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Blubber cortisol qualitatively reflects circulating cortisol concentrations in bottlenose dolphins

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Abstract

Stress hormones, released into circulation as a consequence of disturbance, are classically assayed from blood samples but may also be detected in a variety of matrices. Blubber and fecal samples can be remotely collected from free-ranging cetaceans without the confounding hormone elevations associated with chase, capture, and handling required to collect blood samples. The relationship between cortisol concentrations in circulation with that of blubber and feces, however, is unknown. To assess these associations, we elevated cortisol by orally administering hydrocortisone for five days in five bottlenose dolphins. Voluntary blood and fecal samples were collected daily; blubber biopsies were collected on day one, just prior to hydrocortisone administration, and days three and five of hydrocortisone administration. We evaluated subsequent changes in several circulating stress hormones as well as cortisol and glucocorticoid metabolites in blubber and feces, respectively. There was a significant association between cortisol levels in serum and in blubber ($F_{1,12.7} = 14.3$, $P < 0.01$, $mR^2 = 0.57$) despite substantial variability in blubber cortisol levels. Counterintuitively, fecal cortisol metabolite levels were inversely related to serum cortisol. The relationship between serum and blubber cortisol levels suggests blubber samples from remote sampling may be useful to detect stress loads in this species.

Key words: stress, marine mammal, bottlenose dolphin, *Tursiops truncatus*, HPA axis, conservation.

Animals are frequently exposed to a variety of stressful conditions (Boonstra 2013). Stressors are increasing in many environments, often due to anthropogenic influences (Fair and Becker 2000, Maxwell *et al.* 2013). Measures of stress hormones are

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frequently used to detect stress in wild animals and there is additional interest in monitoring stress in managed care settings. Stress hormones (*e.g.,* glucocorticoids) are typically measured from blood samples but it can be challenging to obtain blood samples from free-ranging animals that represent baseline conditions. These collections must be rapid (*e.g.,* within 3–5 min of initial disturbance in birds (Romero and Reed 2005) or conducted using sedatives that mitigate the stress response in select species (Champagne *et al.* 2012). Neither constraint is feasible in numerous taxa (*e.g.,* free-ranging cetaceans), thus alternative methods for measuring stress hormones in animals are being explored, especially using less invasive collection methods and from more easily accessed tissues and matrices (Sheriff *et al.* 2011, Hunt *et al.* 2013).

Several characteristics suggest that blubber may be an appropriate tissue to assess stress levels in marine mammals. Glucocorticoids (*e.g.*, cortisol and corticosterone) are stable lipophilic compounds amenable to measurement in a variety of tissues and excreta and techniques have been explored in several of these, including urine and feces, dermal structures including fur, feathers and scales, and skin and subcutaneous adipose tissue (*e.g.*, see Wasser *et al.* 2000, Hunt *et al.* 2013, Cattet *et al.* 2014, Atkinson *et al.* 2015). Each of these tissues will have different rates and degrees of incorporation of glucocorticoids. The rate and degree of incorporation may be influenced by a variety of factors, including perfusion, growth rate and metabolic activity, and hormone solubility in different tissues may vary among species and environmental conditions (Bortolotti *et al.* 2009, Cattet *et al.* 2014). Cortisol and corticosterone are highly soluble in adipose tissue (Mead *et al.* 1986) and this subcutaneous tissue can be remotely sampled from free-ranging cetaceans using blubber biopsy darts (Kellar *et al.* 2006, Noren and Mocklin 2012). Similarly, glucocorticoids are excreted in the feces and fecal samples can be collected both on land and in the ocean for several marine mammal species (Hunt *et al.* 2006, Ayres *et al.* 2012, Gobush *et al.* 2014), thus avoiding handling-induced stress responses. Cortisol, the principal glucocorticoid in most marine mammals studied (St. Aubin 2001), circulates primarily bound to carrier proteins (*e.g.,* corticosteroid-binding-globin, CBG, and albumin to a lesser extent (Breuner and Orchinik 2002). Only free, unbound, cortisol is thought to incorporate into blubber (Breuner *et al.* 2013); blubber cortisol concentrations may therefore provide an integrated measure of stress over time that is less influenced by short-term acute stress and brief increases in circulating cortisol concentrations (Hughes *et al.* 2010). Conversely, fecal cortisol metabolites reflect passage of degraded cortisol from the bile into the intestinal system, thereby reflecting more acute changes in cortisol but presumably over time scales that would be unaffected by handling artifact. These features make cortisol assessment of blubber and fecal samples a promising means of evaluating stress in marine mammals—both in free-ranging populations and for comparisons with animals in managed care settings.

For blubber and fecal glucocorticoid measurements to be meaningful, however, we must determine the relationship between blubber and fecal glucocorticoid content and serum cortisol concentrations, as it is these circulating hormones that influence metabolism and energy use (Mendel 1989). We therefore evaluated the incorporation of cortisol into blubber and the excretion of its metabolites into feces in a well-studied marine mammal, the bottlenose dolphin (*Tursiops truncates*). We manipulated circulating cortisol levels by orally administering synthetic cortisol (hydrocortisone) over 5 d and evaluated its impact on blubber cortisol and fecal glucocorticoid metabolites. Additionally, we assessed several hormones—adrenocorticotropic hormone (ACTH), aldosterone, and thyroid hormones—to explore potential influences

of chronically elevated cortisol within the hypothalamic-pituitary-adrenal (HPA) axis and on the thyroid hormone axis.

Methods

Study Subjects and Animal Handling Procedures

Five bottlenose dolphins participated in this study—four male and one female, aged 20–35 yr, with an average mass of 217 (SD = 29) kg. Study animals were housed in open-water netted enclosures within San Diego Bay, California, and maintained by the U.S. Navy Marine Mammal Program (MMP). The MMP is accredited by the Association for the Accreditation of Animal Laboratory Animal Care (AAALAC). Study animals were under the care and supervision of trained clinical staff and fed a fish diet commensurate with their body size; animals experienced normal fluctuations in environmental conditions including day/night cycle, weather, and water temperatures.

Study dolphins were trained to voluntarily allow blood sampling by presenting their fluke. Blood samples were collected from the arteriovenous plexus of the ventral fluke, using a 21 G, 1.25-inch winged sampling needle and extension tube, into chilled serum and EDTA plasma Vacutainers (BD & Company, Franklin, NJ). As part of the behavioral routine for dolphins at the MMP, study dolphins were previously trained to voluntarily beach onto tri-fold padded mats used for dolphin transport. To facilitate adipose tissue sampling, dolphins beached and remained on the padded mat until the blubber biopsy was completed. Blubber biopsies were collected from an area approximately 5 × 5 cm, slightly posterolateral of the dorsal fin and overlying the epaxial muscle. Biopsies were collected using a 16 G biopsy needle set to 33 mm biopsy depth, sampling a tissue core across the same depth relative to the outer skin surface for each sample (Medical Device Technologies, Inc., Gainesville, FL). A cold pack was placed on the skin of the biopsy site for several minutes just prior to the biopsy procedure in order to numb the site. This procedure was better tolerated by the dolphins than was the injection of local anesthetics. Two to three needle punches were required per sample to obtain sufficient blubber for subsequent cortisol assays. To facilitate fecal sample collection, dolphins were conditioned to voluntarily accept a soft, flexible 14 Fr, polyvinylchloride catheter (item #J0695, Jorgensen Laboratories, Loveland, CO), inserted into the anal orifice. Following insertion to ~40 cm depth, fecal matter was gently passed into the catheter. Fecal collections were made daily, placed into a plastic 5 mL vial, and frozen at –80°C until processing. To elevate circulating cortisol levels, study animals were orally administered exogenous cortisol tablets (hydrocortisone, Pfizer Inc., New York, NY) along with 0.5 kg of fish. After the completion of the prescribed dosing regimen (described below) and sampling procedures, hydrocortisone treatment was gradually reduced over 2–3 d until administration ended.

Pilot Trial

To determine the appropriate dose and frequency required to elevate circulating cortisol levels, we conducted a pilot study in two trials using a single dolphin. The dolphin was orally administered a high dose of hydrocortisone (120 mg) on each of two nonconsecutive days (trial 1 and trial 2). Two separate trials were conducted to

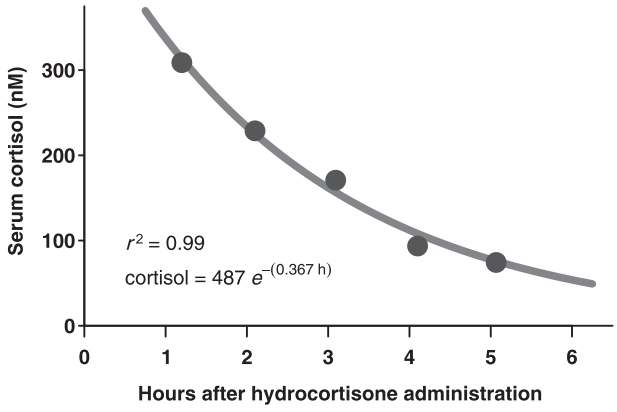

Figure 1. Following an oral hydrocortisone administration of 120 mg, cortisol was cleared from circulation with a half-life ($t_{½}$) of 109 min. This rate of clearance was used to determine the magnitude and frequency of hydrocortisone treatment used in the subsequent investigation. Samples were collected from a single dolphin in two separate trials; samples at hours 1 and 3 were from the first trial and those collected at hours 2, 4, and 5 were from the second trial.

limit the number of blood draws in a day and to ensure that the dolphin's voluntary participation in repetitive blood sampling would not breakdown. Blood samples were collected 1 and 3 h after hydrocortisone administration in trial 1; and 2, 4, and 5 h after administration in trial 2. Blood samples were centrifuged at 4°C and the serum collected and stored at −80°C until further analysis. Serum cortisol concentration was determined by radioimmunoassay (see below for details). We determined that the half-life ($t_{½}$) of circulating cortisol following oral hydrocortisone administration was 109 min (Fig. 1) and used this to establish the frequency and magnitude of dosing for the subsequent experimental procedures.

Study Design

Study animals were orally administered hydrocortisone to elevate circulating cortisol levels during a 5 d experimental protocol. Hydrocortisone tablets (60 mg each dose) were administered every 6 h, at 0600, 1200, 1800, and 2400, beginning after initial sample collections were completed at midday on day 1, and ending at 0600 on day 5 (16 total doses). Feeding at all dosing times, except 1200, was limited to 0.5 kg of fish. Sample times for each subject and sample matrix (blood, blubber, and feces) are shown in Table 1. Initial samples were collected on day 1, prior to the first hydrocortisone administration. Blood samples were collected each day at approximately 0800, 2 h after the most recent hydrocortisone treatment when the animals were presumed postabsorptive. Blubber biopsies were collected on days 1, 3, and 5. We collected blubber biopsies a few hours after collecting blood samples; the timing of these collections varied among study dolphins between 0900 and 1230 (average 1013; mean = 127 ± 67 (SD) min after blood sample collection). This variation was primarily a result of variability in the timing of voluntary participation among study animals. Based on the timing of each dolphin's voluntary participation in the initial biopsy, subsequent biopsy collection times remained reasonably consistent within

Table 1. Description of the study animals and sampling design.

		Study day							
Study dolphin	Sample matrix	1	2	3	4	5	6	Mean	SD
NEH, M, 253	blood sample	0800	0806	0809	0805	0800	—	0803	5
kg, 28 January	blubber biopsy	0915	—	0920	—	920	—	0918	3
2013	fecal sample	1200	1350	1400	1245	1330	1300	1310	62
SAY, F, 245 kg,	blood sample	0802	0800	0802	0915	0810	—	0804	5
13 May 2013	blubber biopsy	1050	—	1050	—	1205	—	1115	43
	fecal sample	1403	*	1440	1410	1300	1230	1354	51
TYH, M, 200	blood sample	0810	0803	0810	0802	0807	—	0809	2
kg, 17	blubber biopsy	0915	—	0930	—	0915	—	0920	9
December	fecal sample	1130	1300	1328	1330	1400	1215	1259	79
2012									
OLY, M, 200	blood sample	0807	0803	0810	0803	0757	—	0804	7
kg, 17	blubber biopsy	0930	—	0935	—	0925	—	0930	5
December	fecal sample	1130	1155	1300	1410	1330	1400	1240	62
2012									
TRO, M, 188	blood sample	0815	0759	0802	0815	0815	—	0810	8
kg, 28 January	blubber biopsy	1220	—	1120	—	1135	—	1145	31
2013	fecal sample	1235	1340	1200	1300	1415	1325	1256	70

Note: Each study dolphin identifier (NEH, SAY, TYH, OLY, and TRO), sex (M/F), their body mass (kg), and the initial sampling dates are shown in the first column. The timing of sample collections for each sample matrix is provided for each study day, along with the mean time of day and standard deviation (in minutes) collection time for each dolphin. "—" indicates no sample was collected; "*" indicates the precise time of this sample is unknown and was imputed for graphical display.

each subject (SD = 18 min; see Table 1). Fecal samples were collected daily between 1130 and 1440 (average 1311, SD = 54 min); this time was several hours after the first daily feeding and was the most consistent window for obtaining fecal samples. Blood samples were centrifuged at 4°C and the serum or plasma was stored at −80°C until further analysis. Blubber biopsies were transferred to preweighed cryovials and stored along with fecal samples at −80°C until further analysis.

Hormone Assays

Serum hormone concentrations were measured using commercially available enzyme- or radio-immunoassay kits (EIA or RIA, respectively) within a single assay run for each hormone. Cortisol and thyroid hormones (each of free and total thyroxine and triiodothyronine; T_4 and T_3, respectively) were measured with dolphin serum using RIA kits with antibody-coated tubes (Siemens Inc, Los Angeles, CA. Siemens has since ceased the production of their coat-a-count RIA kits, but catalog numbers were as follows: cortisol TCO1, free T_4 TKF41, total T_4 TKT41, free T_3 TKF31, total T_3 TKT31. Replacement RIA kits are available from MP Biomedicals). Aldosterone was assayed using an ImmuChem double antibody RIA kit (cat# 07108202, MP Biomedicals, Orangeburg, NY).

All RIA serum assays were processed in duplicate and average CVs between sample replicates were <2.5% for each hormone. ACTH was measured in triplicate with EDTA plasma using an EIA kit (cat# 21-ACTHU-E01, Alpco Inc, Salem, NH). Six of the 25 samples had ACTH concentrations below the stated assay sensitivity of

0.048 pM; these were imputed with a value of 0.048 pM. The average intra-assay CV of the ACTH EIA was 6.5%. The cortisol and thyroid hormone assay kits have been previously validated for use in bottlenose dolphins (Ortiz *et al.* 2010, Houser *et al.* 2011). The ACTH and aldosterone assays were validated for the present and associated studies in bottlenose dolphin. Serially diluted pools of dolphin plasma (ACTH) and serum (aldosterone) showed parallelism with respective assay standard curves (see Fig. S1).

Blubber cortisol levels were 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 250 μL of 1 mole/liter (1 M) phosphate buffered saline and vortexed in the multitube vortexer for 15 min. The cortisol measurements were performed using an EIA kit (cat# K003-H1, Arbor Assays, Ann Arbor, MI); the intra-assay CV was between 6.0% and 14.7%, and the interassay CV was between 7.2% and 10.9%. This steroid hormone extraction and assay kit have been previously validated for use in common dolphins (Kellar *et al.* 2015) and further validated for use with bottlenose dolphins for this study. Parallelism characteristics are summarized in Figure S1. Recovery of cortisol-spiked lipid extracts of varying volume (from 1 μL to 160 μL) showed 99% recovery (SD 10%). Differing from previous work, the blubber cortisol concentration in this study is reported as nanograms of cortisol per gram of lipid extracted (as opposed 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 blubber cortisol measurements were only assayed once.

Fecal glucocorticoid and thyroid hormone metabolite levels were determined as previously described (Wasser *et al.* 2000, 2010). Briefly, RIAs were performed to measure fecal hormone metabolites using RIA kits for glucocorticoids and thyroid hormone (MP Biomedicals, Orangeburg, NY) and for aldosterone (Siemens Inc., Los Angeles, CA). Samples were lyophilized for a minimum of 48 h prior to extraction to remove water from the samples. Freeze-dried fecal samples were then thoroughly homogenized, a 0.1 g subsample was transferred to a 50 mL polypropylene tube, and hormones were extracted with 15 mL 70% ethanol on a pulsing vortexer for 30 min. Samples were then centrifuged at 2,200 rpm for 20 min and aliquots of the supernatant collected for RIA analysis. A portion of the ethanol extract was dried under forced air, resuspended in assay buffer, and run alongside a standard curve and other assay controls. Parallelism and accuracy validations were performed using a fecal pool to ensure the antibodies recognized the targeted fecal metabolites in a predictable manner and without interference. The slopes of serially diluted extracts did not significantly differ from those of the standard curves (glucocorticoids: $F_{1,7} = 0.29$, $P = 0.60$; T_3: $F_{1,9} = 3.9$, $P = 0.08$; aldosterone: $F_{1,8} = 3.9$, $P = 0.08$). All assays exhibited good accuracy at their target dilutions (glucocorticoids: slope = 1.2, $r^2 = 1.0$; T_3: slope = 1.0 $r^2 = 0.98$; aldosterone: slope = 1.1, $r^2 = 1.0$), indicating that substances in fecal extract do not interfere with the hormone binding. Intra-assay CVs between duplicates were less than 3.5% and interassay variation of assay controls were less than 10.5% for all fecal metabolite RIAs.

Data Analyses

We evaluated the influences of hydrocortisone administration on endogenous hormone concentrations using linear mixed models (LMMs) with individual dolphin as a random effect. To determine differences due to hydrocortisone treatment, we compared initial hormone values, before hydrocortisone treatment, with those during the treatment using LMMs. To further assess temporal changes over the duration of the experiment, study day was used as an ordered fixed effect and *post hoc* comparisons were made using Dunnett's test against the initial, day 1, values prior to hydrocortisone administration. Statistical analyses were conducted using R statistical software version 3.1.1 (R Core Team 2013) and the lme4 package (Bates *et al.* 2014). Residual distributions of fitted models were visually inspected for normality and homogeneity of variance; response variables were log-transformed if needed (ACTH was log-transformed for analyses). 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) and Dunnett's *post hoc* comparisons were conducted in the multcomp package (Hothorn *et al.* 2008). Associations between continuous variables (*e.g.,* hormone concentrations) were tested similarly using LMMs and 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).

Results

Repeated oral hydrocortisone administration caused a five-fold increase in serum cortisol concentration; average values increased from approximately 20 nM in the initial samples to nearly 100 nM in the samples collected two hours after hydrocortisone administration ($F_{1,19} = 31.1$, $P < 0.0001$; Table 2). Serum cortisol concentration was increased by the second day of the study and remained elevated for the duration of the study ($F_{4,16} = 12.7$, $P < 0.0001$; Dunnett's *post hoc* test, $P < 0.001$ for each day; Fig. 2). Hydrocortisone administration also led to increased blubber cortisol levels ($F_{1,9} = 8.9$, $P < 0.05$; Table 3) and these levels remained elevated throughout the study ($F_{2,8} = 6.3$, $P < 0.05$; Dunnett's *post hoc* test, $P = 0.0008$ for day 3 while only marginally significant, $P = 0.08$, for day 5; see Fig. 2). We did not detect a relationship between blubber cortisol level and the duration since either the previous hydrocortisone dose ($F_{1,4.9} = 0.52$, $P = 0.5$) or the blood sample collection ($F_{1,3.6} = 0.4$, $P = 0.6$). There was a significant association between the concentrations of cortisol in serum and in blubber tissue ($F_{1,12.7} = 14.3$, $P < 0.01$, $mR^2 = 0.57$; Fig. 3B); a duration-since-blood-sample cofactor was not a significant effect and was not included in the model. Conversely, fecal glucocorticoid metabolite concentration was decreased during hydrocortisone administration ($F_{1,22} = 19.5$, $P < 0.01$, Table 3) and each of days 2–5 differed from the initial day ($F_{5,18.1} = 3.8$, $P < 0.05$; Dunnett's *post hoc* test, $P < 0.05$; Fig. 2). There was an inverse relationship between serum cortisol concentration and fecal glucocorticoid metabolite levels ($F_{1,20.9} = 10.4$, $P < 0.01$, $mR^2 = 0.28$; Fig. 3A).

ACTH concentration decreased with hydrocortisone administration ($\log_{10}$[ACTH]; $F_{1,19} = 113.9$, $P < 0.0001$; Table 2) and remained low throughout the study ($F_{4,16} = 90.2$, $P < 0.0001$; Dunnett's *post hoc* test, $P < 0.0001$ for each study day). The concentrations of both serum ACTH and fecal glucocorticoid metabolites

Table 2. Treatment with oral hydrocortisone had a significant effect on several circulating hormone concentrations in bottlenose dolphins.

	Cortisol nM	ACTH pM	Aldosterone pM	Total T_4 nM	Free T_4 pM	Total T_3 nM	Free T_3 pM
Baseline	20.0 ± 7.8	7.76 ± 3.48	20.93 ± 6.66	106.1 ± 26.7	19.8 ± 3.2	1.36 ± 0.18	1.14 ± 0.73
Hydrocortisone feeding	95.7 ± 41.6	0.15 ± 0.14	15.71 ± 5.21	98.0 ± 20.5	17.2 ± 1.9	1.23 ± 0.12	0.74 ± 0.26
F	**31.1**	**113.9**	**4.7**	2.2	**10.7**	4.2	**7.7**
P	**<0.0001**	**<0.0001**	**<0.05**	n.s.d.	**<0.01**	=0.053	**<0.05**
Fixed-effect estimate	57.8 (14.4)	3.95 (0.39)	18.3 (1.7)	—	18.5 (0.8)	—	0.94 (0.14)
Random-effect variance	52%	5%	25%	—	53%	—	49%

Note: Hormone concentrations are mean ± standard deviations. Baseline represents the initial sample collected prior to hydrocortisone treatment (a single blood sample from each of the five dolphins). Hydrocortisone feeding includes all samples collected during the treatment (four blood samples from each dolphin). Differences were tested using LMMs with dolphin ID as a random effect. Numerator and denominator degrees of freedom were 1 and 19, respectively; *F*-statistic and *P*-values are displayed within each column (significant differences between groups are in bold). Model parameters for the fixed-effect of hormone (standard error) and the percent variance explained by the random effect are also shown. For ACTH, hormone concentration is reported but the statistical tests were conducted on transformed ($\log_{10}$) data to meet assumptions of model fit.

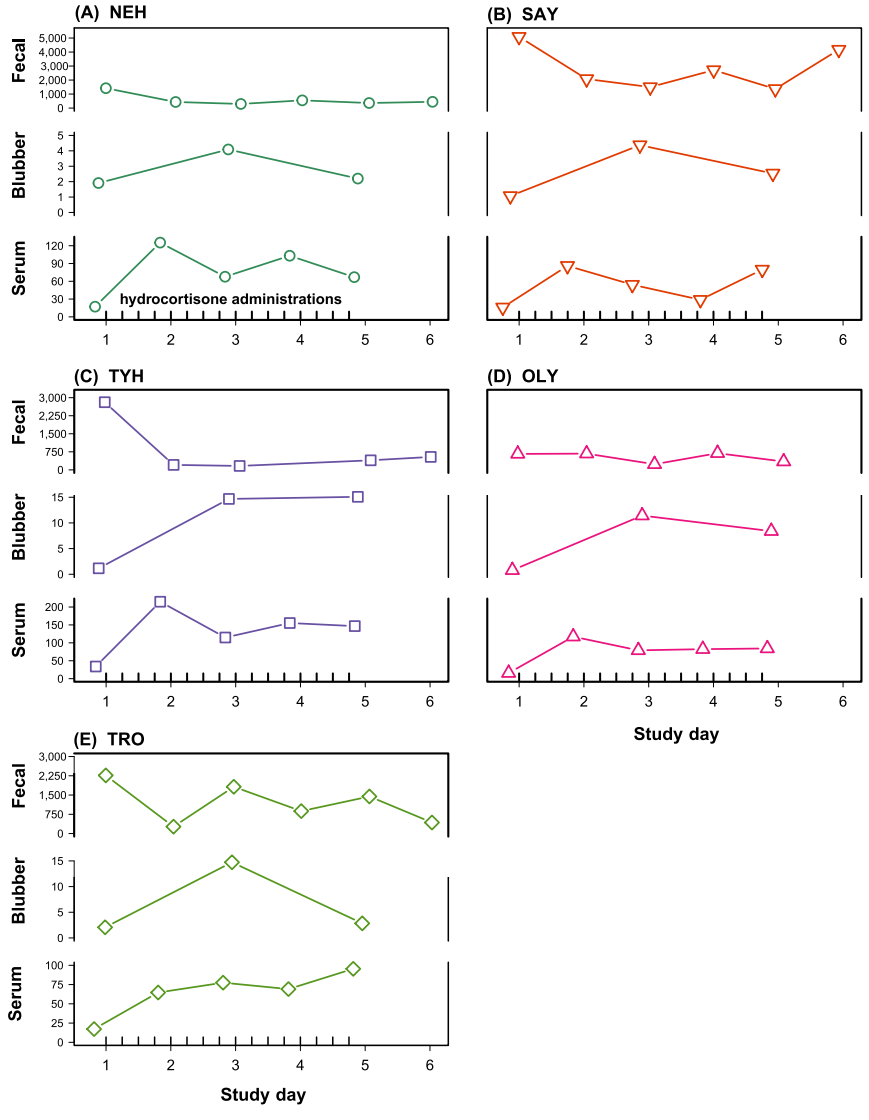

Figure 2. Cortisol values for each sample matrix—fecal glucocorticoids, blubber, and serum cortisol—throughout the study duration are shown for each dolphin (panels A–E). Serum cortisol concentrations were elevated (bottom line in each panel; $F_{4,16} = 12.7$, $P < 0.0001$) using oral administrations of hydrocortisone (timing of administrations shown by the gray ticks on the *x*-axis). The increase in circulating cortisol was associated with increased blubber cortisol levels (middle line; $F_{2,8} = 6.3$, $P < 0.05$) and decreased fecal glucocorticoid levels (top line; $F_{1,22} = 19.5$, $P < 0.01$). Fecal samples were conducted for 6 d (except for subject D, OLY) while blood was collected daily for 5 d, and blubber biopsies were conducted on days 1, 3, and 5. Note that the scale of the hormone axes varies between individuals but not across rows. The top of each panel shows the three-letter dolphin ID; line colors and symbols are coded by subject to maintain consistency with Figures 3 and 4. The units for each variable are: fecal glucocorticoids, ng/g dry fecal mass; blubber cortisol, ng/g lipid wet mass; and serum cortisol, nM.

Table 3. Blubber and fecal glucocorticoid levels were influenced by hydrocortisone treatment in bottlenose dolphins.

	Blubber cortisol ng/g lipid	Fecal glucocorticoids ng/g feces	Fecal aldosterone ng/g feces	Fecal T_3 ng/g feces
Baseline	1.40 ± 0.56	2,449 ± 1,688	6.2 ± 3.4	227 ± 76
Hydrocortisone feeding	8.03 ± 5.48	956 ± 979	4.3 ± 2.1	245 ± 111
F	**8.9**	**19.5**	2.4	0.3
P	**<0.05**	**<0.01**	n.s.d.	n.s.d.
df	1, 9	1, 22	1, 19	1,23
Fixed-effect estimate	4.7 (1.5)	1,682 (446)	—	—
Random-effect variance	23%	63%	—	—

Note: Hormone concentrations are mean ± standard deviations. Baseline represents the initial sample collected prior to hydrocortisone treatment (one blubber and fecal sample from each of the five study dolphins). Hydrocortisone feeding includes all samples collected during the treatment (two blubber samples and five fecal samples per dolphin). Concentrations are given as ng hormone per g of blubber lipid (wet mass) or feces (dry mass). Differences were tested using LMMs with dolphin ID as a random effect. Numerator and denominator degrees of freedom (df), *F*-statistic and *P*-values are displayed within each column (significant differences between groups are in bold). Model parameters for the fixed-effect of hormone (standard error) and the percent variance explained by the random effect are also shown.

declined during hydrocortisone treatment and were similarly associated with one another ($F_{1,18.4} = 26.9$, $P < 0.0001$, $mR^2 = 0.32$). Aldosterone was decreased during the hydrocortisone treatment ($F_{1,19} = 4.7$, $P = 0.043$; Table 2) but only the final, day 5, value differed from the initial concentration ($F_{4,16} = 2.9$, $P = 0.056$; Dunnett's *post hoc* test, $P < 0.05$). Fecal aldosterone metabolites did not significantly vary across the study but were associated with serum aldosterone concentrations ($F_{1,17.6} = 5.6$, $P = 0.029$, $mR^2 = 0.27$).

The hydrocortisone administration had variable influences on thyroid hormones (see Table 2). We did not detect a significant effect of hydrocortisone treatment on total T_4 concentrations ($P > 0.1$) whereas free T_4 was slightly decreased during the treatment ($F_{1,19} = 10.7$, $P < 0.01$). Similarly, total T_3 showed a small and marginally significant decrease ($F_{1,19} = 4.2$, $P = 0.053$) whereas free T_3 concentration was decreased during hydrocortisone treatment ($F_{1,19} = 7.7$, $P < 0.05$). Few differences in thyroid hormone concentrations were detected among the study days; only free T_4 concentrations varied across study days ($F_{4,16} = 4.6$, $P < 0.05$; Dunnett's *post hoc* test $P \leq 0.05$ for days 3–5; Fig. 4C). Fecal T_3 metabolites did not vary across the study days, and there were no relationships detected with circulating free or total T_3 levels.

Discussion

These data demonstrate that blubber cortisol level was substantially elevated in association with serum cortisol concentration during hydrocortisone feeding (Fig. 2, 3B). Blubber cortisol levels were similar to one another in the initial samples, which were collected in the baseline state with low circulating cortisol concentrations. Cortisol levels in subsequent blubber samples that were collected during elevated

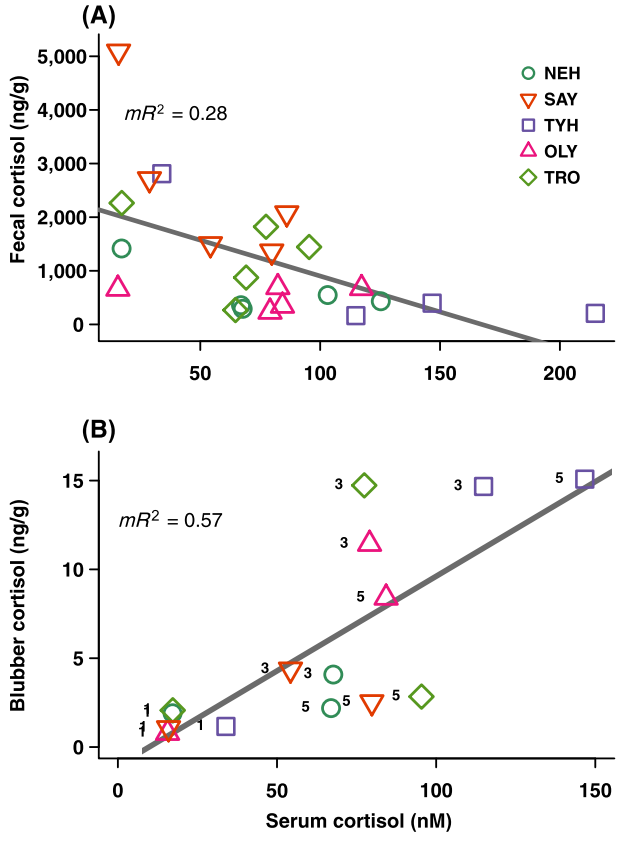

Figure 3. Fecal glucocorticoid (A) and blubber cortisol (B) levels were associated with serum cortisol concentrations collected on the same day. (A) There was a negative relationship between fecal and serum cortisol ($F_{1,20.9} = 10.4$, $P < 0.01$; $n = 25$ data points, a single daily blood and fecal sample from each dolphin). Individual dolphins are shown in different symbols according to the legend. (B) There was a positive relationship between blubber and serum cortisol concentrations (LMM: $F_{1,\ 12.7} = 14.3$, $P < 0.01$; $n = 15$); integers to the left of plot symbols indicate the day of the sample (day 1, 3, or 5) and symbols are as in panel (A). The regression line and marginal R^2 (mR^2) for the LMM fits are shown in each panel; note the scale of the serum cortisol axis differs between panels. The units for fecal glucocorticoids are ng/g dry fecal mass and for blubber cortisol are ng/g lipid wet mass.

circulating cortisol concentrations, however, were highly variable among study subjects. We did not collect blood, blubber, and fecal sample matrices simultaneously. Blood samples were collected as dolphins voluntarily presented their flukes for blood draws while remaining in the water, whereas blubber biopsies required having the dolphin voluntarily beach out of the water. This imposed a delay between these sampling points and the study subjects varied in their timing of beaching and consequent blubber sampling; most of this variation was among rather than within subjects. Additionally, fecal samples were collected later in the day when fecal matter was available. Despite the variability in the timing of sample collections, there was a

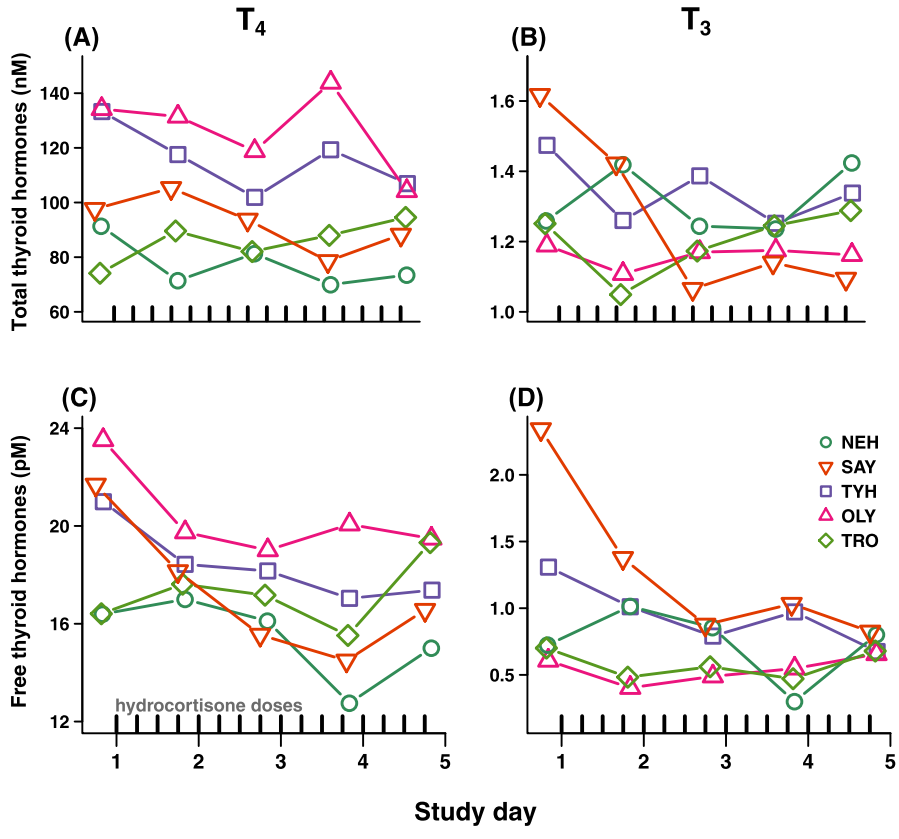

Figure 4. Circulating thyroid hormone concentrations were moderately affected by hydrocortisone treatment. The top row depicts total (free and bound to carrier proteins) thyroxine and triiodothyronine, T_4 and T_3, respectively. There was no detectable influence on total T_4 ($P > 0.1$, panel A) whereas there was a marginally significant decrease in total T_3 concentrations during hydrocortisone administration ($F_{1,19} = 4.2$, $P = 0.053$; panel B). The bottom row shows free T_4 and T_3 (panels C and D, respectively). Both free T_4 and free T_3 concentrations were decreased during hydrocortisone administration (free T_4: $F_{1,19} = 10.7$, $P < 0.01$; free T_3: $F_{1,19} = 7.7$, $P < 0.05$). The timing of the hydrocortisone doses is shown in ticks along the *x*-axes and individual dolphins are shown in different symbols according to the legend.

significant association in cortisol concentrations between blood and blubber: serum cortisol concentration explained 57% of the variation in blubber cortisol (Fig. 3B). Blubber cortisol levels were most variable at intermediate circulating cortisol concentrations, between 50 and 100 nM. The strength of this relationship does not suggest that we could accurately assess circulating cortisol concentration based on blubber cortisol measurements. All blubber samples with high and low cortisol levels, however, were associated with respective high and low serum cortisol concentrations. These data suggest that it may therefore be possible to differentiate individuals with high circulating concentrations of cortisol using blubber cortisol levels, although at this time it would best be used as a qualitative or categorical rather than a

quantitative assessment. This study was conducted in a relatively small sample size of five subjects under managed care, so further research is needed to validate these findings during endogenous cortisol release and in free-ranging animals.

The increased circulating cortisol levels achieved in the present study are likely similar to that exhibited during stressful events in wild odontocetes. Baseline circulating cortisol levels are generally low within the MMP population, typically around 20 nM (this study and DSH, unpublished data). This value is slightly lower than the mean previously reported for this population (St. Aubin *et al.* 1996), but this difference may represent greater cross-reactivity with other compounds using older RIA kits than actual differences between the studies. Oral hydrocortisone treatment increased circulating cortisol to just under 100 nM 2 h after administration (Table 2). Assuming the half-life of cortisol is similar across subjects, cortisol levels may have declined to ~25 nM just prior to the subsequent dosage. The increased cortisol level is similar to what has been reported in other studies in free-ranging dolphins, typically during wild-capture health assessments. Dolphin circulating cortisol concentrations reported elsewhere are surprisingly consistent across a range of sample years and among different research groups: ~70–75 nM in (Fair *et al.* 2014, Hart *et al.* 2015) and (St. Aubin *et al.* 1996); ~77 nM in (Ortiz and Worthy 2000); 139 nM following a chase-encirclement protocol in *Stenella* (from St. Aubin *et al.* 2013); and 50 nM in beluga (*Delphinapterus leucas*) and ~117 nM during induced stress of veterinary examination in three subjects (Schmitt *et al.* 2010). Thus, the manipulated cortisol levels presented here do not represent drastically elevated pharmacological increases in cortisol but rather are similar to levels exhibited in odontocetes under presumably stressful handling conditions.

The relatively high variability in blubber cortisol level was not unexpected. Blubber cortisol may be influenced by several factors other than total circulating cortisol concentration, including the duration of stress experienced, changes in cortisol binding globulin (CBG) concentrations that affect free hormone availability, the degree of perfusion, and the penetration depth of the blubber biopsies during sampling. Kellar *et al* (2015) reported large variation in blubber cortisol among dolphins that had stranded onshore, from near 0 ng/g to 70 ng/g with higher levels in stranded animals than those killed in fisheries bycatch. They speculated that animals killed in bycatch events experienced an acute stress event prior to death, whereas stranded animals may have had elevated circulating cortisol for prolonged periods (*e.g.,* for the duration of a pathology or starvation) and thus exhibited higher blubber cortisol levels. In this study, dolphins received oral hydrocortisone every six hours to elevate circulating cortisol for 5 d. The experimentally elevated blubber cortisol levels in this study, on average 8.0 ng/g lipid, were intermediate to those of bycaught animals, ~4 ng/g, and stranded individuals, ~24 ng/g adipose tissue, from Kellar *et al*. (2015). Surprisingly, blubber cortisol was not highest at the end of this study; levels on day 3 strongly differed from the initial values ($P < 0.001$) but they were only marginally different at the end of the study (initial *vs.* day 5 Dunnett's test, $P = 0.08$). In four of the five study animals blubber cortisol level decreased from day 3 to day 5 despite continued hydrocortisone treatment and persistent elevations in serum cortisol concentrations (see Fig. 2).

Several factors may have potentially contributed to the blubber cortisol patterns observed in this study. Only free cortisol, not bound with carrier proteins, is thought to diffuse into adipose tissue, and most cortisol in circulation, over 90% in most species that have been studied, is bound with CBG (Desantis *et al.* 2013). Increasing the circulating cortisol concentration five-fold with exogenous hydrocortisone likely

dramatically increased the free cortisol in circulation and consequently increased blubber cortisol levels as observed on day 3 of this study. Subsequent synthesis of CBG may have then sequestered cortisol in circulation, reducing the free cortisol available to diffuse into blubber tissue as the study progressed. We did not, however, measure CBG so the influence of this binding globulin remains speculative. Another factor that may have contributed to the variation in blubber cortisol measurements in this study is the stratified nature of marine mammal blubber, with the inner blubber layer thought to be more highly perfused and metabolically active than the outer layer (Koopman 2007, Strandberg *et al.* 2008). The depth of sampling has been shown to influence cortisol levels, with higher concentrations in the inner blubber layer (Trana *et al.* 2015). The use of trained dolphins in this study permitted manual sample collection directly beside the animal; blubber samples were from the outer 33 mm of tissue and likely sampled with greater consistency than what is achieved using remote dart collection in many field studies. Despite consistent sampling depth, the relative depth of the biopsy may have varied among individual dolphins with different body sizes and consequent blubber thicknesses. Visual inspection of blubber biopsies indicated that in the two largest animals (NEH and SAY) the entire blubber depth may not have been sampled and these dolphins had the lowest concentrations of blubber cortisol (Fig. 2, 3B). Additionally, differences in perfusion due to individual and temporal variations in cardiac output and vasoconstriction may have contributed to the varied blubber cortisol levels reported here. The dynamics of perfusion can be altered due to the state of stress of an individual as well as to environmental factors, such as water temperature. Finally, the timing of sample collection after the morning hydrocortisone dose could have also been a contributing factor. Blubber biopsies were performed between 0915 and 1205 on study days 3 and 5 (average of 1013, SD = 66 min), primarily due to variability in voluntary beaching participation by the dolphin. There was, however, no detectable influence of the duration since the hydrocortisone dose on blubber cortisol levels. This lack of association suggests that blubber cortisol level did not vary substantially over the few hours sample difference between subjects. The relationship between cortisol levels in serum and blubber may have nevertheless varied among individual subjects (see Fig. 3B). Our sample size of only three blubber biopsies per individual, however, is insufficient to adequately characterize this relationship by individual with any degree of certainty (*i.e.,* we did not include an interaction between individual and serum cortisol in our model due to limited sample replicates within individuals). Despite these confounding factors, blubber and serum cortisol values were associated with one another and samples displaying the highest blubber cortisol levels all had correspondingly elevated circulating levels. While we did not find a close association between serum and blubber cortisol throughout the range of values in this study; it may be possible to qualitatively distinguish high-stress conditions in animals from measures of blubber cortisol.

The reduction in fecal glucocorticoid metabolites when circulating cortisol is chronically elevated initially seems counter-intuitive; it might be expected that the fecal metabolites would be elevated either from increased excretion of serum cortisol in the bile, or through the incomplete absorption of the administered hydrocortisone and its subsequent passage into feces. Fecal steroid metabolites frequently occur in far greater concentrations than their respective circulating hormones, reflecting an accumulation of excreted hormones (Wasser *et al.* 1994). In this study, however, the administration of hydrocortisone suppressed ACTH production (see below) but did so without a concomitant decrease in serum cortisol as might be expected under a

"natural" stressor with subsequent negative feedback. This administration of substantial free hydrocortisone likely altered the kinetics and excretion of circulating cortisol. Under exogenous cortisol administration in humans subjected to cortisol suppression by dexamethasone, it has been demonstrated that as serum cortisol is elevated, less serum cortisol is bound with CBG and a greater amount of free cortisol is excreted *via* the urine (Beisel *et al.* 1964). Thus, under the conditions of this study, the reduction in fecal cortisol may best be explained by an alteration in cortisol clearance with urinary clearance becoming the dominant route for excretion. This is supported by the observation that fecal T_3 and aldosterone metabolites were unaltered in response to the hydrocortisone administration (see Table 3), suggesting the fecal glucocorticoid results do not reflect a gross alteration in gut dynamics. Alternatively, or in addition to an increase in urinary excretion of free cortisol, a greater amount of conjugated metabolites may be reabsorbed back through the intestinal wall prior to excretion. Unfortunately, the mechanism by which fecal glucocorticoid metabolites are reduced under the specific treatment of this study must remain speculative as no urine samples were collected as part of the study design.

Increasing circulating cortisol concentration elicited negative feedback of the HPA axis. ACTH concentrations were drastically reduced in all study dolphins and remained near the detection limit of the assay for the duration of the study (see Table 2). The ACTH suppression observed here agrees with the typical response in terrestrial mammals (Atkinson *et al.* 2015) as well as previous studies of glucocorticoid administrations in dolphins (Reidarson and McBain 1999). The site of the inhibition in this instance is not known—long-feedback inhibition of release of corticotropin-releasing hormone by the hypothalamus or short feedback of cortisol directly suppressing ACTH release from the anterior pituitary—but is possibly some combination of both. The corticosteroids cortisol and aldosterone are often correlated in marine mammals, both in cetaceans (Thomson and Geraci 1986, St. Aubin and Geraci 1990) and pinnipeds (Ensminger *et al.* 2014, Champagne *et al.* 2015, Keogh and Atkinson 2015). Furthermore, HPA axis activation, through ACTH administrations or handling effects, has been shown to stimulate aldosterone release in these taxa (Thomson and Geraci 1986, Gulland *et al.* 1999, Ensminger *et al.* 2014, Champagne *et al.* 2015, Keogh and Atkinson 2015). From these studies several researchers have speculated that the HPA axis is an important regulator of aldosterone in marine mammals (Ensminger *et al.* 2014, Atkinson *et al.* 2015, Champagne *et al.* 2015, Keogh and Atkinson 2015). Consequently, we predicted that suppressed ACTH would reduce aldosterone levels. This prediction, however, was only weakly supported; we detected a statistically significant decrease in circulating aldosterone concentrations during hydrocortisone treatment but the magnitude of decrease was quite small—decreasing from on average 21 pM in the initial sample to 16 pM during the treatment (see Table 2). While there is strong evidence indicating that aldosterone release is stimulated by ACTH in marine mammals, these data show that baseline levels of aldosterone can be maintained simultaneous with near complete ACTH suppression. This suggests that aldosterone remains under some degree of regulation outside of the HPA axis. Most likely, plasma potassium and the renin-angiotensin-aldosterone system (RAAS) retain some, if not a large, regulatory influence over aldosterone, which likely explains the observed aldosterone levels under the conditions experienced in the present study.

We detected small but statistically significant influences of elevated cortisol on the thyroid hormone axis. The baseline thyroid hormone concentrations observed in this study were similar to those previously reported in this species (St. Aubin 2001, Fair

et al. 2011). Stress typically reduces thyroid hormone concentrations in mammals, probably *via* reduced thyrotropin releasing hormone (TRH) production by the hypothalamus (Kakucska *et al.* 1995). We were unable to validate an assay for thyroid stimulating hormone (TSH) in dolphins, as have previous researchers, likely due to the high degree of peptide sequence variation in this hormone and consequent unavailability of a complementary antibody (St. Aubin 2001). We were therefore unable to assess consequent changes in TSH concentrations in this study. It will require further effort to develop an immunoassay for this tropic hormone in cetaceans and other marine mammals. There was, however, no detectable reduction in total T_4 concentration during hydrocortisone treatment, as would be expected from reduced TRH and TSH release from the hypothalamus and pituitary, respectively. In this study, we administered hydrocortisone to chemically elevate glucocorticoid levels and the dolphins were not stressed *per se*. These thyroid hormone findings are consistent with this interpretation. Glucocorticoids can, however, directly inhibit the conversion of T_4 to T_3 (Bianco *et al.* 1987) and we did detect reduced T_3 concentrations during the hydrocortisone treatment—a marginally significant reduction in total T_3 ($P = 0.053$) and with greater certainty in free T_3 values ($P < 0.05$; see Table 2). Generally, the magnitudes of decrease in thyroid hormones were fairly small relative to their initial concentrations (T_4: total no change and free 13%; T_3: total 10% and free 35%; see Fig. 4). These trends are of a lesser magnitude but consistent with a previous study of the influence of capture stress in beluga whales that showed reductions in both T_4 and T_3 of more than 50% (St. Aubin and Geraci 1988, St. Aubin and Geraci 1992). It therefore seems that there is a small but detectable influence of elevated glucocorticoids on thyroid hormones and that stress itself probably has additional influences on this axis *via* pathways unstudied here.

To our knowledge, these data are the first *in vivo* association between stress hormones in circulation with that of blubber in marine mammals. This study provides context for blubber cortisol levels measured in samples collected from free-ranging cetaceans and suggests that the ability to assess stress from remotely collected samples may offer a tractable mechanism to evaluate stress in these far-ranging and highly mobile taxa of management concern. The timescale of glucocorticoid incorporation into blubber, as well as its removal following recovery from stress, requires further investigation to establish the kinetics of cortisol exchange between serum and blubber that underlies the results of blubber cortisol measurements. Fecal glucocorticoid metabolites have been used in a number of wildlife systems to monitor animal stress; however, perturbations made as part of this study likely altered both the protein binding kinetics and the natural excretory dynamics of free cortisol. Thus, additional work on the relationship between fecal glucocorticoid metabolites and circulating cortisol is needed.

Acknowledgments

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.

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Literature Cited

Atkinson, S., D. Crocker, D. Houser and K. Mashburn. 2015. Stress physiology in marine mammals: How well do they fit the terrestrial model? Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 185:463–486.

Ayres, K. L., R. K. Booth, J. A. Hempelmann, *et al*. 2012. Distinguishing the impacts of inadequate prey and vessel traffic on an endangered killer whale (*Orcinus orca*) population. PLOS ONE 7:e36842.

Barton, K. 2015. MuMIn: Multi-model inference. R package version 1.13.4. Available at https://cran.r-project.org/web/packages/MuMIn/MuMIn.pdf.

Bates, D., M. Maechler, B. Bolker and S. Walker. 2014. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67:1–48.

Beisel, W. R., J. J. Cos, R. Horton, P. Y. Chao and P. H. Forsham. 1964. Physiology of urinary cortisol excretion. The Journal of Clinical Endocrinology and Metabolism 24:887–893.

Bianco, A. C., M. T. Nunes, N. S. Hell and R. M. Maciel. 1987. The role of glucocorticoids in the stress-induced reduction of extrathyroidal 3,5,3′-triiodothyronine generation in rats. Endocrinology 120:1033–1038.

Boonstra, R. 2013. Reality as the leading cause of stress: Rethinking the impact of chronic stress in nature. Functional Ecology 27:11–23.

Bortolotti, G. R., T. Marchant, J. Blas and S. Cabezas. 2009. Tracking stress: Localisation, deposition and stability of corticosterone in feathers. Journal of Experimental Biology 212:1477–1482.

Breuner, C. W., and M. Orchinik. 2002. Plasma binding proteins as mediators of corticosteroid action in vertebrates. The Journal of endocrinology 175:99–112.

Breuner, C. W., B. Delehanty and R. Boonstra. 2013. Evaluating stress in natural populations of vertebrates: Total CORT is not good enough. Functional Ecology 27:24–36.

Cattet, M., B. J. Macbeth, D. M. Janz, A. Zedrosser, J. E. Swenson, M. Dumond and G. B. Stenhouse. 2014. Quantifying long-term stress in brown bears with the hair cortisol concentration: A biomarker that may be confounded by rapid changes in response to capture and handling. Conservation Physiology 2(1):cou026.

Champagne, C. D., D. S. Houser, D. P. Costa and D. E. Crocker. 2012. The effects of handling and anesthetic agents on the stress response and carbohydrate metabolism in northern elephant seals. PLOS ONE 7:e38442.

Champagne, C., M. Tift, D. Houser and D. Crocker. 2015. Adrenal sensitivity to stress is maintained despite variation in baseline glucocorticoids in moulting seals. Conservation Physiology 3(1):cov004.

Desantis, L. M., B. Delehanty, J. T. Weir and R. Boonstra. 2013. Mediating free glucocorticoid levels in the blood of vertebrates: Are corticosteroid-binding proteins always necessary? Functional Ecology 27:107–119.

Ensminger, D. C., D. A. Somo, D. S. Houser and D. E. Crocker. 2014. Metabolic responses to adrenocorticotropic hormone (ACTH) vary with life-history stage in adult male northern elephant seals. General and Comparative Endocrinology 204C:150–157.

Fair, P., and P. Becker. 2000. Review of stress in marine mammals. Journal of Aquatic Ecosystem Stress and Recovery 7:335–354.

Fair, P. A., E. Montie, L. Balthis, J. S. Reif and G. D. Bossart. 2011. Influences of biological variables and geographic location on circulating concentrations of thyroid hormones in wild bottlenose dolphins (*Tursiops truncatus*). General and Comparative Endocrinology 174:184–194.

Fair, P. A., A. M. Schaefer, T. A. Romano, G. D. Bossart, S. V. Lamb and J. S. Reif. 2014. Stress response of wild bottlenose dolphins (*Tursiops truncatus*) during capture–release health assessment studies. General and Comparative Endocrinology 206:203–212.

Gobush, K. S., R. K. Booth and S. K. Wasser. 2014. Validation and application of noninvasive glucocorticoid and thyroid hormone measures in free-ranging Hawaiian monk seals. General and Comparative Endocrinology 195:174–182.

Gulland, F. M. D., M. Haulena, L. J. Lowenstine, C. Munro, P. A. Graham, J. Bauman and J. Harvey. 1999. Adrenal function in wild and rehabilitated Pacific harbor seals (*Phoca vitulina richardii*) and in seals with phocine herpesvirus-associated adrenal necrosis. Marine Mammal Science 15:810–827.

Hart, L. B., R. S. Wells, N. Kellar, *et al*. 2015. Adrenal hormones in common bottlenose dolphins (*Tursiops truncatus*): Influential factors and reference intervals. PLOS ONE 10 (5):e0127432.

Hothorn, T., F. Bretz and P. Westfall. 2008. Simultaneous inference in general parametric models. Biometrical Journal 50:346–363.

Houser, D. S., L. C. Yeates and D. E. Crocker. 2011. Cold stress induces an adrenocortical response in bottlenose dolphins (*Tursiops truncatus*). Journal of Zoo and Wildlife Medicine 42:565–571.

Hughes, K. A., R. M. Reynolds, R. Andrew, H. O. Critchley and B. R. Walker. 2010. Glucocorticoids turn over slowly in human adipose tissue *in Vivo*. The Journal of Clinical Endocrinology and Metabolism 95:4696–4702.

Hunt, K. E., R. M. Rolland, S. D. Kraus and S. K. Wasser. 2006. Analysis of fecal glucocorticoids in the North Atlantic right whale (*Eubalaena glacialis*). General and Comparative Endocrinology 148:260–272.

Hunt, K. E., M. J. Moore and R. M. Rolland, *et al*. 2013. Overcoming the challenges of studying conservation physiology in large whales: A review of available methods. Conservation Physiology 1(1):cot006.

Kakucska, I., Y. Qi and R. M. Lechan. 1995. Changes in adrenal status affect hypothalamic thyrotropin-releasing hormone gene expression in parallel with corticotropin-releasing hormone. Endocrinology 136:2795–2802.

Kellar, N. M., M. L. Trego, C. I. Marks and A. E. Dizon. 2006. Determining pregnancy from blubber in three species of delphinids. Marine Mammal Science 22:1–16.

Kellar, N. M., K. N. Catelani, M. N. Robbins, M. L. Trego, C. D. Allen, K. Danil and S. J. Chivers. 2015. Blubber cortisol: A potential tool for assessing stress response in free-ranging dolphins without effects due to sampling. PLOS ONE 10(2):e0115257.

Keogh, M. J., and S. Atkinson. 2015. Endocrine and immunological responses to adrenocorticotrophic hormone (ACTH) administration in juvenile harbor seals (*Phoca vitulina*) during winter and summer. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 188:22–31.

Koopman, H. 2007. Phylogenetic, ecological, and ontogenetic factors influencing the biochemical structure of the blubber of odontocetes. Marine Biology 151:277–291.

Kuznetsova, A., P. B. Brockhoff and R. H. B. Christensen. 2013. lmerTest: Tests for random and fixed effects for linear mixed effect models. Available at https://cran.r-project.org/web/packages/lmerTest/lmerTest.pdf.

Maxwell, S. M., E. L. Hazen, S. J. Bograd, *et al*. 2013. Cumulative human impacts on marine predators. Nature Communications 4(2688).

Mead, J. F., R. B. Alfin-Slater, D. R. Howton and G. Popjak. 1986. Lipids: Chemistry, biochemistry, and nutrition. Plenum Press, New York, NY.

Mendel, C. M. 1989. The free hormone hypothesis: A physiologically based mathematical model. Endocrine Reviews 10:232–274.

Nakagawa, S., and H. Schielzeth. 2013. A general and simple method for obtaining R^2 from generalized linear mixed-effects models. Methods in Ecology and Evolution 4:133–142.

Noren, D. P., and J. A. Mocklin. 2012. Review of cetacean biopsy techniques: Factors contributing to successful sample collection and physiological and behavioral impacts. Marine Mammal Science 28:154–199.

Ortiz, R. M., and G. A. J. Worthy. 2000. Effects of capture on adrenal steroid and vasopressin concentrations in free-ranging bottlenose dolphins (*Tursiops truncatus*). Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 125:317–324.

Ortiz, R. M., B. Long, D. Casper, C. L. Ortiz and T. M. Williams. 2010. Biochemical and hormonal changes during acute fasting and re-feeding in bottlenose dolphins (*Tursiops truncatus*). Marine Mammal Science 26:409–419.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Reidarson, T. H., and J. F. Mcbain. 1999. Hematologic, Biochemical, and endocrine effects of dexamethasone on bottlenose dolphins (*Tursiops truncatus*). Journal of Zoo and Wildlife Medicine 30:310–312.

Romero, L. M., and J. M. Reed. 2005. Collecting baseline corticosterone samples in the field: Is under 3 min good enough? Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 140:73–79.

Schmitt, T. L., D. J. St. Aubin, A. M. Schaefer and J. L. Dunn. 2010. Baseline, diurnal variations, and stress-induced changes of stress hormones in three captive beluga whales, *Delphinapterus leucas*. Marine Mammal Science 26:635–647.

Sheriff, M. J., B. Dantzer, B. Delehanty, R. Palme and R. Boonstra. 2011. Measuring stress in wildlife: Techniques for quantifying glucocorticoids. Oecologia 166:869–887.

St. Aubin, D. J. 2001. Endocrinology. Pages 165–192 *in* L. A. Dierauf and F. Gulland, eds. Marine mammal medicine. CRC Press, New York, NY.

St. Aubin, D. J., and J. R. Geraci. 1988. Capture and handling stress suppresses circulating levels of thyroxine (T_4) and triiodothyronine (T_3) in beluga whales *Delphinapterus leucas*. Physiological Zoology 61:170–175.

St. Aubin, D. J., and J. R. Geraci. 1990. Adrenal responsiveness to stimulation by adrenocorticotropic hormone (ACTH) in captive beluga whales (*Delphinapterus leucas*). Canadian Bulletin of Fisheries and Aquatic Sciences 224:149–157.

St. Aubin, D. J., and J. R. Geraci. 1992. Thyroid hormone balance in beluga whales, *Delphinapterus leucas*: Dynamics after capture and influence of thyrotropin. Canadian Journal of Veterinary Research 56:1–5.

St. Aubin, D. J., S. H. Ridgway, R. S. Wells and H. Rhinehart. 1996. Dolphin thyroid and adrenal hormones: Circulating levels in wild and semidomesticated *Tursiops truncatus*, and influence of sex, age, and season. Marine Mammal Science 12:1–13.

St. Aubin, D. J., K. A. Forney, S. J. Chivers, *et al*. 2013. Hematological, serum, and plasma chemical constituents in pantropical spotted dolphins (*Stenella attenuata*) following chase, encirclement, and tagging. Marine Mammal Science 29:14–35.

Strandberg, U., A. Käkelä, C. Lydersen, K. M. Kovacs, O. Grahl Nielsen, H. Hyvärinen and R. Käkelä. 2008. Stratification, composition, and function of marine mammal blubber: The ecology of fatty acids in marine mammals. Physiological and Biochemical Zoology 81:473–485.

Thomson, C. A., and J. R. Geraci. 1986. Cortisol, aldosterone, and leucocytes in the stress response of bottlenose dolphins, *Tursiops truncatus*. Canadian Journal of Fisheries and Aquatic Sciences 43:1010–1016.

Trana, M. R., J. D. Roth, G. T. Tomy, W. G. Anderson and S. H. Ferguson. 2015. Influence of sample degradation and tissue depth on blubber cortisol in beluga whales. Journal of Experimental Marine Biology and Ecology 462:8–13.

Wasser, S. K., S. L. Monfort, J. Southers and D. E. Wildt. 1994. Excretion rates and metabolites of oestradiol and progesterone in baboon (*Papio cynocephalus cynocephalus*) faeces. Journal of Reproduction and Fertility 101:213–220.

Wasser, S. K., K. E. Hunt, J. L. Brown, *et al*. 2000. A Generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. General and Comparative Endocrinology 120:260–275.

Wasser, S. K., J. C. Azkarate, R. K. Booth, *et al*. 2010. Non-invasive measurement of thyroid hormone in feces of a diverse array of avian and mammalian species. General and Comparative Endocrinology 168:1–7.

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Supporting Information

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Figure S1. Validations of (A) ACTH, (B) aldosterone, and (C) blubber cortisol assays used in this study. Serial dilutions of plasma (ACTH), serum (aldosterone), and lipid extracts (blubber cortisol) were processed (shown in gray) along with standards from the kits (shown in black); and each serial dilution was in parallel with the standard curve. *X*-axes are hormone concentrations; *y*-axes are response metrics—absorbance at 450 nm wavelength for ACTH and percent bound relative to the maximum binding tube for aldosterone and blubber cortisol (see kit protocols for detail). The pooled sample dilutions are shown relative to their dilution factor (secondary *x*-axis at top).