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Variability of Hormonal Stress Markers and Stress Responses in a Large Cross-Sectional Sample of Elephant Seals

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Award Number: N000141110434

LONG-TERM GOALS

Physiological indicators of stress in wild marine mammals, the interrelationships between different stress markers and assessment of the biological effects of stress can be used to estimate the impact of anthropogenic stressors on marine mammal populations. Currently, there are no large cross-sectional datasets of stress markers in free ranging marine mammal populations. Without these data there is no context with which to interpret the biological significance of variation in stress markers in individuals. The United States Navy, as part of its environmental stewardship, can utilize stress markers to assess the acute and chronic impacts that its actions might have on marine mammals. This approach would permit better mitigation of potential impacts and ensure that Navy activities do not come at a deleterious cost to marine mammal populations.

OBJECTIVES

The objectives of this effort are to: 1) determine the variation in glucocorticoid hormones (GC), aldosterone (A), thyroid hormones (TH), and catecholamines within a free-ranging northern elephant seal population and its dependence upon gender, age, seasonality, time of day, reproductive state and fasting duration; 2) establish relationships between serum GC levels and levels found in fur and blubber; 3) perform adrenocorticotropic hormone (ACTH) and thyroid stimulating hormone (TSH) challenges and characterize the activation of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-thyroid (HPT) axes across multiple matrices.

APPROACH

Task 1 – Natural variations in hormones across multiple matrices
Baseline characterization of hormones will be conducted during all four years (36 months) of the study with the highest effort in the first year. We will obtain 260 matched blood, blubber and fur samples in year one. We will obtain samples from 80 known-aged females and 60 adult males, either early or late in their natural fasts during the breeding or molt haulouts. Similarly, we will sample 80 known-age juvenile seals early or late in either of their haulouts and 40 weaned pups early and late in their post-weaning developmental fast. During year two of the study we will sample 40 known age adult females, 40 adult males, 40 known age juveniles and 40 weaned pups early and late in the haulout as above. We will target sampling times to complete a broad sample for assessing diel variation. During year three of the study we will repeat the process. The complete effort will achieve a total sample size of 580 individuals. As a value added component of the study we will obtain blood samples only for analysis of stress hormones from animals involved in other ongoing elephant seal research projects (approximately 100-150 additional individuals per year).

Serum samples will be processed for ACTH, cortisol, aldosterone, catecholamines (epinephrine, norepinephrine), and TH (T3 and T4) via radioimmunoassay (RIA). All of these hormones are measured routinely in our lab and the assays have been validated for use in this species. Field collected samples for catecholamine analysis will be centrifuged on site and immediately frozen in liquid nitrogen prior to transport. A multi-step biphasic organic solvent extraction will be used to isolate the corticosteroids from the blubber tissue (Kellar *et al.*, 2006; Kellar *et al.*, 2009). The hormones will be measured using a commercially available enzyme immunoassay (EIA) and parallel processed via HPLC to verify method performance. Hair samples will be collected from the anterior back region of seals for determination of cortisol as a measure of chronic stress (Davenport et al., 2006). Hair shaft cortisol will be determined using a technique recently validated for use in free-ranging terrestrial mammals (Macbeth et al., 2010). Briefly, hair samples will be washed to remove external contamination and then ground to a fine powder with a mixer mill. Cortisol will be extracted into methanol, reconstituted in phosphate buffer, and measured using a commercially available enzyme-linked immunosorbent assay (Macbeth et al., 2010). Blood chemistries will be run for all individuals as part of a health assessment at the time of sampling

Task 2 – Adrenocortical sensitivity and temporal pattern in matrices.
Adrenocortical sensitivity and the relationship between activation of the HPA axis and reflection of this activation in serum and other matrices will be determined during second and third years of the study. In year two, we will complete pilot work for the ACTH, cortisol and TSH studies, working out appropriate dosages for the challenge work in year 3. We will complete 4-6 ACTH and cortisol challenges early and late in the fasts in yearlings and breeding adult females. Initial baseline samples

will be taken for all challenges. ACTH slow-release gel will be intramuscularly implanted to permit time-controlled and sustained release of ACTH. Repeat samples will be taken daily to determine the relationship between the time course of serum GC increase and ACTH dose. The number of days over which sampling will occur will depend on the results of pilot work conducted during the second year of the study. Serum, blubber and fur samples will be processed as described in Task 1.

Direct cortisol challenge will be used to raise cortisol levels independent of HPA axis up-regulation and to potentially allow elevation of cortisol if adrenocortical sensitivity is reduced. Furthermore, the time course of the ACTH challenge may be insufficient for serum cortisol elevations to be observed in the blubber. Therefore, in year 2 small cortisol implants that release cortisol over 21 days will be implanted in 2 weaned pups and 2 juveniles. Two dose rates will be used for the pups, with the same dose rates used for the females. Blood samples will be taken 0, 12, 14 and 16 days after implantation for plasma cortisol measurements, and blubber samples will be taken at 12 and 16 days after implantation for blubber cortisol measurements. In year 3 we will perform ACTH challenges in 10 weaned pups and in 10 juveniles. We will perform cortisol implant challenges in 5 weaned pups and 5 juveniles.

Task 3 – TSH challenges
Thyroid hormones (thyroxin, T4 and triiodothyronine, T3) are released from the thyroid gland and are responsible for regulating a number of metabolic functions, including the regulation of catecholamine activity through permissiveness. Individuals will receive an intramuscular injection of thyroid-stimulating hormone, TSH (Genzyme Corporation, Cambridge, MA, USA). Baseline blood samples will be collected prior to the first injection and then every 30 minutes for 4 hrs from the time of injection. We will perform 2-3 pilot challenges in year 2 and challenges in 5 weaned pups and 5 breeding females in year 3.

Task 4 - Biological significance of baseline hormone values.
We will leverage the large existing research effort on northern elephant seals to assess the biological significance of the variation in hormone levels including survival impacts in juveniles and reproductive impacts in adults. Survival and natality at varied hormone levels will be compared to the larger annual samples of juvenile survival and female natality rates. Furthermore, where information on foraging success can be obtained (i.e. returning tagged females), relationships to foraging success and stress markers will be performed. This might be a particularly fruitful area for exploring relationships between energy acquisition and stress, as recent observations suggest that elevated cortisol is correlated with reduced energy gain at sea in adult female elephant seals and that elevated cortisol levels at implantation or during gestation may play an important role in determining natality in a given year. We will examine the relationship of stress responses to reproductive hormones to better undertsand the mechanism by which stress responses may impact reproduction.

WORK COMPLETED

Task 1 – Natural variation in stress hormones across multiple matrices.
We have completed all of the proposed sampling during the sampling periods of the project, successfully sampling 580 animals across all matrices. In addition to these samples blood samples were obtained fron an additional 246 animals involved in other studies. The total blood baseline sample size is currently 826 animals. Over 5200 individual hormone assays have been completed for the project icluding complete analysis of cortisol, aldosterone, fT3, tT3, fT4, and tT4 for all samples. We are working on completing ACTH, NE and Epi analyses. Blubber cortisol analysis is complete for

over half the samples and is proceeding. Hair cortisol analysis was completed for a subsample of the fur samples and based on the results we moved to analyzing cortisol in the vibrissae as a better matrix. Vibrissae cortisol analysis is ongoing.

Task 2 - Adrenocortical sensitivity and temporal pattern in matrices.
We completed 8 pilot trials to find the appropriate ACTH dose for elephant seals and to better understand the duriation of HPA axis activation. Based on these pilots we performed ACTH challenges in 15 juveniles during the molt haul-out and in 18 adult males during the breeding season and molt. For these animals we measured changes in cortisol, aldosterone and endogenous ACTH to the challenge as well as impacts on blood chemistries including fat mobilization, sugar production and electrolytes. We performed ACTH challenges in 12 weaned pups with repeated blubber sampling to examine the time course of changes in blubber cort profiles with an acute and prolonged stressor.

Task 3 - Pilot studies for TSH have been completed.
Trials will be completed in the 2014 breeding season.

Task 4 - Biological significance of baseline hormone values.
We analyzed stress hormone profiles (cortisol, aldosterone, thyroid hormones) in 530 blood samples for 291 adult females tracked as part of the TOPP project. We measured sex hormones (estradiol and progesterone) in molting females to examine reproductive impacts of stress. We related stress responses to measures of foraging success while at sea and breeding decisions by females.

RESULTS

Task 1 - As of this report, analysis of the blood samples for cortisol, aldosterone, and 4 thyroid hormones (tT4, fT4, tT3 and fT3) are complete for all samples. ACTH, epinephrine and norepinephrine are still being analyzed. Complete blood chemistries have been run for a subset of animals. Analyis of hair samples (and vibrissae) is underway at University of Saskatchewan and analysis of blubber samples is continuing at SWFS. Numerous novel findings are evident in the preliminary analysis from the blood samples. Patterns of change across life history stages have been determined for a ll hormones (e.g. Figure 1). Cortisol and aldosterone levels are correlated in all study groups (Figure 2), suggesting the potential importance of aldosterone as a stress hormone in phocids. Aldosterone levels had significant effects on Na^+ levels suggesting potential for stress impacts on salt balance. Similar results in the parallel dolphin studies suggest this may be a major difference between the stress response in marine mammals and terrestrial species Thyroid hormones show strong negative relationships to cortisol, particularly T3, suggesting a strong suppression of the thyroid axis in response to stress (Figure 3). Preliminary analysis suggests this may occur through promotion of production rT3, a thyroid receptor blocker, from T4.

Initial analysis of juvenile hair samples shows that cortisol levels in hair are low compared to terrestrial carnivores but are measureable. Variation in hair cortisol was significantly related to serum cortisol levels, suggesting hair has utility for stress measurements in phocids. We are focusing on vibrissae as a time signature of stress exposure. We learned that adult female blubber samples contain extremely high progesterone levels which require use of an analysis platform with no cross-reactivity to progesterone. Adult female blubber extractions are being reanalyzed using an assay that meets this requirement. In adult males and juveniles blubber cortisol was strongly related to serum cortisol levels except for the late molt period (Figure 4).

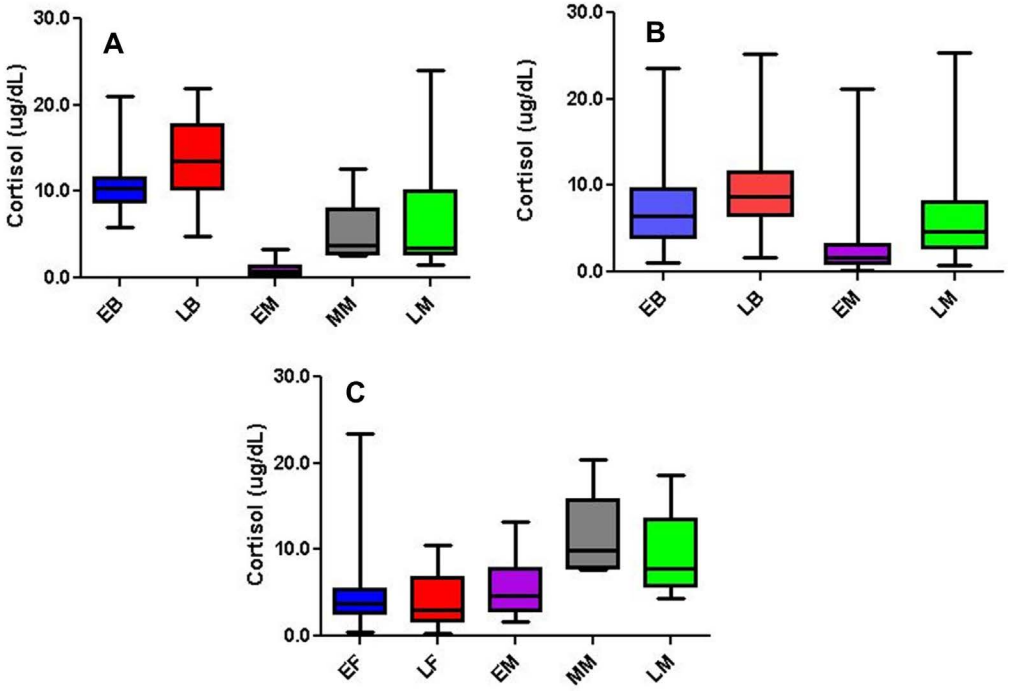

Figure 1. Annual patterns of serum cortisol in A: adult males; B: adult females, and C: juveniles.

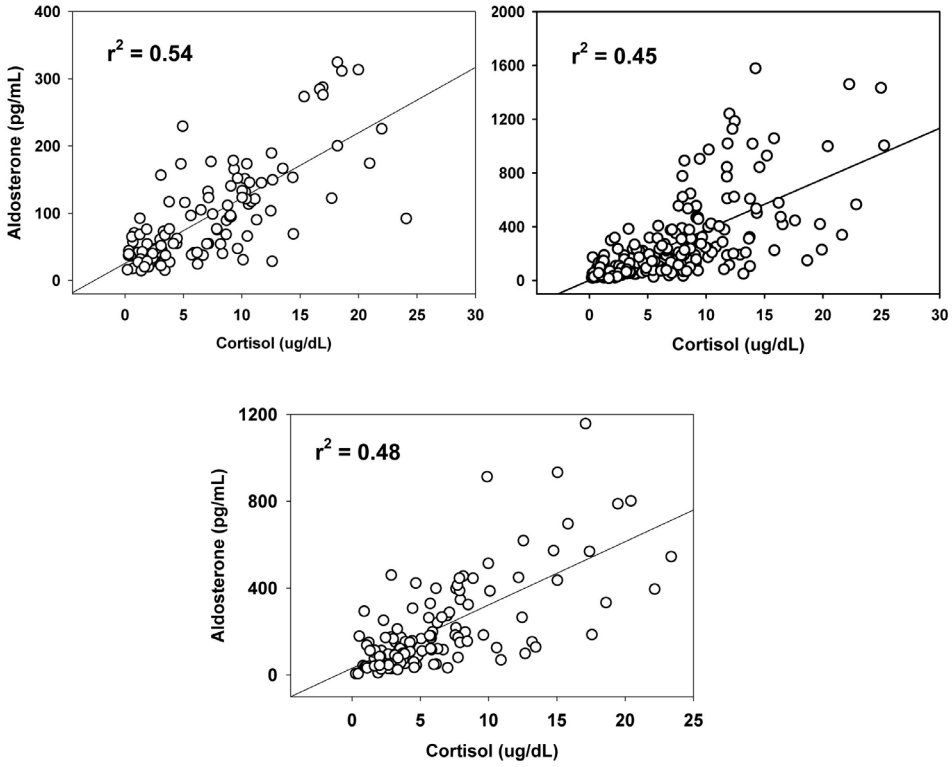

Figure 2. Relationship between serum cortisol and aldosterone in A: adult males; B: adult females, and C: juveniles.

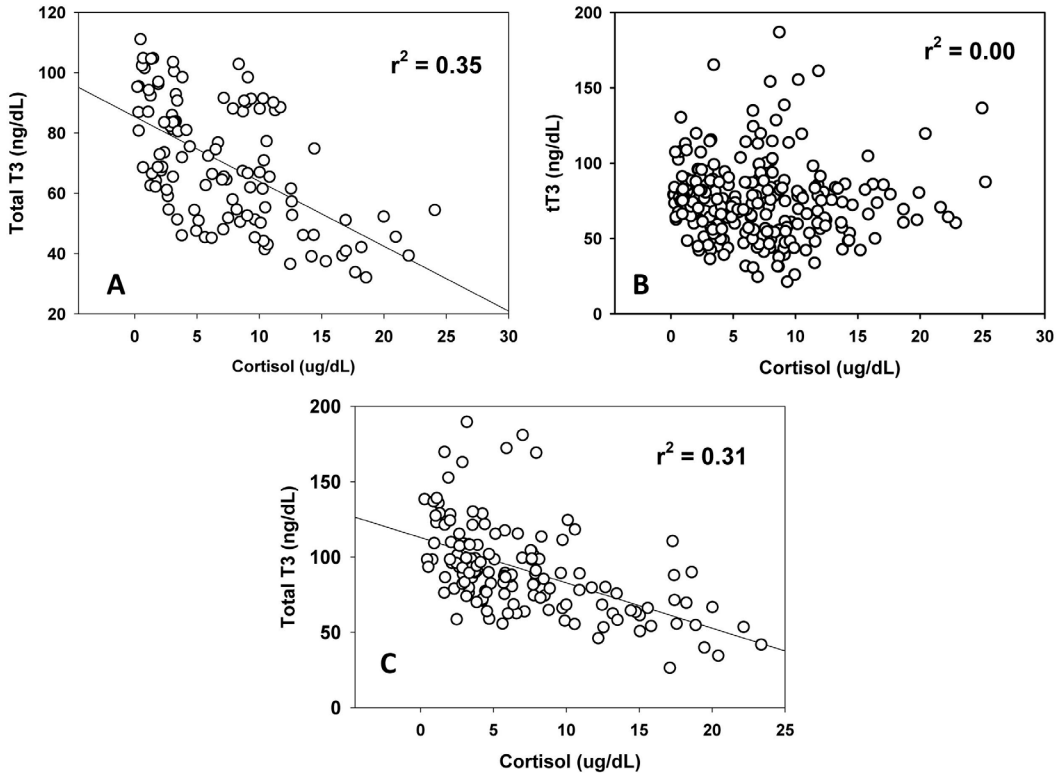

Figure 3. Relationship between serum cortisol and triiodothyronine in A: adult males; B: adult females, and C: juveniles.

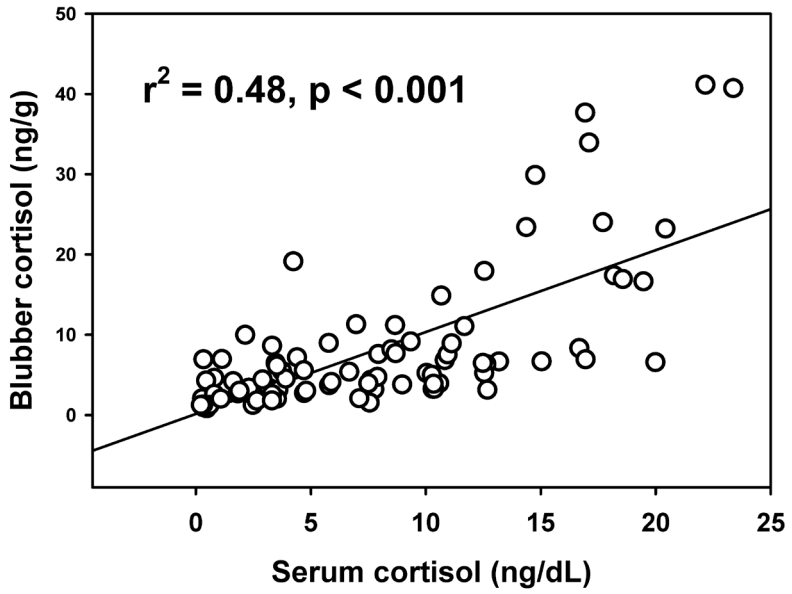

Figure 4. Relationship between blubber cortisol levels and serum cortisol levels in adult male and juvenile elephnat seals.

Task 2 - We found that the HPA axis of elephant seals was very sensitive to ACTH, despite their lack of stress response to handling. ACTH gel elevated cortisol to 10 times baseline for 48-72 hrs. ACTH was as strong a secretagogue for aldosterone as it was for cortisol (Figure 5). Elevations in cortisol and aldosterone impacted glucose, lactate, NEFA , BUN and electrolyte levels. These data provide novel information on the physiological and metabolic impacts of acute alterations in stress hormones in phocids and provide an excellent mechanism to examine variation in stress measures across matrices in response to acute and sustained stress. We are currently analyzing blubber cortisol levels in response to ACTH challenges.

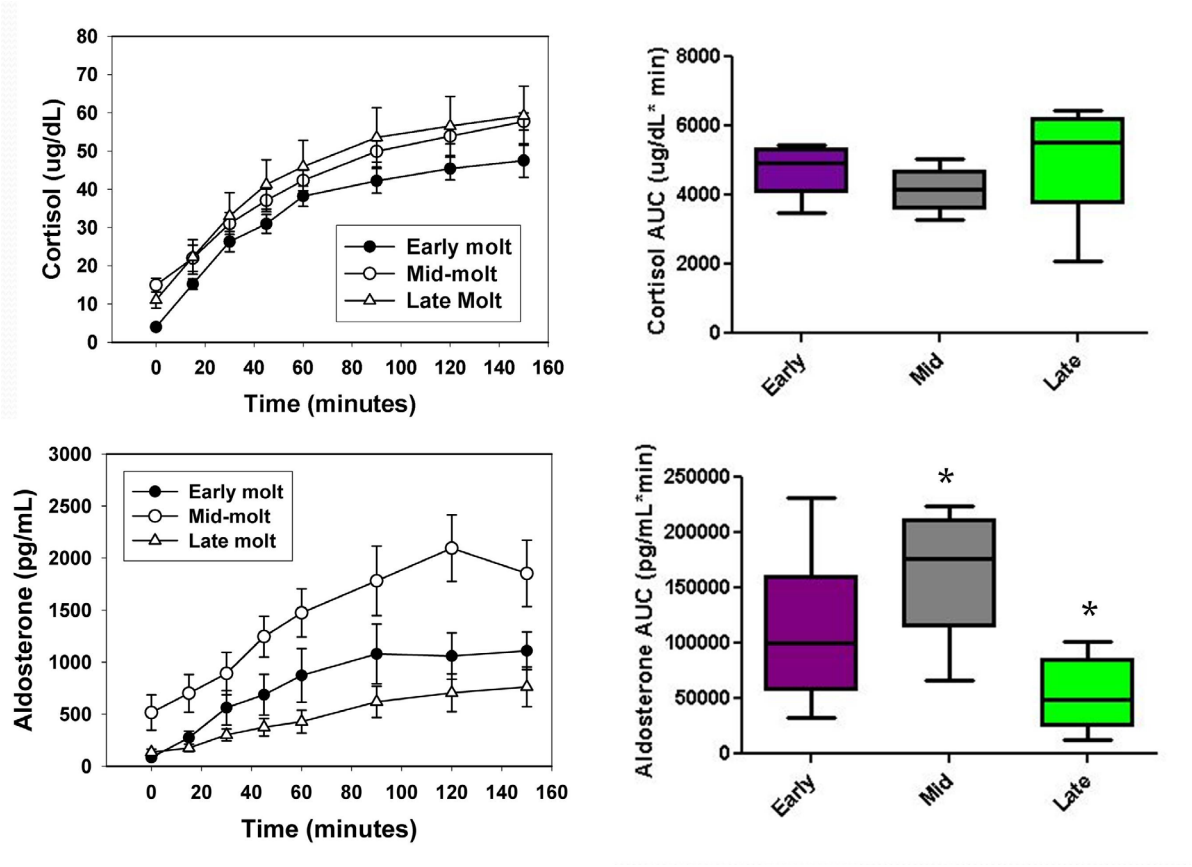

Figure 5. Summary of effects of ACTH challenge on cortisol and aldosterone in juveniles.

Task 3 – We have determined appropriate TSH dosages for trials.

Task 4 - We have found strong relationships between serum cortisol levels at recovery and long-term foraging success in females. Cortisol levels directly predicted natality and influenced levels of progesterone during the implantation period. These data suggest elephant seals may use natural stress responses to nutritional status to make breeding decisions as mediated by the interaction of cortisol with the gonadal axis. This analysis is finished and we will submit a manuscript in this next 6 months.

IMPACT/APPLICATIONS

The ability to identify stress markers and their relationship to the health of marine mammal populations is critical to understanding the impact of anthropogenic activities upon those populations.

The baseline characterization of stress marker variation in elephant seals as a function of seasonality, gender, age, fasting duration, health and reproductive status is important to assessing measurements made in wild pinnipeds and other species, including understanding acute natural variation in stress hormones in contrast to sustained stress reponses resulting in biological impacts. Information on levels and dynamics of stress markers between different matrices will provide better estimates of the overall condition of marine mammals sampled in the wild from either blubber biopsies or hair samples. In addition, an understanding of the function of the HPA and HPT axis and variation in axis function across life histories will provide fundamental information on the mechanics of stress responses in these marine mammals, which may differ significantly from that of the terrestrial mammals. An increased understanding of the mechanisms by which stress hormones interact with physiological variables and reproductive status will provide critical information to understanding when stress impacts become biologically significant to populations of marine mammals.

RELATED PROJECTS

Project: Variability of Hormonal Stress Markers Collected from a Managed Dolphin Population

PI: Dorian Houser

This project examines variation in stress hormone markers across several matrices in a captive dolphin population, allowing intensive longitudinal sampling in contrast to the broad, cross-sectional sampling of our study.

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Macbeth, B. J., Cattet, M. R. L., Stenhouse, G. B., Gibeau, M. L., and Janz, D. M. 2010. Hair cortisol concentration as a non-invasive measure of long-term stress in free ranging grizzly bears: Technique development and considerations with implications for other wildlife. Canadian Journal of Zoology. 88(10):935-949.

PUBLICATIONS IN PREPARATION

4 papers are in development for submission in the next 6 months:

D.C. Ensminger, D.A. Somo, D.S. Houser, D.E. Crocker. Metabolic impacts of HPA axis activation varies with life-history stage in adult male elephant seals. (Targeted for submission to General Comparative Endocrinology).

C.D. Champagne, M.S. Tift., D.S. Houser, D.E. Crocker. Changes in HPA axis responsiveness and impacts during molting in juvenile northern elephant seals. (Targeted for submission to Physiological and Biochemical Zoology).

D.E. Crocker and D.P. Costa. Foraging success mediates reproduction through cortisol levels in female northern elephant seals. (Targeted for submission to Journal of Animal Ecology).

D.E. Crocker, M.S. Tift, J.S. Sharick, D.C. Ensminger, D.S. Houser. A comprehensive profile of adrenal function in a large cross-sectional sample of northern elephant seals. (Targeted for submission to General Comparative Endocrinology).

We anticipate additional manuscripts in the following year focusing on:

- A comprehensive profile of thyroid function (Crocker lead)
- Relationship of blubber cortisol to serum cortisol (Cockrem lead)
- Timing of blubber cortisol changes in response to an acute stress activation. (Cockrem lead)
- Patterns in hair and vibrissae cortisol (Janz lead)
- TSH stimulation responses (Houser lead).