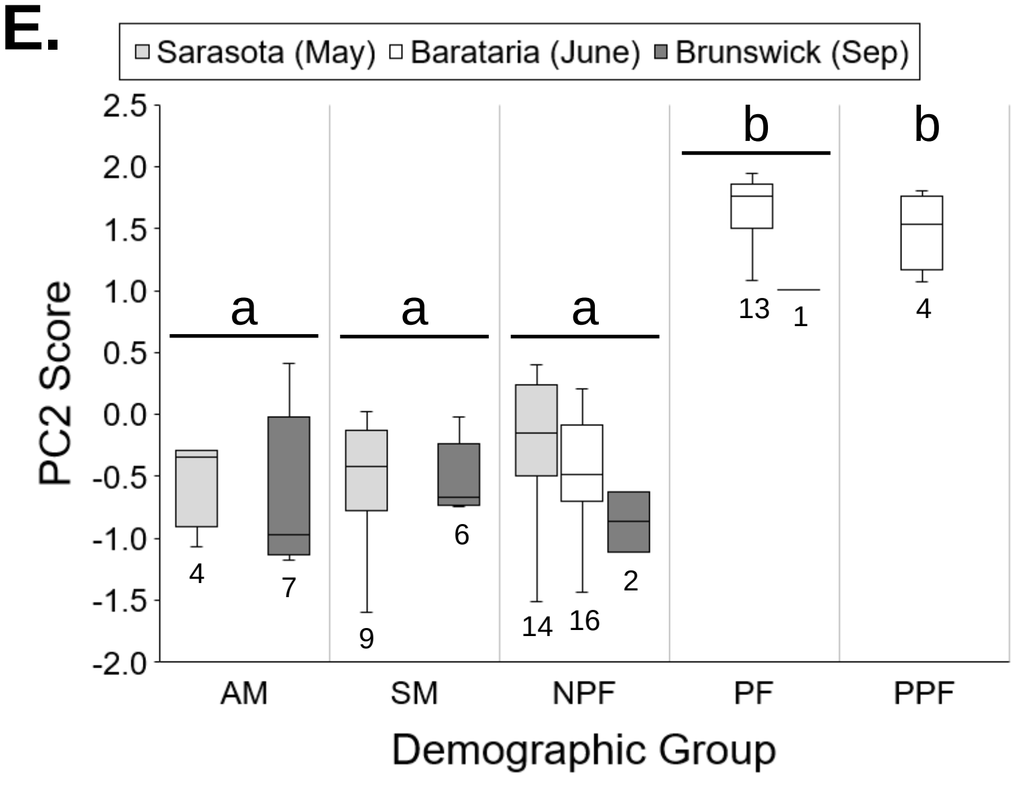
C.


Sarasota (May)
Barataria (June)
Brunswick (Sep)
PC1 Score
AM
SM
NPF
PF
PPF
Demographic Group

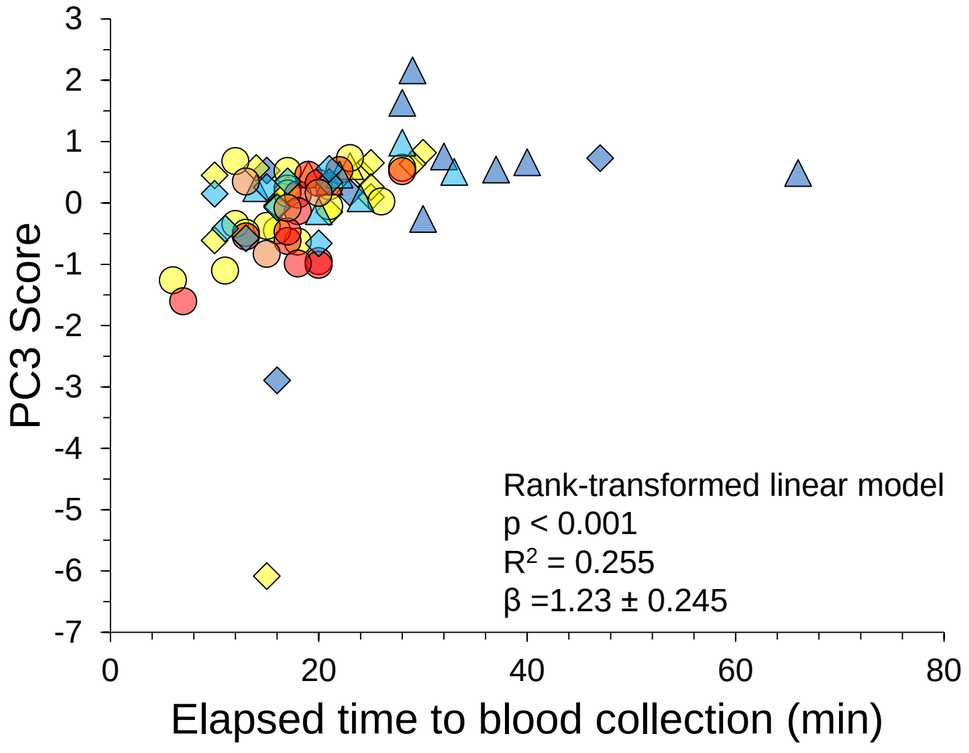
D.


Sarasota (May)
Barataria (June)
Brunswick (Sep)
a
b
c
d
PC1 Score
AM
SM
NPF
PF
PPF
Demographic Group

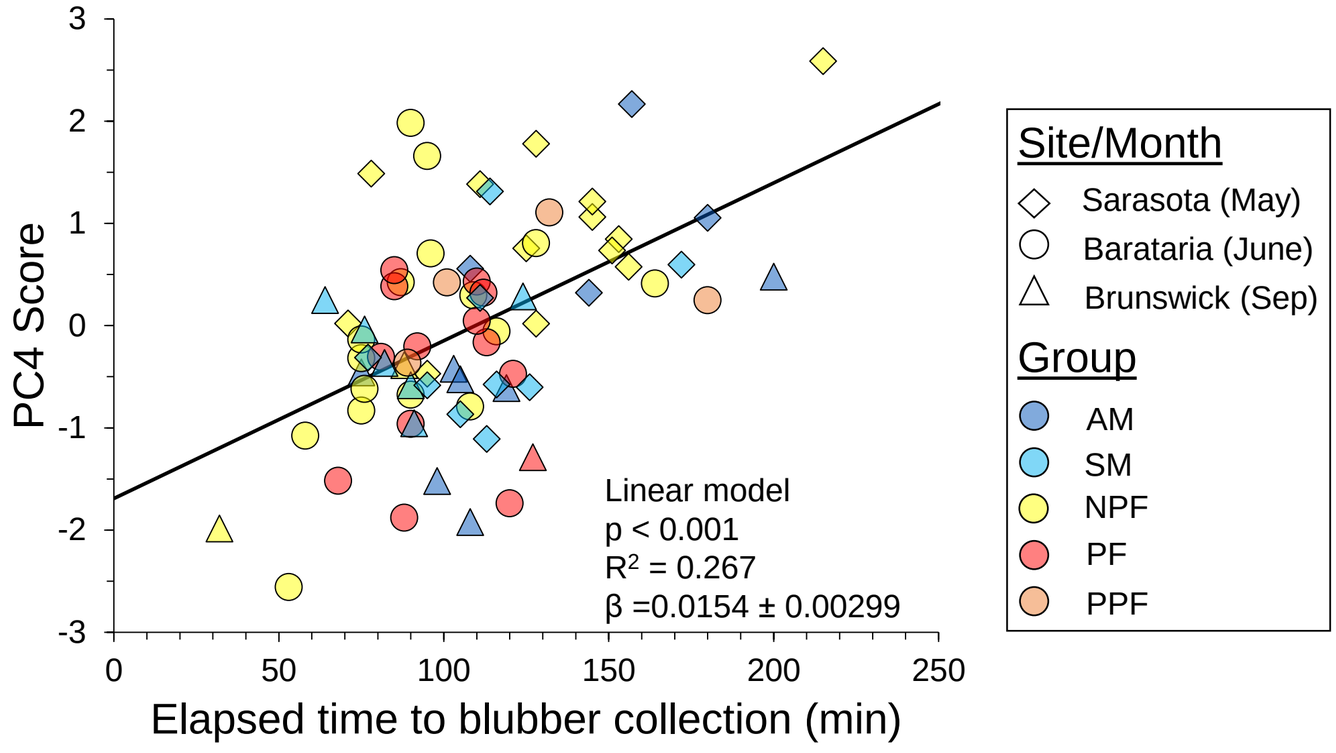
E.


Sarasota (May)
Barataria (June)
Brunswick (Sep)
a
b
PC2 Score
AM
SM
NPF
PF
PPF
Demographic Group

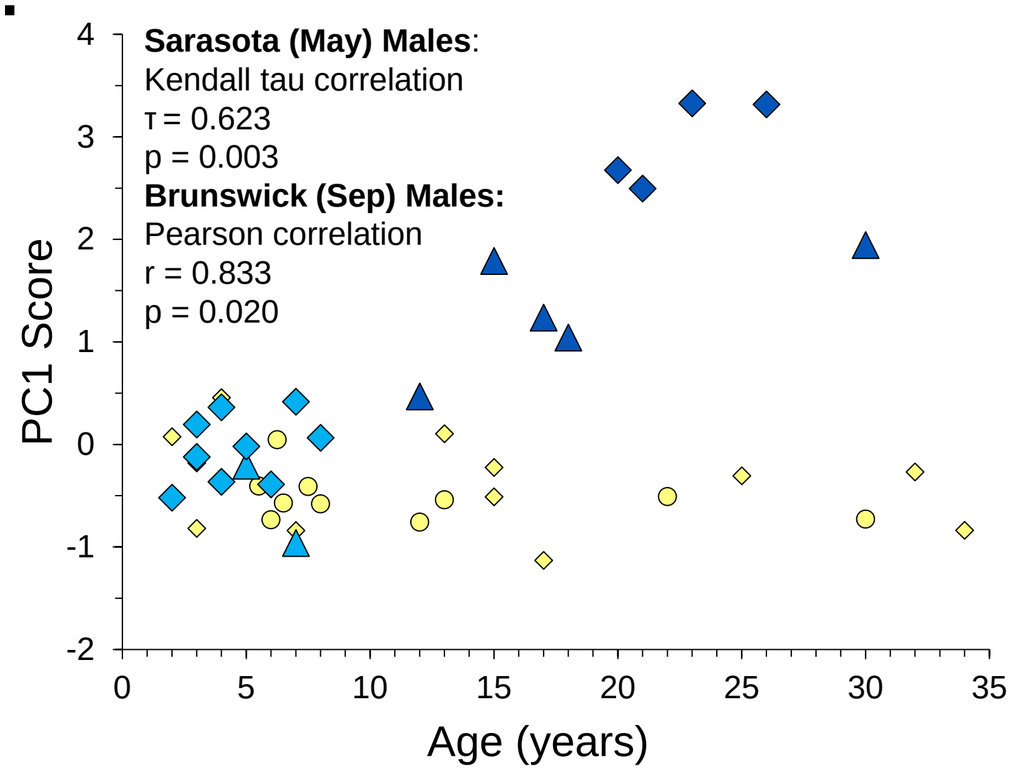
A.


PC3 Score
Elapsed time to blood collection (min)
Rank-transformed linear model
p < 0.001
R2 = 0.255
β =1.23 ± 0.245

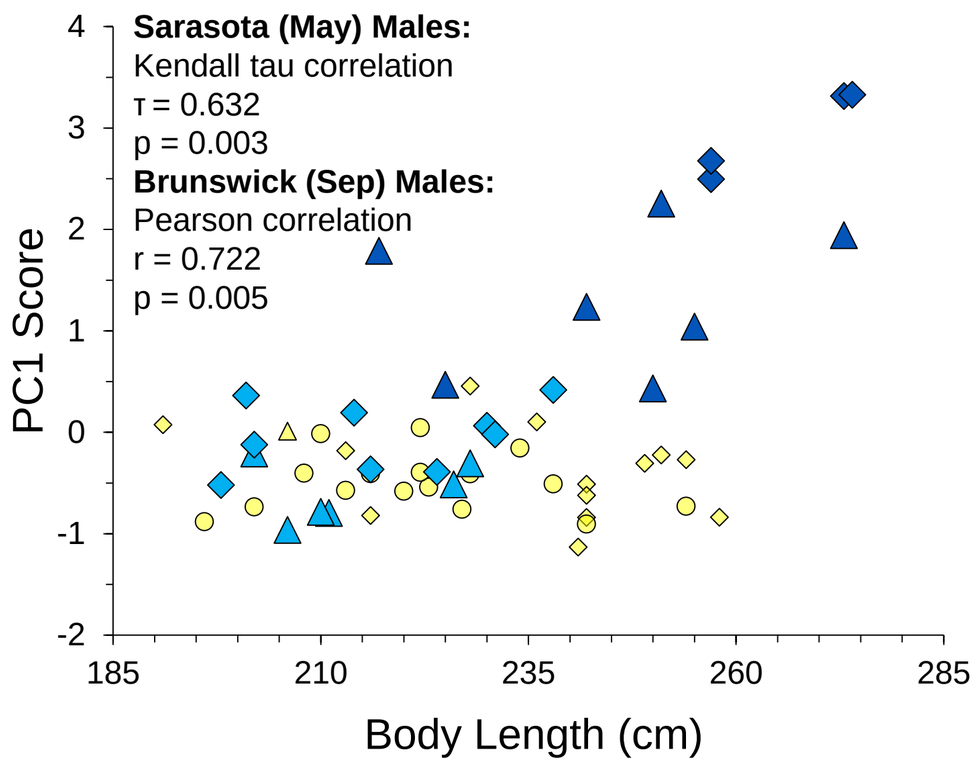
B.


PC4 Score
Elapsed time to blubber collection (min)
Linear model
p < 0.001
R2 = 0.267
β =0.0154 ± 0.00299
Site/Month
Sarasota (May)
Barataria (June)
Brunswick (Sep)
Group
AM
SM
NPF
PF
PPF

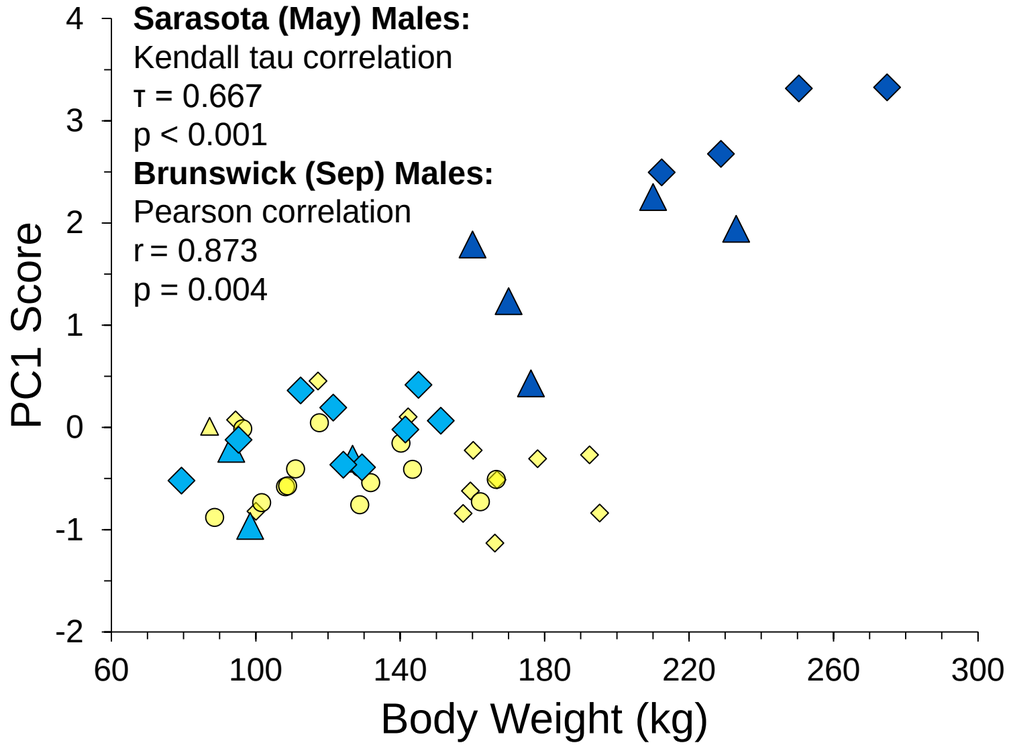
A.

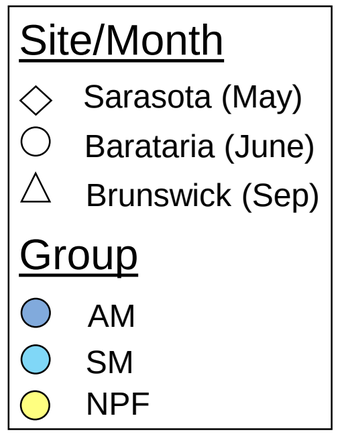

Sarasota (May) Males:
Kendall tau correlation
τ = 0.623
p = 0.003
Brunswick (Sep) Males:
Pearson correlation
r = 0.833
p = 0.020
PC1 Score
Age (years)

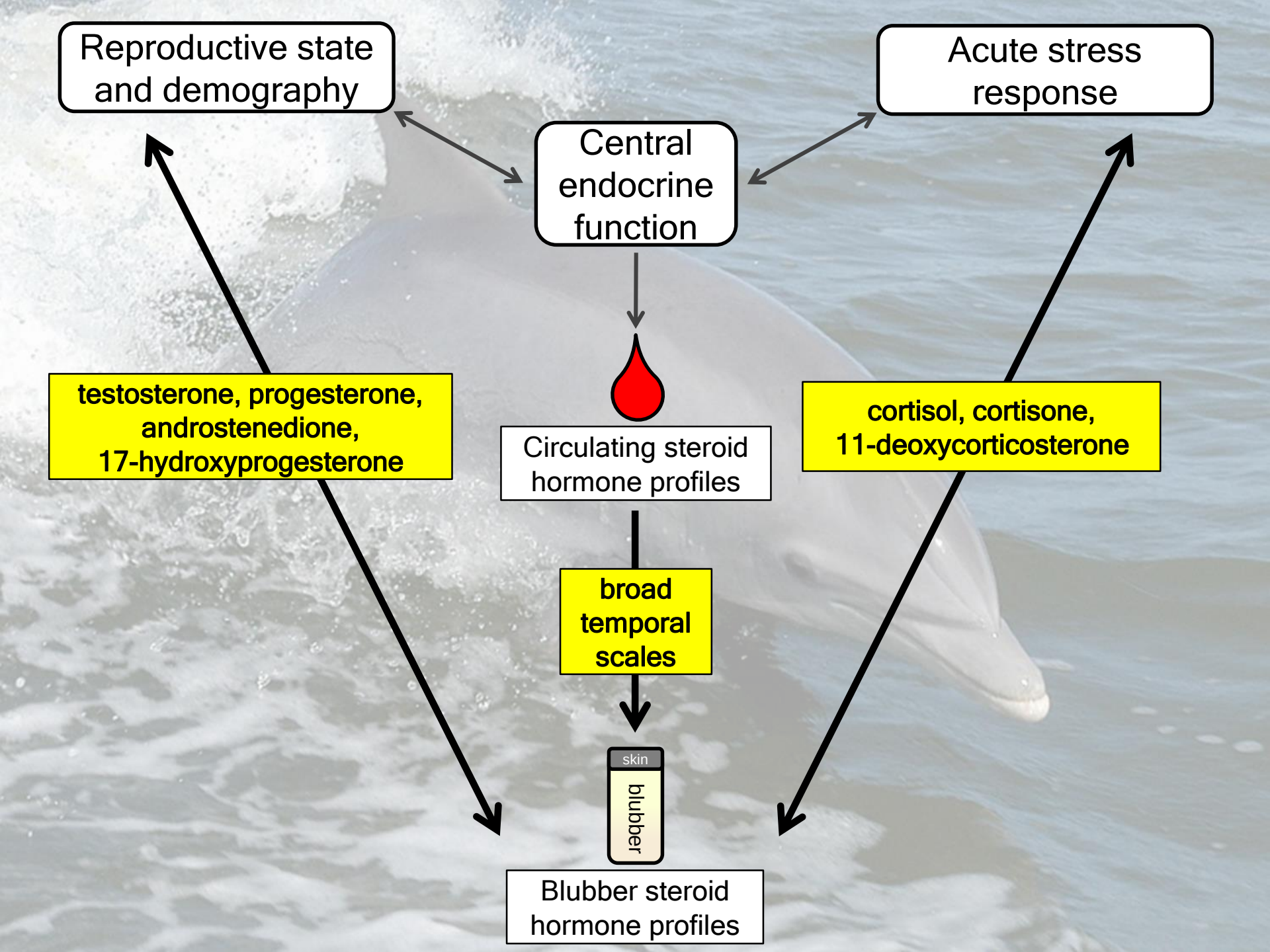
B.


Sarasota (May) Males:
Kendall tau correlation
τ = 0.632
p = 0.003
Brunswick (Sep) Males:
Pearson correlation
r = 0.722
p = 0.005
PC1 Score
Body Length (cm)

C.


Sarasota (May) Males:
Kendall tau correlation
τ = 0.667
p < 0.001
Brunswick (Sep) Males:
Pearson correlation
r = 0.873
p = 0.004
PC1 Score
Body Weight (kg)


Site/Month
Sarasota (May)
Barataria (June)
Brunswick (Sep)
Group
AM
SM
NPF


Reproductive state and demography
Acute stress response
Central endocrine function
testosterone, progesterone, androstenedione, 17-hydroxyprogesterone
cortisol, cortisone, 11-deoxycorticosterone
Circulating steroid hormone profiles
broad temporal scales
skin
blubber
Blubber steroid hormone profiles