Comprehensive Endocrine Response to Acute Stress in the Bottlenose Dolphin from Serum, Blubber, and Feces

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Highlights

- Induced stress activated the HPA axis with typical increases in stress hormones (e.g. cortisol).
- Serum corticosteroids rapidly returned to baseline levels when dolphins were returned to the water.
- Elevated cortisol was detectable in blubber as well as in feces.
- These alternate matrices may be useful indicators of acute stress in dolphins.

Abstract

Several hormones are potential indicators of stress in free-ranging animals and provide information on animal health in managed-care settings. In response to stress, glucocorticoids (GC, e.g. cortisol) first appear in circulation but are later incorporated into other tissues (e.g. adipose) or excreted in feces or urine. These alternative matrices can be sampled remotely, or by less invasive means, than required for blood collection and are especially valuable in highly mobile species, like marine mammals. We characterized the timing and magnitude of several hormones in response to a stressor in bottlenose dolphins (*Tursiops truncatus*) and the subsequent incorporation of cortisol into blubber, and its metabolites excreted in feces.

We evaluated the endocrine response to an acute stressor in bottlenose dolphins under managed care. We used a standardized stress protocol where dolphins voluntarily beached onto a padded platform and remained out of water for two hours; during the stress test blood samples were collected every 15 minutes and blubber biopsies were collected every hour (0, 60, and 120 min). Each subject was studied over five days: voluntary blood samples were collected on each of two days prior to the stress test; 1 and 2 hours after the conclusion of the out-of-water stress test; and on the following two days after the stress test. Fecal samples were collected daily, each afternoon.

The acute stressor resulted in increases in circulating ACTH, cortisol, and aldosterone during the stress test, and each returned to baseline levels within 2 hours of the dolphin's return to water. Both cortisol and aldosterone concentrations were correlated with ACTH, suggesting both corticosteroids are at least partly regulated by ACTH. Thyroid hormone concentrations were generally unaffected by the acute stressor. Blubber cortisol increased during the stress test, and fecal GC excretion was elevated on the day of the stress test. We found that GCs in bottlenose dolphins can recover within hours of acute stress, and that cortisol release can be detected in alternate matrices within a few hours—within 2 h in blubber, and 3.5 - 5 h in fecal samples.

abbreviations

ACTH – adrenocorticotropic hormone
CBG – cortisol binding globulin
GC – glucocorticoids
GCm – GC metabolites
HPA – hypothalamic-pituitary-adrenal [axis]
HPG – hypothalamic-pituitary-gonadal [axis]
MMP – Marine Mammal Program
SNS – sympathetic nervous system
T4 – thyroxine
T3 – triiodothyronine
rT3 – reverse T3

1. Introduction

Animals are exposed to disturbances from myriad sources, both natural and anthropogenic (Butchart et al., 2010; Maxwell et al., 2013). Stress hormones, especially the glucocorticoids (GC, e.g. cortisol), can be useful indicators of disturbance-induced stress in animals (Dantzer et al., 2014; McCormick and Romero, 2017; Sheriff et al., 2011). GC variability has been associated with environmental disturbance in several terrestrial (Madliger et al., 2016; Wikelski and Cooke, 2006) and marine animals (Ayres et al., 2012; Rolland et al., 2012), including bottlenose dolphin (*Tursiops truncatus*) in the Gulf of Mexico following the Deepwater Horizon oil spill (Smith et al., 2017) and in animals living in chronically contaminated environments (Fair et al., 2017). GCs are routinely measured from blood samples but are also found in alternative matrices (e.g. blubber and excreta), which offer less invasive means of monitoring GC variability.

Disturbance-induced stress from capture and handling activates the hypothalamic-pituitary-adrenal (HPA) axis in most free-ranging species, including marine mammals, complicating the interpretation of circulating hormone levels (Fair et al., 2014; Ortiz and Worthy, 2000; Schwacke et al., 2014; St. Aubin et al., 2013). Adrenocorticotropic hormone (ACTH) stimulates the release of GCs, and also the mineralocorticoid aldosterone in many marine mammals, including bottlenose dolphins (Thomson and Geraci, 1986), other odontocetes (St. Aubin and Dierauf, 2001), and pinnipeds (Champagne et al., 2015; Ensminger et al., 2014; Keogh and Atkinson, 2015). Natural stressors, such as temperature, have also been linked to stimulation of the HPA axis in dolphins (Houser et al., 2011).

Perceived threats and other rapid onset stressors activate the sympathetic adrenomedullary (SAM) system, which results in the release of catecholamines. The catecholamines, epinephrine and norepinephrine, are core constituents of the "fight or flight" response. In terrestrial mammals, these hormones are responsible for upregulating heart rate, cardiac output, and increasing oxygen consumption, and glucose oxidation and production. In marine mammals, the exact role of the catecholamines remains to be determined and their action might be modulated through vagal regulation, as has been observed during dive bradycardia in seals (Hance et al., 1982; Hochachka et al., 1995).

The thyroid hormone axis may also be affected by stress (Chrousos, 2007; St. Aubin and Geraci, 1988, 1992; St. Aubin et al., 1996). Thyroxine (T4) released by the thyroid is deiodinated at target tissues to the biologically active triiodothyronine (T3), which has wide ranging metabolic influences (Mullur et al., 2014). T4 also can be converted to the inactive, reverse T3 (rT3), which binds with T3 receptors but does not result in altered gene expression, thus acting as a T3

antagonist (Bianco and Kim, 2006). Dolphins, and marine mammals in general, have high levels of rT3 (St. Aubin, 2001) compared with terrestrial mammals (Atkinson et al., 2015), although the reason for this is not known.

The interpretation of stress hormone data collected under wild and laboratory conditions is complicated by several factors, including a limited understanding of the stress response relative to a baseline, unstressed state, as well as how representative animals under human care are of wild counterparts. Furthermore, understanding the dynamics of free hormones, which bind with receptors and influence target tissues (Breuner et al., 2013; Perogamvros et al., 2012), is limited because little information exists on the binding capacity and levels of their respective carrier proteins (e.g. cortisol and cortisol binding globulin, CBG) in marine mammals. Interpretation is particularly complicated for cetaceans, where little information is known on the variation in corticosteroids and thyroid hormones as a function of life history status.

Collecting blood from free-ranging animals to assess stress hormones is difficult, especially in cetaceans. Blood collection often requires complicated animal capture and restraint to acquire blood samples, with the associated handling stress confounding measurements of unperturbed animal states (Romero and Reed, 2005). An alternative is to use remote or non-invasive sampling techniques, or collect alternative matrices that can provide information on an animal's stress state. The stable, lipophilic properties of steroid hormones allow GCs to diffuse into a variety of tissues and matrices including cutaneous structures (e.g. skin, scales, feathers), adipose tissue (e.g. blubber), and excreted substances (e.g. saliva, urine, feces). These matrices offer promising alternatives to assess stress hormone levels from subjects while minimizing confounds due to handling (Hunt et al., 2013; Kellar et al., 2015). The time course and extent that stress hormones released into circulation appear in these alternative matrices is, however, unknown for most marine mammal species. To make alternative sample matrices most useful, the relationship between stress hormone dynamics in circulation and in alternative matrices must be known.

Our objective was to evaluate the response to, and recovery from, an acute stressor in a marine mammal—the bottlenose dolphin. Study animals were under managed-care at the U.S. Navy Marine Mammal Program (MMP). Dolphins were exposed to a standardized "stress test" by being held out of water for two hours while repetitive samples of blood and blubber were collected. We evaluated the hormone response of catecholamines (epinephrine and norepinephrine), the HPA axis (ACTH, cortisol, and aldosterone), and thyroid hormones (free and total T4 and T3, and rT3) to the stress test, as well as recovery following the test. We also quantified the levels of the binding protein CBG to determine levels of biologically active free plasma cortisol. We concurrently

measured the incorporation and excretion of cortisol into alternate matrices—blubber and feces—to assess their use in the detection of elevated cortisol.

2. Methods

2.1 Study Animals

Five bottlenose dolphins participated in this study, aged 13 – 49 yrs with an average mass of 202 (sd 19) kg. Each dolphin was identified by a unique 3-character identifier (1 female: BLU, and 4 males: TRO, NEH, COL, TYH) and individual dolphin characteristics are provided in Table 1. Study animals were housed in open-water netted enclosures within San Diego Bay, CA, and were maintained by the MMP. Animals experienced normal fluctuations in environmental conditions including day/night cycle, weather, and water temperatures. Dolphin enclosures were connected by underwater gates so that animal care staff could control social mixes; males and females, though kept within 10 m of one another, were not allowed to mix in order to control breeding. Social mixes within the female and male groups were managed daily based on research objectives, animal motivation and interaction. The female dolphin, BLU, had been independent of her last calf for several years at the time of this study. Study animals were under the care of trained clinical staff and fed a fish diet commensurate with their body size. All dolphins were fed a mixed diet of herring (*Clupea harengus*), mackerel (*Scomber scombrus*), squid (*Doryteuthis opalescens*), and capelin (*Mallotus villosus*) throughout the study period. Samples were collected in July and August to minimize seasonal hormone variability.

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Biosciences Division, Space and Naval Warfare Systems Center Pacific and the Navy Bureau of Medicine and Surgery. The treatment of animal subjects followed all applicable U.S. Department of Defense guidelines for the care of laboratory animals. The MMP is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2.2 Experimental Design

We employed a standardized out-of-water "stress test" to evaluate the acute stress response in bottlenose dolphins. Each dolphin was sampled over a five-day study period and food intake was strictly controlled during the study (see Figure 1 for an overview of the study design). Voluntary samples were collected daily for two consecutive days before and after the stress test (days 1, 2, 4, and 5), and the stress test was conducted on day 3 (with one exception: the stress test was delayed one day for study dolphin BLU). Dolphins were trained to voluntarily participate in blood sampling by presenting the ventral surface of their fluke in exchange for a fish reward. Dolphins were requested to present the ventral surface of their fluke and blood samples were collected from the arteriovenous plexus using a 21G, 1.25" winged sampling needle and extension tube into chilled

serum and EDTA plasma vacutainers (BD & Co., Franklin NJ; Champagne et al., 2017). Voluntary blood collections were conducted at 09:00 (sd 6 mins), three hours (sd 2 mins) after feeding a 0.5 kg fish meal, which was sufficient time for the dolphins to reach a post-absorptive state (Yeates and Houser, 2008). Blood samples were briefly stored in a cooler at ~4 °C, carried to the on-site facility, centrifuged at 1,090 *g* for 15 min and stored at -80 °C until subsequent analysis. After voluntary blood samples were collected, dolphins were fed as normal for the remainder of the day. Fecal samples were also collected through voluntary participation; dolphins were trained to permit a flexible 14 Fr polyvinylchloride catheter (Jorgensen Laboratories, Loveland CO) to be inserted into the anal orifice to ~40 cm depth where fecal material passed into it (Champagne et al., 2017; Houser et al., 2017). Fecal samples were collected daily, each afternoon (average time was 13:36, sd 53 mins).

On the day of the stress test, dolphins were fed a 0.5 kg fish meal at 06:00. Instead of collecting a voluntary blood sample at 09:00, each study dolphin was subjected to the stress test. Dolphins voluntarily beached onto a padded mat covered by a wooden hut shielding subjects from the sun (at average time 09:08 sd 16 mins). The dolphin then remained out of water for two hours while repeated blood samples and blubber biopsies were collected. Beaching is routine for MMP dolphins (Champagne et al., 2017); it is conditioned through positive reinforcement (food reward) and is itself not considered a stressor to the dolphin. The subsequent repeat blood sampling and blubber biopsies (described below), however, were expected to induce a stress response. The duration of the sampling was deemed sufficient to allow hormone to appear in all matrices sampled.

Just after beaching, initial blood and blubber samples were rapidly collected. Blood samples were collected by venipuncture of the peduncle vessel using an 18 G, 1.5" blood collection needle (initial, time 0 sample collected 5.4 sd 1.1 mins after beaching); this venipuncture was maintained for the duration of the stress test to permit repeated blood sampling every 15 minutes over 2 h (see Figure 1). Blubber was biopsied from an approximately 5 x 5 cm area just posterolateral of the dorsal fin and overlying the epaxial muscle using a 16G biopsy needle (Medical Device Technologies, Inc., Gainesville, FL; Champagne et al., 2017) set to the maximum depth of 33 mm. To collect sufficient tissue, two to three co-located needle insertions (within ~2 cm of one another) were required. The initial biopsy was performed an average of 9.4 sd 1.9 mins after beaching. A cold pack was placed on the site of the biopsy for several minutes prior to sampling to numb the site. Blubber biopsies were repeated one and two hours after beaching (62 sd 3 mins and 121 sd 1 min, respectively). The initial blood and blubber samples collected immediately after beaching are both referred to as time 0 samples. Throughout the out-of-water stress test, dolphins were kept

wet by applying seawater. At the completion of the stress test, dolphins were returned to the water and resumed their normal feeding schedule. Voluntary blood samples were collected one and two hours after their return to the water and for the next two days, as described above.

2.3 Circulating Hormone Assays

Circulating hormone concentrations were measured using commercially available enzyme- or radio-immunoassay kits (EIA or RIA, respectively). ACTH was measured in triplicate with EDTA plasma using an EIA kit (Alpco Inc., Salem NH, cat# 21-ACTHU-E01), previously validated for use in bottlenose dolphin (Champagne et al., 2017); the average coefficient of variation (CV) of sample replicates was 3.9%. Cortisol and free and total T4 and T3 were measured in duplicate from serum using antibody-coated tube RIA kits (from Siemens, Inc., Los Angeles, CA; since discontinued); average replicate CVs were 2.8, 1.9, 2.8, 2.1, and 1.9% for cortisol, fT4, tT4, fT3, and tT3, respectively. Each of these kits have been validated for use in the bottlenose dolphin (cortisol: Houser et al., 2011; Ortiz and Worthy, 2000) and (thyroid hormones: Ortiz et al., 2010). Reverse T3 was measured in duplicate from serum using an EIA kit (Alpco Inc. cat# 38-RT3H-R125). One-fourth of the recommend sample volume was used to bring the unknown percent bound values into the standard range (0 to 3000 pM) and a correction for altered sample volume was applied; this kit and modified procedure has been previously validated in our lab (Houser et al in prep). The average replicate CV for rT3 was 2.8%.

Circulating aldosterone concentrations are typically very low in cetaceans, especially in managed-care populations where voluntary participation and positive reinforcement can be used to mitigate handling stress (Champagne et al., 2017; St. Aubin, 2001; St. Aubin et al., 1996). Aldosterone was therefore assayed by first extracting steroids from a known serum volume (~1 mL) into an organic solvent (methylene chloride, Sigma cat# 650463) in three extraction series of 4 mL each (i.e. three washes each using 4 mL methylene chloride) prior to conducting the RIA. The solvent containing the extracted aldosterone was evaporated to dryness under vacuum on a vacuum centrifuge. The sample was assayed using an antibody-coated tube RIA kit (Siemens, Inc.; since discontinued) by reconstituting the dried sample in steroid-free serum matrix (500 µL, provided separately by the kit manufacturer, Siemens, Inc.) and following the kit protocol. The average CV between sample replicates was 1.8%. We previously validated this extraction procedure and kit for use in the bottlenose dolphin (Houser et al in prep). Tests of extraction efficiency using known concentration standards in dolphin serum matrix found greater than 90%

recovery of sample using this extraction; extractions of negative controls (both manufacturer-supplied zero matrix and steroid-stripped dolphin serum) tested undetectable for aldosterone.

Epinephrine and norepinephrine were simultaneously assayed in duplicate from EDTA plasma using a Bi-Cat EIA kit (Alpco, Inc., cat# 17-BCTHU-E02-RES); the average replicate CVs were 3.6 and 2.9% for epinephrine and norepinephrine, respectively. The Bi-Cat assay was validated for use in bottlenose dolphin; serially diluted pools of dolphin plasma were parallel with the kit standard curve (supplementary Figure S1). Sex steroids—testosterone in the four male dolphins, and progesterone in the single female dolphin—were measured from a subset of samples during (0, 60, and 120 min samples) and following the stress test (+1 h, + 2h recovery samples; and +1 and +2 d post samples). Sex steroids were measured in singlet by automated immunoassay on a Cobas 6000 (Roche Diagnostics, Indianapolis, IN; Krasowski et al., 2014).

2.4 Binding Capacity of CBG and Calculation of Free Cortisol

We measured the maximum binding capacity of CBG for cortisol using a 96-well microdialysis plate (HTDialysis, Gales Ferry, CT). Each well was comprised of two compartments—a "serum side" and a "buffer side"—separated by a vertical dialysis membrane (Spectra/POR 2, 10-14 kD MWCO, Spectrum Labs, Rancho Dominguez, CA). The serum side of each well received 150 µL of a mixture of 10% serum and 27 nM cortisol (10% 1,2,6,7-^{3}H-cortisol, Perkin Elmer, Waltham, MA, and 90% non-labeled cortisol, cat. no. C-106, Sigma-Aldrich, Mississauga, Canada) in phosphate buffered saline with 0.1% gelatin (PBS). The buffer side received 150µL of 27 nM cortisol in PBS (we determined from our calculation of the binding affinity of CBG that 27 nM would saturate the CBG in 10% serum at 4 °C). Each serum sample was run in 5 wells: 3 wells to measure total binding and 2 wells to measure nonspecific binding (primarily binding by albumin). In the nonspecific binding wells, an additional 4 µM unlabeled cortisol was added to the PBS on both sides of the well. Once all samples were added to the plate, the unit was left to equilibrate for at least 4 hours at 4°C. After reaching equilibrium, 100 µL was removed from each side of each well and counted in 3 mL of scintillation fluid in a Beckman LS6500 scintillation counter. Because proteins like CBG and albumin are too large to pass through the membrane, the serum side of the well contained CBG-bound and albumin-bound cortisol whereas the remaining free cortisol diffused evenly across the membrane. Thus, the total binding (CBG and albumin) was calculated by subtracting the counts of the buffer side from those of the serum side. In the nonspecific binding wells, the excess of nonlabelled cortisol caused the CBG to saturate with nonlabelled cortisol, but the albumin (which is nonsaturable at these concentrations) was still able to bind the labeled cortisol. Thus, nonspecific

binding is calculated by subtracting the buffer side counts from the serum side counts of the nonspecific binding wells. Specific binding by CBG is therefore equal to the difference between total and nonspecific binding. The specific binding (in nM) was determined by measuring the total counts in 100 µL of the 27 nM solution and adjusting for the serum dilution. On occasion, CBG values were inconsistent with values from adjacent samples (i.e. an increase or decrease in CBG aberrant from the general trend of the adjacent blood samples for an individual). In these cases, all or some of that animal's samples were reassayed. In most cases the sudden changes in CBG levels were confirmed on the second assay; we therefore used the average binding capacity for samples in which more than one estimate was made. The interassay CV was 5.0% and the mean intra-assay CV was 8.5% (range: 2.3%-24.2%).

Once the CBG binding capacity was known for a given sample, the free hormone concentration was calculated using the equation of Barsano and Baumann (1989). To calculate free hormone concentrations, one must also know the species-specific binding affinity of CBG. We calculated this to be 2.64 nM at 37°C.

2.5 Blubber Hormone Assay

Blubber cortisol level was determined as described previously (Kellar et al., 2015). Briefly the blubber was first mechanically homogenized (Omni Bead Rupter-24, cat# 19-040; Omni International, Kennesaw, GA). Tissue debris was removed in a series of ethanol (100%), ethanol:acetone (4:1), and diethyl ether (100%) rinses in which the supernatant was recovered after each solvent rinse. The resulting lipid residue was mixed twice with acetonitrile and hexane (two immiscible solutions), each time collecting the acetonitrile layer. The final acetonitrile layer was dried down and stored at –20°C until the extract was ready to be assayed. To prepare the extracts for the EIA, they were suspended in 125 µL of 1 M phosphate buffered saline and vortexed in a multitube vortexer for 15 min. Cortisol assays were performed using an EIA kit (cat# K003-H1, Arbor Assays, Ann Arbor, MI); the measured intra-assay CV was between 9.6% and 17.4% and the interassay CV was between 13.1% and 20.1% (these CV values are inclusive of variation between extractions). The steroid hormone extraction and measurement procedures have been validated for use in common dolphins (Kellar et al., 2015) and further validated for use with bottlenose dolphins (Champagne et al., 2017). The blubber cortisol concentration in this study is reported as nanograms of cortisol per gram of lipid extracted (quantified gravimetrically on an OHAUS pioneer microbalance, model# PA214); this differs from some prior reports that reference to per gram of blubber tissue (which includes nonlipid components of the blubber, such as proteins and water).

Because of the limited tissue available from each sample, all but 5 blubber cortisol measurements were only assayed once. As such, the CV values reported above were generated from both these 5 samples and conspecific reference blubber tissue that was extracted and measured concurrently.

2.6 Fecal Hormone Assays

Fecal GC metabolites (GCm), aldosterone, and thyroid (T4 and T3) metabolites were determined as previously described (Champagne et al., 2017; Wasser et al., 2010; Wasser et al., 2000). RIAs were conducted to measure fecal hormone metabolites using commercially available kits (GC and thyroids: MP Biomedicals, Orangeburg, NY; fecal aldosterone: Siemens, Inc., Los Angeles, CA). Each of these assay kits has been previously validated for use in this species (Champagne et al., 2017). Briefly, fecal samples were lyophilized for at least 48 h to remove water before hormone extraction. Dried samples were then homogenized and a 0.1 g subsample was transferred to a 50 mL polypropylene tube. Hormones were extracted into 15 mL 70% ethanol on a pulsing vortexer for 30 min. Samples were then centrifuged at 1,092 *g* for 20 min and aliquots of the supernatant collected for analyses. A portion of the ethanol extract was dried under forced air, resuspended in assay buffer, and assayed in parallel with a standard curve and assay quality controls. Intra-assay CV between sample replicates were less than 3.5% and inter-assay variation was 5.6%.

2.7 Statistical Treatment

Statistical analyses were conducted using R statistical software version 3.2 (R Core Team, 2016). Linear mixed models (LMM) with individual dolphin as a random effect were used to assess responses using the lme4 package (Bates et al., 2014). We tested for variation among samples (pre samples: -2 and -1 day before the stress test; the initial, time 0 sample collected immediately after beaching; stress samples collected at times: +15, +30, +45, +60, +75, + 90, +105, and +120 mins; recovery samples collected +1 and +2 h after the conclusion of the stress test; and post samples collected +1 and +2 d after the stress test, as outlined in Figure 1) and, if significant differences were detected, we followed with a Dunnett's post-hoc test against the pre samples (binned into a single group). We similarly explored relationships between hormones using LMM with individual as a random effect; we evaluated whether the relationship varied among individuals using a random slope model, and tested this against a fixed slope model using likelihood ratio tests. The goodness of fit was determined with marginal r-squared (mR^2) statistics for mixed models (Nakagawa and Schielzeth, 2013) implemented in the MuMIn package (Barton, 2015). Residual

distributions of fitted models were visually inspected for normality and homogeneity of variance; responses were log-transformed if needed. Appropriate degrees of freedom within LMMs were estimated using the Kenward-Rogers approximation and p-values were calculated in the lmerTest package (Kuznetsova et al., 2013); post-hoc comparisons were conducted using the multcomp package (Hothorn et al., 2008).

3. Results

3.1 Serum Hormone Response

Circulating concentrations of HPA axis hormones and CBG were significantly influenced by the out-of-water stress test (Table 2, and Figures 2 and 3). ACTH concentrations were elevated 45 min into the stress test and remained higher than baseline concentrations through the +1 hr recovery sample (LMM: $F_{13,57} = 24.6$, $p < 0.0001$; Dunnett's post-hoc test: $p < 0.01$, Figure 2a). Aldosterone concentrations were elevated from 45 mins to the end of the stress test, returning to baseline by the +1 hr recovery sample (LMM: $F_{13,57} = 12.9$, $p < 0.0001$; Dunnett's: $p < 0.01$; Figure 2b). Total serum cortisol showed a significant increase by the 15-min sample and remained elevated through the +1 hr recovery sample (LMM: $F_{13,57} = 32.0$, $p < 0.0001$; Dunnett's, $p < 0.01$; Figure 3a). Free serum cortisol showed a similar response, increasing within 15 min of beaching (LMM: $F_{13,57} = 15.9$, $p < 0.0001$; Figure 3c), but had returned to baseline levels at the +1 h recovery sample (Dunnett's test, $p > 0.1$).

CBG binding capacities were relatively stable during the study period and did not significantly vary with sample time (LMM: $p = 0.08$, Figure 3b); when binned by sample group, however, CBG did show a small but statistically significant difference between stress test and post test samples (collected 1 and 2 days after the stress test; LMM: $F_{4,66} = 4.9$, $p < 0.01$; Tukey's post-hoc test: $p < 0.01$; Table 2). The proportion of CBG bound with cortisol increased dramatically during the stress test (LMM: $F_{13,57} = 28.8$, $p < 0.0001$, Figure 3c); values were greater than the pre-samples from the 15 min sample through the +1 h recovery sample (Dunnett's: $p < 0.01$). In the pre-samples, representing the baseline state, 42% of the CBG was bound with cortisol but this varied greatly among individuals (sd 19%). During the stress test, CBG approached saturation in all subjects (92 sd 5% bound; Table 2 and see Figure 3d).

There were strong associations among HPA axis hormones: both total cortisol and aldosterone were positively associated with ACTH (LMM: total serum cortisol: $F_{1,4.3} = 22.5$, $p < 0.01$, $mR^2 = 0.59$; aldosterone: $F_{1,4.8} = 33.1$, $p < 0.01$, $mR^2 = 0.65$; Figure 4a & c) and with one another ($F_{1,4.4} = 12.3$, $p < 0.05$, $mR^2 = 0.41$; Figure 4b). The associations between total cortisol and ACTH, and total cortisol and aldosterone varied by individual dolphin; whereas the relationship between aldosterone and ACTH did not (based on likelihood ratio tests). Similarly, free cortisol was also associated with ACTH, and varied by individual (LMM, random slope model: $F_{1,4.3} = 14$, $p = 0.02$; mR2 = 0.55; relationship not shown).

Epinephrine concentrations varied among the sample times (LMM: $F_{13,57} = 2.7$, $p < 0.01$, Figure 5a) but only the initial sample differed from the pre samples (Dunnett's: $p < 0.05$). Norepinephrine

304 concentrations also varied due to the stress test (LMM: $F_{13, 57} = 2.7$, $p < 0.05$); these concentrations
305 were higher than baseline from 45 to 90 minutes during the stress test (Figure 5b). Epinephrine
306 and norepinephrine concentrations were not associated with one another (LMM: $p > 0.1$).
307 Free and total thyroid hormones (both T4 and T3) were not markedly influenced by the stress
308 test (LMM: $p > 0.05$ for all four of these models; Figure 6). There was, however, an influence of the
309 stress test on rT3 concentration (LMM: $F_{9, 36} = 3.0$, $p < 0.01$; Figure 6e). The +1 h and +2 h recovery
310 samples immediately after the stress test, and the +1 d post sample all showed elevated rT_3
311 concentration relative to the pre sample (Dunnett's, $p < 0.05$); by +2 d, rT3 concentration had
312 returned to baseline levels (no detectable difference in rT3 between pre and +2 d samples, $p > 0.1$).
313 The concentration of rT3 always exceeded that of total T3 (the mean rT3 : total T3 ratio was 6.2 (sd
314 1.1), range: 4.4 to 8.8). There was, however, no detectable change in the ratio of rT3 : total T3
315 (LMM: $p > 0.1$), nor a detectable association between rT3 and total T3 (LMM: $p > 0.1$).
316 Sex steroids, testosterone and progesterone, were only measured in select samples: at time 0,
317 60, and 120 min during the stress test, at the +1 and +2 h recovery samples, and at the +1 and +2 d
318 post samples. Testosterone was measured in the four male dolphins whereas progesterone was
319 measured in the single female dolphin (BLU). Progesterone concentration for BLU ranged from 1.4
320 to 2.9 nM (mean 2.2 sd 0.6 nM); as this was the only female in the study, sex steroid data for BLU
321 were not included in statistical analyses. Testosterone concentration decreased following the
322 stress test (LMM: $F_{6, 17} = 3.6$, $p < 0.05$; Figure 7); both +1 and +2 h recovery samples were lower
323 than the initial, time 0 sample (Dunnett's: $p < 0.05$). Testosterone concentration did not differ from
324 the initial sample in the +1 or +2 d post samples (Dunnett's: $p > 0.05$), suggesting that levels had
325 recovered within 24 hours of the stress test. We did not detect an association between testosterone
326 and total or free cortisol concentrations (LMM: $p > 0.1$).
327
328 *3.2 Fecal and Blubber Hormones*
329 Fecal samples were collected each afternoon at approximately 13:30; on day 3 this was 4.5 (sd
330 0.9) h after the onset of the stress test (Table 3). Fecal GCm was highly variable, ranging from 154
331 to 4450 ng / g dry fecal mass (with one outlying sample at 21,000 ng / g on the day of the stress
332 test, in NEH; see supplementary Figure S2). We detected significant variation during the five-day
333 study period (LMM, log-transformed fecal GC: $F_{4, 16} = 3.8$, $p < 0.05$); fecal GCm was higher on the day
334 of the stress test than on days two and five (Tukey's: $p < 0.01$) but no other days were significantly
335 different from one another. There were no significant differences in fecal aldosterone metabolites

(Figure S3) nor thyroid hormone (T4 and T3) metabolites among study days (LMM: $p > 0.1$ for all three hormone metabolites).

One study subject, BLU, was an outlier with respect to blubber cortisol concentrations. She had substantially greater initial blubber cortisol levels (see supplemental Figure S4) and her fecal GCm level suggested she may have experienced an unknown stressor in the days prior to the stress test (see supplemental Figure S2). We therefore omitted BLU from the statistical analyses of blubber cortisol variation. The following describe changes in blubber cortisol for the remaining four study subjects (for comparison we report the statistical analysis including BLU in supplementary Figure S5). Mean blubber cortisol concentrations were 3.4 (sd 1.3), 6.8 (sd 3.6), and 16.9 (sd 14.3) ng per g lipid at sample times 0, 60, and 120 min, respectively. Blubber cortisol increased during the stress test (LMM, log-transformed blubber cortisol: $F_{2, 6} = 17.4$, $p < 0.01$; see Figure 8a) and concentrations were higher in both the 60 and 120 min samples than in the initial time 0 sample (Dunnett's, $p < 0.05$). Each blubber sample was collected with a nearly simultaneous blood sample (times 0, 60, and 120 min) and blubber cortisol was significantly associated with total serum cortisol (LMM: log-transformed blubber cortisol, $F_{1, 8.3} = 9.1$, $p = 0.02$, $mR^2 = 0.38$; Figure 8b), and with free serum cortisol (LMM: log-transformed blubber cortisol, $F_{1, 8.5} = 5.8$, $p = 0.04$, $mR^2 = 0.29$; Figure 8d).

4. Discussion

We characterized the endocrine response to, and recovery from, an acute stress event in bottlenose dolphins—including the timing and magnitude of hormone responses in circulation, the incorporation of cortisol into blubber, and the excretion of hormone metabolites in feces. Many stress hormones responded predictably: ACTH, cortisol, and aldosterone increased at the onset of the stress test and rapidly declined following its conclusion (Figures 2 and 3). Serum CBG binding capacity was not substantially influenced by the stress test. CBG buffered the exposure of peripheral tissues to freely available cortisol, but free cortisol still increased by nearly ten-fold during the stress test (Figure 3). We detected increased blubber cortisol levels within two hours of the onset of the stress test, and fecal GCm levels were elevated at least four-fold five hours after the stress test. Blubber and fecal samples can be collected less invasively than blood sampling and these data suggest that they reflect serum cortisol levels over relatively recent time periods: under these study conditions, increased blubber cortisol was detectable within 2 h, whereas increased fecal GCm excretion was detected ~5 h after the stress test.

There are a few caveats to consider when interpreting the results of this study, especially if generalizing to wild cetaceans. (1) The study subjects used here were relatively homogenous—five dolphins within the same managed-care population, composed of four males and a single female, all adult and not currently breeding. Despite the similarities among these individuals, we nevertheless observed individual variation in stress responses. Wild populations, comprised of individuals of different ages, experience, social status, etc., may exhibit greater variation in the timing and magnitude of their responses than those reported here (Cockrem, 2013). (2) This study initiated a single, relatively brief (2 hr) stress event and assessed the subsequent acute response in animals under managed care with few other stressors present. Wild subjects may be under a wider array of influences that may or may not have cumulative impacts on their health, body condition, immune, and stress responses (but see Boonstra (2013) for a perspective on chronic stress in wild animals). (3) Dolphins were out of water during the stress test and this facilitated the simultaneous sampling of both blood and blubber tissues throughout the stress response. Being held out of water, however, may be associated with other impacts, including on ventilation (Fahlman et al., 2015), and on circulation (due to the effects of gravity and the differing thermoregulatory challenges in air) that are not present in freely-swimming cetaceans. Collectively, these considerations warrant caution when extrapolating to different populations of wild populations of bottlenose dolphin.

4.1 Response of the HPA axis

387 The out-of-water stress test activated the HPA axis, but we were surprised to detect significant
388 increases in cortisol, as well as aldosterone, *prior to* a significant increase in ACTH (cortisol and
389 aldosterone increased within 15 minutes of beaching, whereas we did not detect an increase in
390 ACTH until 45 minutes after beaching; see Figures 2 & 3). Inspection of individual ACTH plots,
391 however, suggest that ACTH consistently trended upward within the first 15 minutes of the stress
392 test. It is likely that a small increase in ACTH concentration has a substantial initial influence on
393 corticosteroid concentration, and then is subsequently buffered through feedback mechanisms.
394 ACTH, cortisol, and aldosterone all remained elevated for the duration of the stress test and there
395 were close associations among these hormones (Figure 4) reflecting their shared regulatory
396 pathway. The variation in slopes among the subjects (with respect to the relationships between
397 cortisol and both ACTH and aldosterone) indicated that the cortisol response (in timing and
398 magnitude relative to ACTH and aldosterone release) varied among study subjects. This is an
399 important consideration in the measurement and interpretation of stress indicators, as an
400 assessment of the impact of a stressor on a population should consider the variability in the
401 response of individuals to the stressor.
402 Total serum cortisol concentration increased to 100 (sd 33) nM during the out-of-water stress
403 test (range 42 to 166, see Table 2), which is similar to other studies of dolphins placed under
404 stressful conditions, including those under managed care (e.g. veterinary exam: 117 nM, Schmitt et
405 al., 2010), and in wild-caught subjects undergoing routine health assessments (e.g. 70 – 75 nM, Fair
406 et al., 2014), and up to 120 nM during more prolonged capture events (Hart et al., 2015); although
407 somewhat less than the total cortisol concentrations found in beluga (*Delphinapterus leucas*)
408 following relocation to a new facility (~190 nM, Spoon and Romano, 2012). The general similarities
409 in stress hormone concentrations among these studies suggest that the out-of-water stress protocol
410 used in this study elicited a stress response that resembles acute stress in free-ranging dolphins.
411 Thus, the time course and magnitude of the response observed here should inform the potential
412 impact that the capture and handling of wild animals can induce.
413 The circulating concentrations of ACTH and corticosteroids rapidly declined to baseline within
414 1 – 2 h (see Figures 2 and 3) of the end of the stress test and the return of the dolphins to water.
415 Negative feedback on the HPA axis likely occurred after the end of the stress test and possibly
416 during its course. The degree to which negative feedback mechanisms were operational, however,
417 is difficult to determine because the cessation of ACTH production results in a concomitant decline
418 in corticosteroids due to the clearance of excess hormone. Nevertheless, the duration of the stress
419 test was similar to health assessments conducted in wild dolphin populations (Fair et al., 2014) and

suggests that, absent any additional stressors, healthy wild-caught dolphins might be capable of rapidly recovering from handling in these efforts.

4.2 Cortisol Binding Globulin (CBG)

Although we detected a slight reduction in CBG binding capacity in the post-stress test samples (collected over the two days after the stress test), CBG binding capacity was generally stable throughout the study period and unaltered in response to acute stress. Both CBG binding capacity and total serum cortisol showed substantial variability among individual dolphins (see Figure 3 a and b). In the pre-samples, representing the unstressed state, 4.5 % of cortisol was free (not bound with CBG, see Table 2). This is similar to most other mammals studied, with greater than 90% of cortisol bound with circulating carrier proteins (Perogamvros et al., 2012), with a few extreme exceptions (Desantis et al., 2013). The high proportion of hormone binding sequesters most cortisol in circulation and prevents binding with GC receptors at target tissues (Mendel, 1989). During the stress test, cortisol release nearly saturated the available binding capacity of CBG (Figure 3d), occupying over 90% of the available binding sites (Table 2), and producing a dramatic increase in free cortisol. While total cortisol increased four-fold (from 24 nM prior to the stress test to ~100 nM during the stress test), free cortisol levels increased by ~15-fold (from 1.2 to 17.9 nM during the same period). Thus, tissue glucocorticoid receptors were exposed to a relative increase in bioactive cortisol greater than would be detected based on total cortisol concentration changes alone. Furthermore, binding by CBG also resulted in a more rapid reduction in free cortisol than total cortisol—total cortisol remained elevated for at least an hour after the conclusion of the stress test, whereas free cortisol returned to baseline levels by the +1 h recovery sample (Figures 3 a and c). Thus, the interaction between cortisol and its carrier protein facilitated both amplified response to, and rapid recovery from, an acute stressor.

Aldosterone does not have a specific binding protein, although a small proportion (<20%) of aldosterone will bind to CBG. The lack of a specific binding protein may explain the more rapid clearance of aldosterone following cessation of the stress test. Aldosterone returned to within baseline limits within one hour after completing the stress test.

4.3 Blubber Cortisol

Despite substantial interest in using cetacean blubber cortisol values as an indicator of stress (Hunt et al., 2013), there is relatively little published data on blubber corticosteroid levels in marine mammals in general (Beaulieu-McCoy et al., 2017b; Champagne et al., 2017; Kellar et al., 2015;

Kershaw and Hall, 2016; Kershaw et al., 2017; Trana et al., 2015, 2016). Complicating matters, the reference denominator varies among reports (cortisol per mass of blubber tissue or per extracted lipid mass) making direct comparisons difficult. Nevertheless, blubber cortisol levels increased during the stress test and were within the range reported for free-ranging dolphins that stranded and died (~4 - 70 ng / g blubber), presumably following a relatively long period of stress (Kellar et al., 2015; see Table 4).

We recently described cortisol incorporation into blubber in dolphins with total serum cortisol that was elevated by orally administering hydrocortisone over five days (Champagne et al., 2017); and three dolphins in that study also participated in the present study. Baseline blubber cortisol values (time 0) reported here are higher than those previously reported in unstressed dolphins (5.6 vs 1.4 ng / g lipid in Champagne et al., 2017) but this value includes study dolphin BLU that had higher blubber cortisol levels and we suspect experienced an unknown stressor prior to the stress test (indicated by elevated fecal GCm, see supplementary Figure S3). Blubber cortisol values during the two-hour stress test were nearly twice as high as those measured following a prolonged, five-day, experimental elevation of serum cortisol (see Table 4). This difference may reflect the influences of environmental temperature on peripheral perfusion and consequent blubber cortisol levels. The blubber sampling methods differed between these studies in an important manner: blubber samples from Champagne et al. (2017) were collected immediately after the dolphins beached out of the water, whereas each dolphin was out of the water for two hours for sampling in the current study. Over the two-hour course of the stress test peripheral perfusion likely increased to compensate for a reduced ability by the dolphin to dissipate body heat, thus making the circulating cortisol more available for blubber uptake. Additionally, marine mammal blubber is stratified with the inner layer displaying higher cortisol concentrations in some circumstances (Kershaw et al., 2017; Trana et al., 2015). We collected biopsy samples to a consistent depth of 33 mm using a 16 G biopsy needle, and it might be expected that animals with the deepest blubber depths (the largest animals) would have the lowest cortisol levels due to incomplete sampling of blubber depth. The largest dolphins (BLU and NEH), however, did not possess the lowest blubber cortisol levels, supporting the suggestion that individual differences in peripheral perfusion influenced cortisol uptake.

Thermoregulatory challenges from being held out of water may have influenced peripheral perfusion, as ambient air temperature potentially exacerbated thermoregulatory challenges beyond dissipating metabolic heat. We did not adequately consider the potential influence of air temperature during sample collection and did not monitor environmental air temperatures. This

study was conducted in the summer (July – Aug) and stress tests were conducted in the morning, while subjects were out of water and air temperatures were increasing (range 22 – 27 °C). To explore the potential influence of air temperature, we retrospectively obtained temperature data from a nearby weather station, taken to the nearest hour (San Diego Airport, approximately 5 km from the study site). We determined the air temperature deviation (difference between air temp and the mean air temp at the time of each biopsy sample)[1] and evaluated its relationship to blubber cortisol level (see Figure 8c); specifically, we assessed the fixed effects of sample time (0, 60 and 120 m) and air temperature deviance (°C) on blubber cortisol, with dolphin ID as a random effect. We tested for an interaction between sample time and air temperature deviance, but it was not significant, so we removed the interaction from the model. Both sample time and air temperature affected blubber cortisol (LMM, subject BLU removed, log-transformed blubber cortisol; sample time: $F_{2, 5.4} = 22.4$, $p = 0.002$; air temp: $F_{1, 5.8} = 6.7$, $p = 0.04$; $mR^2 = 0.80$; Figure 8c); and this resulted in a significantly better model fit than sample time alone (likelihood ratio test; $\chi^2 = 10.0$, $p < 0.01$). This association is likely mediated by peripheral perfusion: changes in ambient temperature influenced peripheral vasoconstriction/dilation and consequent perfusion of blubber tissue and exposure to circulating cortisol. When diving, heart rate and perfusion to the periphery are dramatically reduced in marine mammals, but during surface intervals and especially after exercise, heart rate increases (Noren et al., 2012; Williams et al., 2015) and likely peripheral perfusion as well (Noren et al., 1999). The rate and degree that blubber cortisol reflects serum cortisol will be influenced by these and other factors. We detected increased blubber cortisol within 2 h of the onset of stress, but the time course of accumulation may vary in other environmental conditions and with wild animals that are unconditioned to being out of the water (in the case of stranded samples).

This, and a previous study (Champagne et al., 2017), detected associations between concentrations of cortisol in blubber and total cortisol in circulation. In the present study, we also assessed CBG binding capacity and calculated free serum cortisol concentrations. It is generally thought that free cortisol diffuses into adipose tissue (Breuner et al., 2013) and thus, blubber cortisol should reflect free serum cortisol concentrations that are integrated over some previous time period. Blubber cortisol was associated with total serum cortisol concentrations from

[1] Because the stress tests were conducted on different days there were different air temperatures during each collection of the 0, 60, and 120m blubber samples. For each of these three time points the air temperatures were averaged across all animals. The deviance therefore was the difference between the air temperature during an individual's blubber sample collection at time x (x = 0, 60, or 120min) and the mean across all individuals at that time. The advantage here is that this temperature metric has no inherent confounding relationship with duration of stress test before samples were taken.

simultaneously collected tissue samples (LMM, log-transformed blubber cortisol: $mR^2 = 0.38$, $p = 0.016$; Figure 8b). Surprisingly, blubber cortisol was only marginally associated with free serum cortisol ($p = 0.04$, $mR^2 = 0.29$) with much of the variation attributed to individuals (conditional $R^2 = 0.67$, Figure 8d). Nevertheless, this and other studies have detected increased adipose cortisol levels during stress (Beaulieu-McCoy et al., 2017a; Kellar et al., 2015; Kershaw and Hall, 2016; Kershaw et al., 2017; Trana et al., 2015, 2016) or increased circulating cortisol levels (Champagne et al., 2017). We did not continue blubber biopsy collection after the stress test, so unfortunately, we do not know if blubber cortisol continued to increase after the stress test (due to a time lag in uptake) nor do we know the rate of removal after dolphins were returned to the water and serum cortisol returned to baseline.

BLU, the single female participant in this study, had a slightly different sample timeline than the other study dolphins. Although BLU was the oldest subject in the study and an experienced research participant, she did not volunteer for beaching on the scheduled day of the stress test (although a fecal sample was collected). BLU beached the following day and we conducted the out-of-water stress test. Fecal GCm analysis (discussed below) indicated that BLU had elevated fecal GCm in the days prior to the stress test, but no increase was seen in her serum cortisol concentration (see figure S2). Furthermore, BLU had uncharacteristically high blubber steroid levels (cortisol and progesterone; progesterone data not shown) at the start of the stress test (see Figure S4). Based on these data we suspect that in the days(s) before the stress test, some unknown factor (e.g. social mixing) caused a cortisol release that was reflected in higher blubber cortisol levels at the beginning of the stress test; although, BLU's serum cortisol concentration suggests that any stress event was transient. Consequently, BLU appears as an outlier in her blubber cortisol measurements—she was the only subject that did not show an increasing trend in blubber cortisol across the three sample time points (0, 60, and 120 mins) and her blubber cortisol was the highest among the study subjects in the first two blubber samples. We, therefore, removed BLU from the analyses of blubber cortisol variation (but we report the analyses including BLU in supplementary Figure S5).

4.4 Fecal hormone excretion

We detected elevated fecal GCm levels in samples collected five hours after the stress test. Fecal GCm concentration varied over the study period and the interpretation of how the stress test influenced fecal GCm was unclear (day 3 differed from days 2 and 5 but not from days 1 and 4). Nevertheless, the highest fecal GCm values were consistently detected on either the day of the

548 stress test, or the following day (day 3 or 4; Table 3, and see supplementary Figure S2). Our
549 sampling technique differed from that of most fecal GCm studies: rather than sampling voided feces,
550 we extracted feces using a catheter. This influenced fecal hormone measures in two ways: (1) We
551 dictated the sample timing, based on when fecal matter was present and the natural passage time
552 may have been slightly longer. (2) We sampled the distal portion of the rectal lumen; this is not a
553 homogenized fecal sample and may not properly represent the fecal pool. Fecal samples were
554 collected at similar times each day and, on the day of the stress test, were collected only 4.5 h (sd 52
555 min) after the start of the test. This suggests a more rapid rate of fecal steroid hormone excretion
556 than has been found in other mammals. Wasser et al (2000) reported fecal GCm elevations
557 following ACTH challenges in a wide variety of mammals. The time of the GC peak varied among
558 species: most species showed a fecal GC peak 24-36 hours after ACTH administration, but a few
559 mammals (e.g., sea otters, gerenuk) showed fecal GCm excretion within the time frame observed
560 here (4 – 6 hrs). A series of investigations used intramuscular administrations of ACTH to evaluate
561 the stress response in another marine mammal—the Steller sea lion (Mashburn and Atkinson,
562 2004, 2007, 2008). These studies did not detect increased fecal GCm until 9 – 12 h after ACTH
563 administration, with fecal GCm excretion peaking between 9 and 28 h, and recovering to baseline
564 levels 52 h after administration (but the time of peak response and recovery varied seasonally in
565 juvenile sea lions). The passage of feces following feeding has been observed to be on the order of 6
566 hours in dolphins, which is a time scale more rapid than often presumed for other mammals but
567 which may apply to other odontocetes (Ayres et al., 2012; Ridgway, 1972). Day 4 fecal samples
568 were collected ~29 h after the start of the stress test, and at least 24 h after serum cortisol had
569 returned to baseline levels. The fecal GCm levels on day 4, however, were intermediate—not
570 differing from those before the stress test (days 1 and 2), or on the day of the stress test (day 3).
571 Peak fecal GCm concentration likely occurred between sampling time points, and probably between
572 day 3 (stress test) and day 4 fecal sample collections. Thus, the time course of fecal sampling used
573 in this study was not sufficient to adequately characterize the dynamics of GC excretion in feces.
574 Fecal GCm values ranged from 150 to 4450 ng/g (with one outlier at 21,780 ng/g, see Figure S2
575 for detail). These values are similar to those reported in free-ranging orca (~500 – 5,000 ng/g and
576 these varied seasonally) and a single stranded individual had a fecal GCm value over 25,000 ng/g,
577 presumably following prolonged stress (Ayres et al., 2012). Reported fecal GCm values, however,
578 vary substantially among marine mammals. Values in healthy North Atlantic right whales
579 (*Eubalaena glacialis*) are typically < 100 ng/g but are substantially elevated during chronic stress
580 (~1,800 ng/g in entangled whales and >5,000 ng/g in a stranded individual; (Rolland et al., 2017).

581 Comparisons with the relatively few measures of fecal GCm in cetaceans is complicated by several
582 factors. First, serum cortisol can vary as a function of age, season, time of day, life history stage, and
583 degree of perceived or actual stress in mammals. It follows that these same factors would affect the
584 excretion of fecal GCm and add to the variability of measured values during baseline collections.
585 Second, we used a somewhat fixed sampling time for fecal collections and did not wait for natural
586 voiding. As a result, our sampling regime might not have adequately captured peak passage of GCm.
587 Additionally, the distribution of GCm in feces is not uniform and pockets of concentrated or dilute
588 GCm might exist if only a portion of excreted feces is analyzed. This was the case here, since
589 completely voided fecal samples were not collected but rather a subsample was collected through
590 insertion of a catheter into the anal orifice. Because of the sampling approach, it is feasible that
591 pockets of highly concentrated GCm (21,780 ng/g) were sampled, or that dilute pockets of GCm
592 (150 ng/g) were sampled. Reductions in variability could be obtained if the entire fecal mass were
593 sampled, but this was deemed impractical for this study because dolphin feces are dilute and do not
594 float once excreted.
595 Despite large increases in serum aldosterone concentrations, we failed to detect increased
596 aldosterone metabolites in fecal samples. A number of factors may have contributed to our
597 inability to detect any change in fecal aldosterone excretion. Aldosterone and cortisol are both
598 excreted by conjugation and renal filtration or metabolism and excretion in bile (Norris and Carr,
599 2013). Wasser et al (unpublished data) compared a variety of aldosterone kits in orca (*Orcinus*
600 *orca*) feces and found them to vary widely. It is possible that the antibody used in the present study
601 had low affinity to the dominant aldosterone metabolites in dolphin feces. We did not collect urine
602 samples in this study, so we are unable to discern the route of aldosterone excretion. Very low
603 circulating aldosterone concentrations are typical in cetaceans (Hart et al., 2015; St. Aubin, 2001)
604 including this managed care population (Champagne et al., 2017); aldosterone concentrations
605 increased to only a few hundred pM even under stress (Figure 2b). It may be that low circulating
606 levels are insufficient to permit the detection of small changes in fecal aldosterone metabolite
607 levels. A recent study, however, reported fecal aldosterone metabolite concentrations in North
608 Atlantic right whales (*Eubalaena glacialis*) that are similar to those reported here and detected
609 differences according to age and reproductive state (Burgess et al., 2017). Dedicated studies into
610 the excretion routes of corticosteroids into urine and feces (using labeled corticosteroids, Wasser et
611 al., 1994) and frequent sampling of these matrices to elucidate the time course of excretion (see:
612 Kersey and Dehnhard, 2014; Sheriff et al., 2010), are needed to better characterize these factors
613 and provide context for investigations of fecal hormone metabolites in cetaceans.

614

615 *4.5 The influence of acute stress on other hormone axes—catecholamines, thyroid hormones, and*
616 *testosterone*

617 We found varying catecholamine levels during this study, with much of the variability due to
618 individual differences among study subjects (Figure 5). Epinephrine varied during the stress test,
619 but there was a high degree of individual variability in the baseline samples collected in the days
620 prior to the stress test (Figure 5a). Epinephrine concentration was increased in the initial sample
621 collected immediately after beaching, but these levels rapidly returned to baseline and remained
622 there for the duration of the stress test. Norepinephrine also varied during the study and was
623 higher in the middle of the stress test (45 to 90 min) than prior to the test. Norepinephrine also
624 varied among the study subjects during the stress test—two individuals had relatively stable
625 concentrations throughout the study period while the remaining three subjects had highly variable
626 concentrations during the stress test (see Figure 5b). The role of catecholamines is of particular
627 interest because they affect peripheral vasoconstriction during diving (Hurford et al., 1996; Suzuki
628 et al., 2017). Elevations in epinephrine immediately following beaching and the beginning of the
629 stress test could be related to a stress-induced peripheral vasoconstriction with a rapid return to
630 baseline mediated by thermoregulatory demands. However, epinephrine was not found to be
631 elevated following short-duration dives in the bottlenose dolphin (Suzuki et al., 2017).
632 Nevertheless, since vasodilation would likely be critical for a beached dolphin, understanding
633 whether catecholamines have similar roles in cetaceans would enlighten the patterns of serum
634 catecholamines observed here.

635 Acute stress had little effect on thyroid hormones. Thyroid hormone concentrations were
636 generally similar to other reports in this species (Champagne et al., 2017; Fair et al., 2011; St. Aubin,
637 2001); free T4 concentrations were somewhat lower here (~10 pM) than in other reports but still
638 fell within the reference range reported by Fair, et al (2011). Previous studies have, however,
639 shown seasonality of thyroid hormone levels in odontocetes (D. Houser, unpublished data; St.
640 Aubin et al., 2001), which might have been a factor affecting initial levels reported here. Our
641 previous study of chronically elevated serum cortisol in this species led to small reductions in
642 thyroid hormone concentrations (Champagne et al., 2017), which might be related to metabolic
643 suppression as a result of chronic stress. This study of acute stress, characterized by a brief
644 increase in serum cortisol, did not have the same effect on thyroid hormones, suggesting a limited
645 influence of short-term stress on the thyroid hormone axis.

Thyroid hormones have been widely measured in marine mammals and circulating concentrations generally do not substantively differ from terrestrial mammals (Fair et al., 2011; St. Aubin, 2001), with the notable exception of rT3. The concentrations of rT3 are generally very high in marine mammals—both cetaceans (St. Aubin et al., 2013; St. Aubin et al., 1996) and pinnipeds (Champagne et al., 2015; Ensminger et al., 2014). Reference values for humans, for example, are below 1 nM (< 0.5 ng/mL; (Melmed et al., 2011); and for domestic dogs typical values are <0.4 nM (Ferguson and Peterson, 1992). Here, rT3 concentration ranged from 4.7 to 10.7 nM (mean: 7.0, sd 1.7 nM) across all samples; averaged 6.5 (sd 1.5) nM in the pre-samples (although these were only evaluated 1 d prior to the stress test), and increased to 7.5 (sd 1.7) nM in the recovery samples (LMM, $p < 0.05$, see Figure 6e). Reverse T3 remained elevated in the +1 d post sample and returned to baseline levels by the next day (+2 d post sample did not differ from pre-levels, LMM: $p > 0.1$). Previous studies have also detected stress-induced increases in rT3 in odontocetes (St. Aubin and Geraci, 1988), and pinnipeds (Ensminger et al., 2014), and a positive association with serum cortisol (Champagne et al., 2015). Because T4 is the precursor to rT3, these hormones are usually inversely proportional (Norris and Carr, 2013); this association, however, was not detected here possibly due to the relatively small increase in rT3 concentrations following the stress test (i.e. thyroid hormones were essentially stable across the study). Reverse T3 has been hypothesized to function as a T3 hormone antagonist, binding with thyroid hormone receptors without upregulating gene expression (Bianco and Kim, 2006). With this possibility in mind, we think the function of consistently elevated rT3 in marine mammals warrants further study.

We detected significant reductions in testosterone in association with the stress test (Figure 7). We did not intend a detailed investigation of the influence of stress on the hypothalamus-pituitary-gonadal (HPG) axis, but opportunistically assessed sex steroids (testosterone in the four male subjects and progesterone in the only female subject) from a subset of samples collected during and after the stress test. The single female subject (BLU) present in the study precluded statistical analysis of progesterone, but visual inspection did not indicate an influence of the stress test on progesterone. Stress, and GCs, generally suppress reproductive behavior and the HPG axis, gonadotropins and gonadal steroids (Kirby et al., 2009; Melmed et al., 2011), but with some exceptions (Van Hout et al., 2010). Here, we found that even brief stress and GC increase resulted in notable reduction in serum testosterone, although the reduction was transient and recovered by the next morning.

4.6 Conclusion

679 We used an out-of-water stress protocol to characterize the timing and magnitude of hormone
680 responses to acute stress in bottlenose dolphins under human care. The HPA axis responded in a
681 classical mammalian pattern but with a substantial aldosterone response typical of marine
682 mammals (Atkinson et al., 2015). Circulating corticosteroids recovered rapidly when animals were
683 returned to water after the stress test. Cortisol increased in blubber samples during the stress test,
684 concomitant with increased serum cortisol, and this increase was detectable within two hours.
685 Similarly, fecal GCm was elevated within hours of the stress test. These findings suggest that both
686 blubber and feces reflect relatively recent elevations in serum cortisol: detectable within 2 h in
687 blubber and 5 h in feces. This study provides estimates of the time-frame by which these
688 alternative matrices reflect serum cortisol levels, but further work is required to determine the
689 dynamics more precisely. Given our sampling design, we were unable to capture declining cortisol
690 levels in either blubber or feces. It remains unclear how long cortisol remains elevated in marine
691 mammal blubber following a stress event.

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Table 1. Age, sex, mass, and date of the stress test for dolphins used in this study.

identifier	sex	Mass (kg)	study date	Age (yr)
TYH	M	188	16-Jul-2014	33
COL	M	198	23-Jul-2014	13
TRO	M	182	30-Jul-2014	22
NEH	M	231	6-Aug-2014	36
BLU	F	210	14-Aug-2014	49

Table 2. Summary of HPA axis hormones and CBG buffering of circulating cortisol.

Serum Feature	sample group:	pre	initial	stress	recovery	post	Statistical Result	
	sample times:	-2 d, -1 d	0	15 - 120 m	+1 h, +2 h	+1 d, + 2 d	F	p
	n:	10	5	40	10	10		
ACTH, pM	mean	6.0	13.1	51.9	25.3	5.7	19.9	< 0.0001
	sd	2.4	5.5	28.5	12.6	2.0		
		a	a	b	a	a		
total serum cortisol, nM	mean	24.2	28.5	100.8	44.0	22.1	62.8	< 0.0001
	sd	9.9	6.2	32.9	21.0	10.6		
		a	a	b	a	a		
free serum cortisol, nM	mean	1.2	1.3	17.9	3.3	0.9	32.4	< 0.0001
	sd	1.1	0.9	9.9	3.0	0.8		
		a	a	b	a	a		
free serum cortisol	mean	4.5	4.6	17.1	6.5	3.6	43.9	< 0.0001
(as % of total cortisol)	sd	3.0	2.5	6.1	3.8	1.7		
		a	a	b	a	a		
CBG binding capacity,								
nM	mean	54.9	53.6	57.6	54.0	50.5	4.8	< 0.01
	sd	14.3	11.1	15.4	16.2	12.0		
				a		b		
CBG bound	mean	41.9	48.8	92.4	66.2	41.7	78.3	< 0.0001
	sd	19.2	13.6	4.9	20.2	20.5		
(% CBG occupied)		a	a	b	c	a		

Circulating adrenocorticotropic hormone (ACTH), total cortisol, free cortisol, the percent of cortisol that was free, the binding capacity of cortisol binding globulin (CBG), and the percent of CBG bound with cortisol before, during, and after the out-of-water stress test in five bottlenose dolphins. Data are binned by sample group (pre, initial, stress, recovery, and post samples; see Figure 1 for a description of sample groups and collection times) and the total number of samples in each group is shown. Statistical results from the linear mixed-model (LMM) analyses are shown at right (numerator and denominator degrees of freedom were 4 and 66, respectively; F-statistic and p-value are shown for each LMM). Different letters within a row indicate significant differences among sample groups (Tukey's post-hoc test, $p < 0.05$).

The stress test was associated with releases of ACTH and cortisol into circulation. Free serum cortisol showed the greatest relative change (> 10-fold), due to the interaction with cortisol's transport protein, CBG. In the unstressed state, >90% of serum cortisol was bound with CBG, while less than half of CBG binding sites were occupied. Conversely, during acute stress, only ~83% of serum cortisol was bound with its carrier (17% free), while >90% of CBG binding sites were bound with cortisol.

Table 3. Fecal hormone metabolites.

Hormone Metabolite		Study Day 1	2	3	4	5	Grand Mean
GCm	mean	2314	1501	6961	1901	1316	2756
	sd	746	1677	8297	962	806	4038
			a	b		a	
aldosterone	mean	10	5	12	7	7	8
	sd	9	3	5	4	3	6
T4	mean	1725	1732	1901	1967	1246	1747
	sd	751	620	338	859	927	720
T3	mean	221	262	238	279	189	243
	sd	116	77	30	118	102	93
	Average Collection Time	13:21	14:00	13:43	13:54	12:50	13:33
	sd, mins	76	39	53	50	22	54

Average fecal hormone metabolite levels are shown for each study day; units for all hormones are ng hormone metabolite per g dry fecal mass. The stress test was conducted on day 3 (adjusted for BLU, see Methods for detail); daily collection times (in local time) and sd (in minutes) are also shown. On the day of the stress test, fecal samples were collected 275 (sd 52) minutes (~4.5 hours) following the start of the stress test. Fecal GCm was the only fecal hormone to show a significant change during the study: (LMM, log-transformed fecal GC: $F_{4, 16} = 3.8$, $p < 0.05$; values were higher on day 3 than on days 2 and 5, shown as different letters in this row; Tukey's post-hoc test, $p < 0.01$).

Table 4. Comparison of blubber cortisol values measured in marine mammals.

Species	Subjects Free-ranging or under Managed Care	Sample Collection Description	sample size	Blubber Cortisol	Units	Reference
Bottlenose dolphin *(Tursiops truncatus)*	managed care	immediately following voluntary beaching, time 0	5	5.6	ng / g lipid	present study
		beached 60 min	5	10.2	ng / g lipid	
		beached 120 min	5	15.6	ng / g lipid	
Bottlenose dolphin	managed care	immediately following voluntary beaching	5	1.4	ng / g lipid	Champagne et al 2017
		during hydrocortisone administration	10[*1]	8.0	ng / g lipid	
Short-beaked common dolphin *(Delphinus delphis)*	free-ranging	deceased, fishery bycatch	23	4.0	ng / g blubber	Kellar et al 2015
		deceased, stranded	40	24.3	ng / g blubber	
Harbor porpoise *(Phocoena phocoena)*	free-ranging	deceased, stranded	20 [*2]	~80	ng / g blubber	Kershaw et al 2017
Harbor seal *(Phoca vitulina)*	free-ranging	live captured, non-molting	74	~150	ng / g blubber	Kershaw and Hall, 2016
		live captured, molting	41	~1000	ng / g blubber	
Beluga *(Delphinapterus leucas)*	free-ranging	harvested following ice-entrapment	29	1.8	ng / g blubber	Trana et al, 2016
		harvested in open water	27	0.3	ng / g blubber	
California sea lion *(Zalophus californianus)*	free-ranging	deceased, fishery bycatch	55	8	ng / g blubber	Beaulieu-McCoy et al, 2017
		stranded, dead	18	256	ng / g blubber	
		stranded, live	17	235	ng / g blubber	

Samples were collected from animals that were either free-ranging (wild) subjects, or under managed care at U.S. Navy Marine Mammal Program. The mean values reported for the present study include data from subject BLU, which was omitted from statistical analyses (see text for detail).

*[1] ten samples from five subjects

*[2] multiple samples collected at various locations from stranded individuals to assess variation with sample location (and depth). See reference for detail.

Figures

Figure 1. Blood sample and blubber biopsy sample collection timeline for each dolphin. Samples were collected over five days, and the stress test was conducted on day 3, beginning at 09:00. Fecal samples were collected daily, each afternoon (not shown). See Methods description for detail. For study dolphin BLU, the stress test was delayed one day.

Figure 2. Adrenocorticotropic hormone (ACTH, panel a), and aldosterone (panel b) responses to the stress test. Each subject is represented by a different symbol according to the legend. Pre-samples were collected 2 and 1 days prior to the test (-2d & -1d, respectively, except for study dolphin BLU where the stress test was delayed one day but data still shown to align with other subjects). Samples collected during the stress test (every 15 minutes for two hours) are shown by the shaded region. Recovery samples were collected 1 and 2 hours after dolphins were returned to the water (+1h & +2h, respectively), and post-samples were collected 1 and 2 days following the stress test (+1d & +2d). See text and Figure 1 for sample collection details. Sample concentrations that significantly differed from the pre samples are shown by the horizontal grey line (Dunnett's test, $p < 0.01$).

Figure 3. Serum cortisol and cortisol binding globulin (CBG) before, during, and following an acute stressor in bottlenose dolphins; shaded regions show samples collected during the stress test. Horizontal bars indicate samples significantly different from pre samples (-2d and -1d; significance based on Dunnett's tests). Each subject is represented by a different symbol according to the legend. Total serum cortisol increased substantially during the stress test (panel a), while there was no significant variation in CBG binding capacity among sample times (panel b). Based on CBG binding capacity, we determined free serum cortisol levels (panel c), which returned to baseline values within 1 h of returning to the water. The cortisol release during the stress test caused near saturation of CBG binding sites (panel d). Saturation of CBG resulted in increased percent of free cortisol available in circulation during the out-of-water stress test (panel e). Note that the stress test for study dolphin BLU was delayed one day (pre samples were collected at -3d and -2d) but data are shown to align with other subjects.

Figure 4. Circulating concentrations of ACTH, aldosterone, and cortisol were associated with one another (marginal r-squared, mR^2, goodness of fits are shown for each whole model). Data from each individual is shown as a different symbol according to the legend in panel (c). If the relationship varied among individuals, slopes for each individual are shown (panels a and b); the model coefficients (intercept and slope) for each individual are shown in the bottom right.
(a) Total serum cortisol was associated with ACTH and this relationship varied among individuals (LMM random slope model: $F_{1, 4.3} = 22.5$, $p < 0.01$; note that two subjects, TYH and COL, have nearly identical model coefficients so their regression lines overlay so only 4 lines are seen in the panel). (b) Total serum cortisol was also associated with aldosterone concentrations and this relationship also varied among individuals (LMM random slope model: $F_{1, 4.4} = 12.3$, $p < 0.05$). (c) Serum aldosterone and ACTH were significantly associated but the nature of association did not vary among individuals (LMM fixed slope model: $F_{1, 4.8} = 33.1$, $p < 0.01$).

Figure 5. Catecholamine (epinephrine (a) and norepinephrine (b)), response to the stress test. Each subject is represented by a different symbol according to the legend. (a) Epinephrine concentration varied among the samples (LMM: $F_{13, 57} = 2.7$, $p < 0.01$) but only the initial, time 0 sample differed from the pre samples, and this was a marginally significant difference (*Dunnett's test, $p = 0.047$). (b) Norepinephrine concentration was increased during the stress test (LMM: $F_{13, 57} = 2.2$, $p < 0.05$; sample concentrations that significantly differ from pre samples are shown by the horizontal gray line, 45 through 90 min samples, Dunnett's test, $p < 0.05$). Visual inspection suggests this is due to three of the five subjects that had highly variable norepinephrine concentrations during the stress test (TRO, NEH, BLU) whereas the other two subjects showed relatively stable concentrations throughout the study. Note that the stress test for study dolphin BLU was delayed one day (pre samples were collected at -3d and -2d) but data are shown to align with other subjects.

Figure 6. Thyroid hormones were not strongly affected by the stress test. Total thyroid hormones are shown in the top row (T4 and T3, panels (a) and (b), respectively). Free thyroid hormones are shown in the middle row (T4 and T3, panels (c) and (d), respectively). Only reverse T3, (rT3) significantly varied during the study ($F_{9, 36} = 2.95$, $p < 0.01$); the +1 and +2 h recovery, and +1 d post samples were higher than the -1 d pre sample (Dunnett's test, $p < 0.05$). Note that the stress test for study dolphin BLU was delayed one day (pre samples were collected at -3d and -2d) but data are shown to align with other subjects.

Figure 7. Testosterone decreased during the stress test (LMM: $F_{6, 17} = 3.6$, $p < 0.05$); +1 and +2 h recovery samples were lower than the initial, time 0, sample collected at the beginning of the stress test (Dunnett's test, $p < 0.05$). Each dolphin is represented by a different symbol according to the legend; only the four male subjects are included, and testosterone was only measured in a subset of samples.

Figure 8. The stress test influenced blubber cortisol levels in bottlenose dolphins. This analysis excludes study dolphin BLU (see text for detail). Individuals are shown in different symbols according to the legend in panel (a).
a) Blubber cortisol significantly increased during the stress test (LMM, log-transformed blubber cortisol: $F_{2, 6} = 17.4$, $p < 0.01$). Blubber biopsy samples were collected just after beaching, 60 minutes after beaching, and 120 minutes after beaching (0, 60, and 120 min, samples, respectively). Blubber cortisol level was greater in each of the 60 and 120 min sample than at time 0 (*Dunnett's test, $p < 0.05$).
b) Blubber cortisol level was associated with total serum cortisol concentrations (LMM, log-transformed blubber cortisol: $F_{1, 8.3} = 9.1$, $p = 0.016$, $mR^2 = 0.38$). Small numbers by each data point designate sample collection time (0, 60, or 120 min).
c) There was a significant influence of air temperature on blubber cortisol levels. We evaluated fixed effects of sample time and air temperature deviance (difference between air temp and the mean air temp at the time of each biopsy sample), with dolphin ID included as a random effect in the model. Both fixed-effects significantly influenced blubber cortisol (sample time: $F_{2, 5.4} = 22.4$, $p = 0.002$; temperature deviance: $F_{1, 5.8} = 6.7$, $p = 0.04$, $mR^2 = 0.80$). The displayed line of fit and

associated mR^2 contains both features and thus does not appear as a line-of-best-fit with air temperature deviation.

d) Blubber cortisol was a marginally significant association between blubber cortisol and free serum cortisol concentrations (LMM, log-transformed blubber cortisol: $F_{1, 8.5} = 5.9$, $p = 0.04$, $mR^2 = 0.29$).

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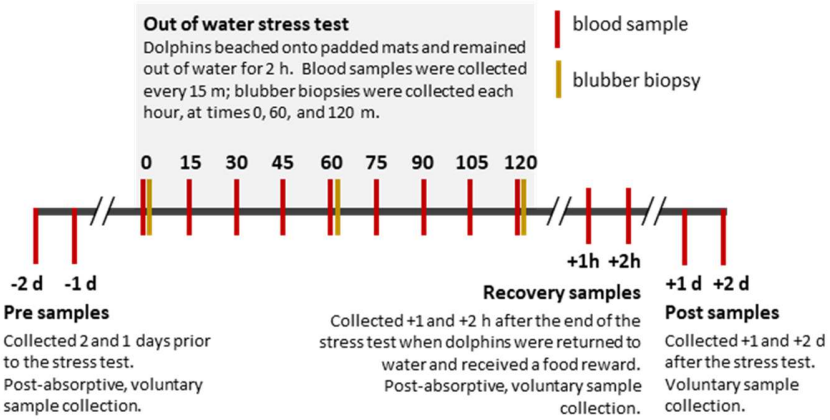

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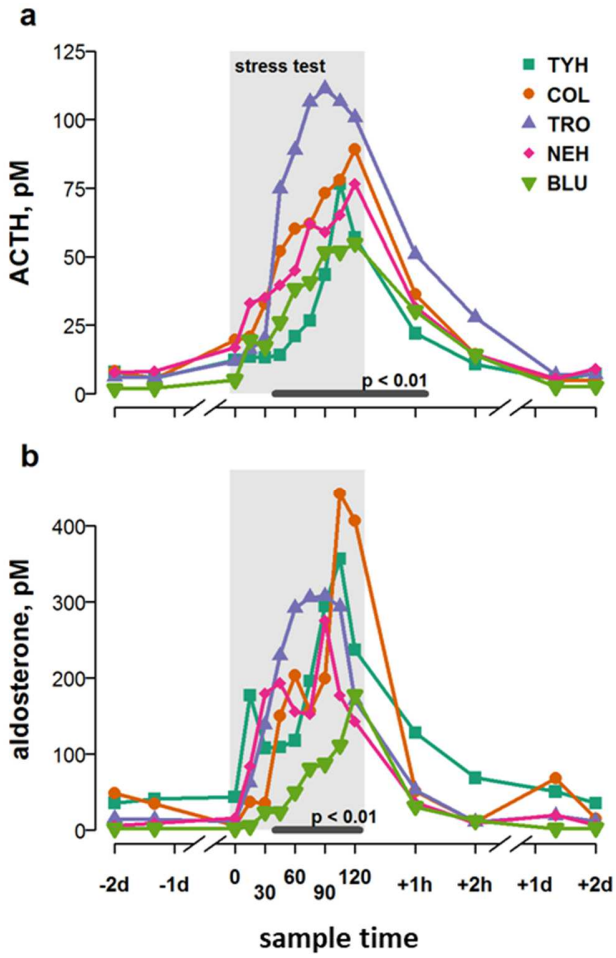

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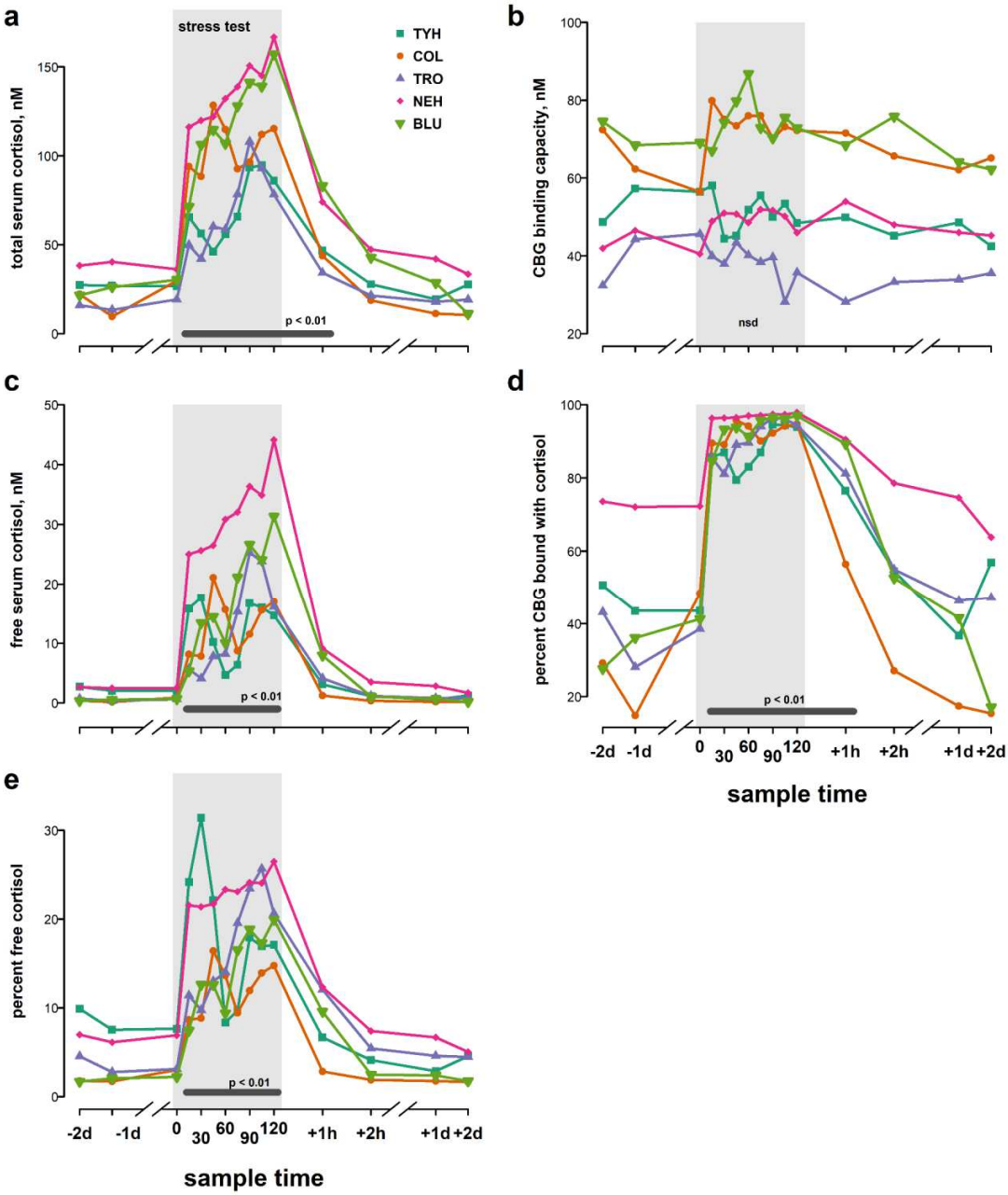

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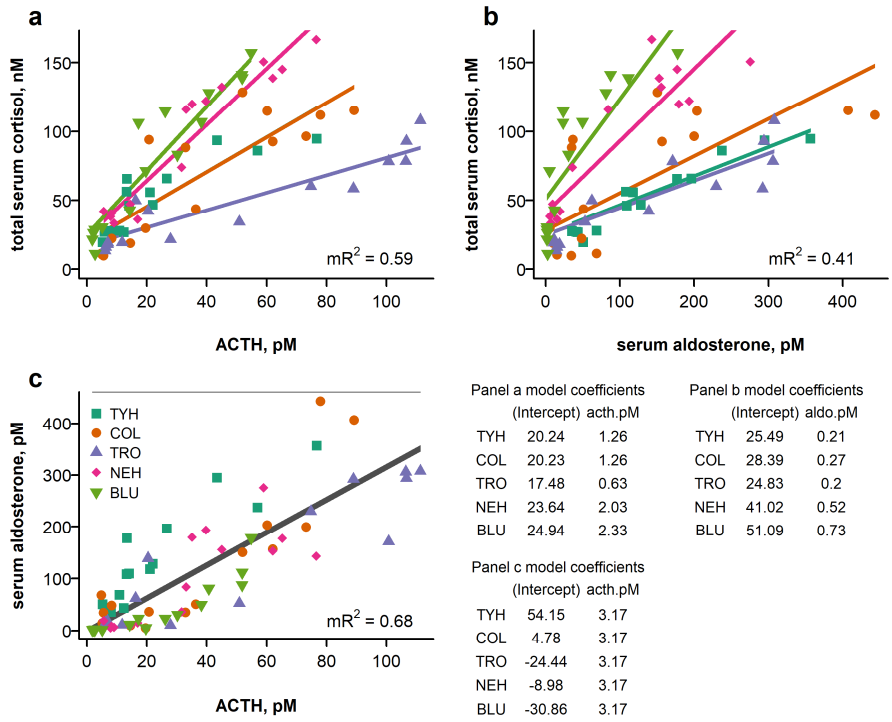

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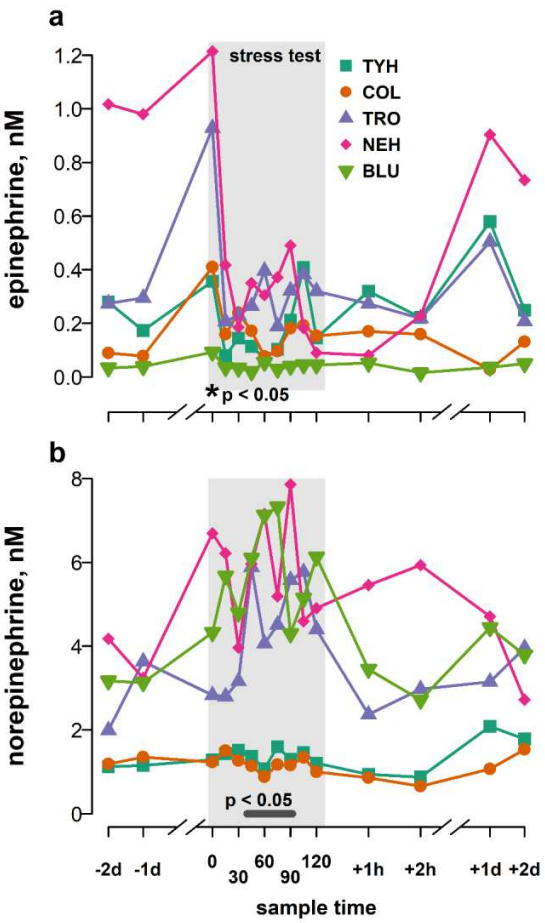

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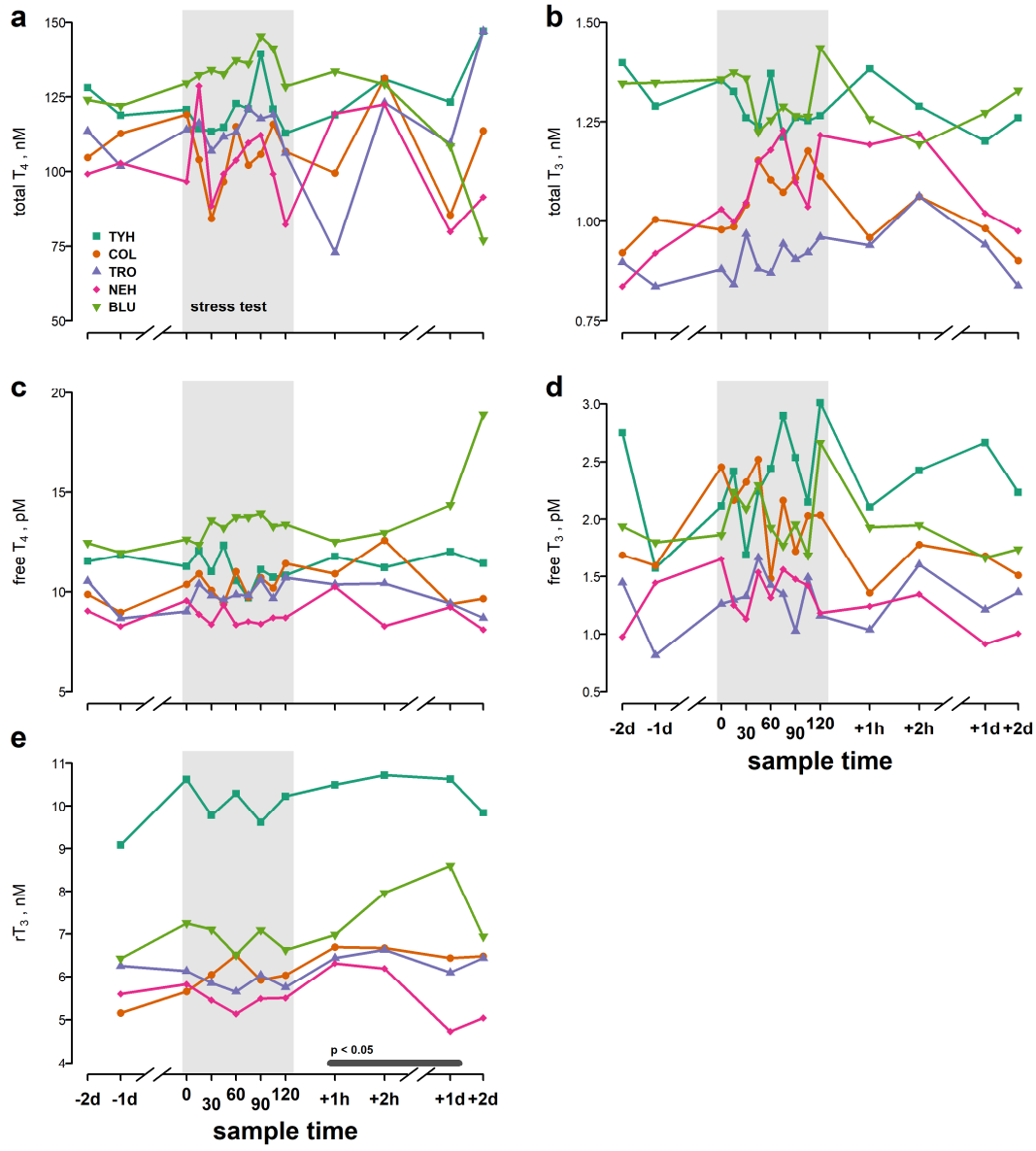

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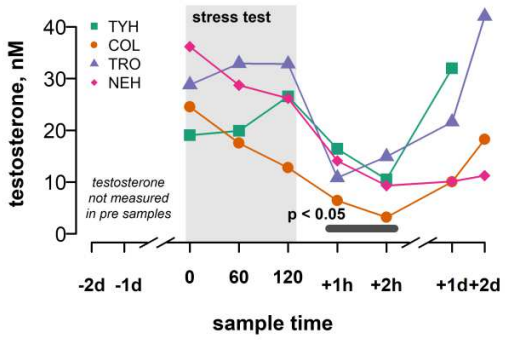

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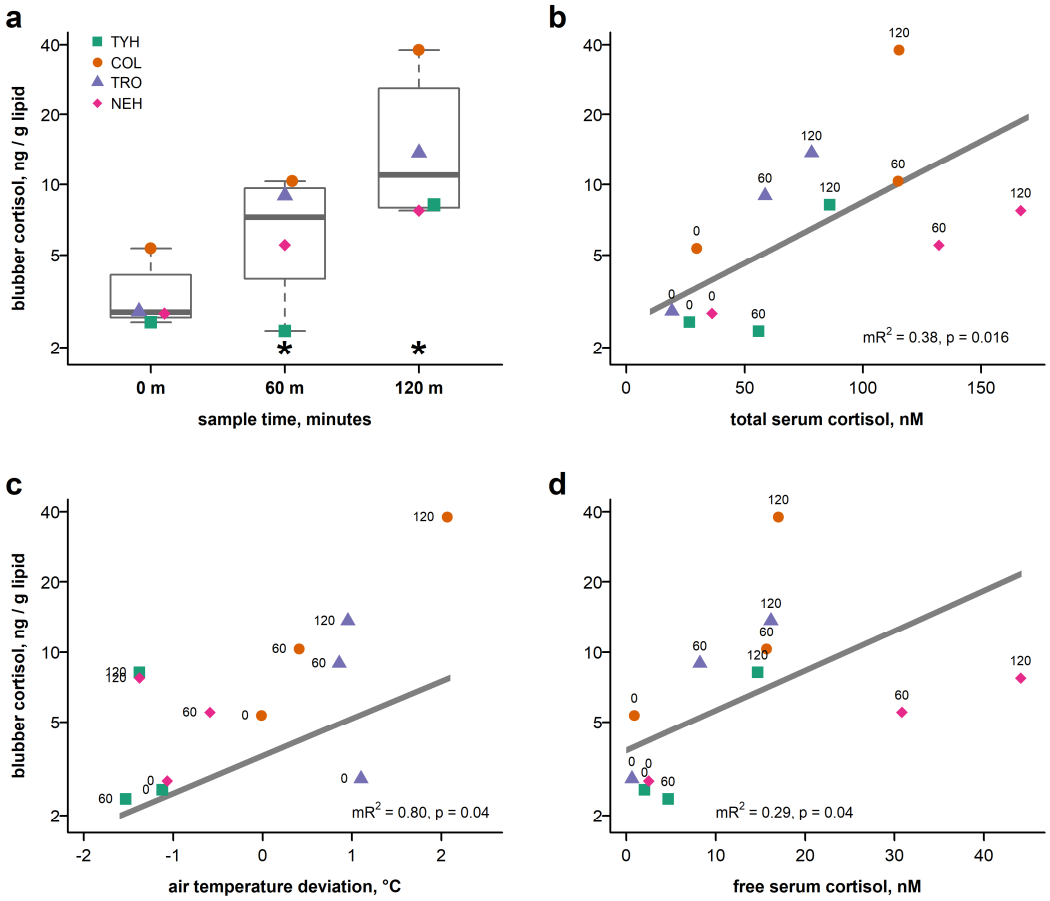

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