

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

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### **LONG-TERM GOALS**

The Office of Naval Research recently funded a multi-year, collaborative research project to investigate baseline hormone variations in northern elephant seals (*Mirounga angustirostris*) and studies of the hypothalamus-pituitary-adrenal (HPA) and hypothalamus-pituitary-thyroid (HPT) axes (grant # N000141110434). These investigations described natural variation in corticosteroid, thyroid, and catecholamine hormones in northern elephant seals across multiple matrices, and assessed the sensitivity of HPA and HPT axes. Using the large sample bank from this project a number of opportunities to better understand the stress response in pinnipeds, and marine mammals overall, are available. These include the relatively low cost ability to better understand the role the binding globulins play in modulating the bioavailability of stress hormones and the mechanisms by which stress hormones interact with energy metabolism, salt balance, reproductive and immune systems. These research goals will increase our understanding of when stress has biologically significant effects on animals and identify potential mediators and mechanisms of those effects.

### **OBJECTIVES**

The objectives of this effort are to: 1) determine the bioavailability of cortisol in serum samples and variability of cortisol binding globulin (CBG) with life-history stage and gender in elephant seals; 2) determine the impact of variation in baseline cortisol on thyroid hormones and function in elephant seals; 3) determine the impact of baseline variation in aldosterone on electrolyte balance in elephant seals; 4) determine the natural life-history variation in sex hormones for both genders and impact of variation in baseline cortisol on reproductive hormones; and 5) examine the ecoimmunology of breeding and molting in elephant seals and determine the impact of variation in cortisol on immune function.

## APPROACH

### ***Task 1 – Corticosteroid Binding Globulin (CBG)***

Most circulating cortisol is bound to carrier proteins—primarily corticosteroid binding globulin (CBG). Only free (unbound) cortisol is thought to be biologically active and capable of interacting with target tissue receptors. Therefore, the metabolic influence of cortisol on target tissues is mediated by carrier protein expression, primarily that of CBG. The exact role of CBG in stress responses is not well understood. It may enhance transport and delivery of corticosteroids to specific target tissues or, conversely, act as a buffer mediating alterations in circulating corticosteroids. Nevertheless, assessing the level of circulating CBG and its variability over time permits far greater understanding of circulating corticosteroid levels and their magnitude of effect on target tissues. CBG may, in fact, be an accurate marker of long term stress as it does not seem to vary with acute stress, like capture shock, in some species. Ultimately, CBG levels may directly influence the bioavailability of cortisol and modulate its biological effects. Variation in CBG may help explain some of the several orders of magnitude variation in cortisol levels observed among marine mammal species. We have validated a radioimmunoassay (RIA) for CBG in elephant seals (Immuno-Biological Laboratories, Inc, USA). We originally were developing a H<sup>3</sup>tracer based measure of total plasma cortisol binding capacity. We had the opportunity to interact with Rudy Boonstra at the ONR program review who offered his collaboration and expertise in this task. Dr. Boonstra has been funded by ONR to spearhead the effort to measure variability in cortisol binding capacity among marine mammal species. We are providing samples from numerous pinniped species for that effort and collaborating to measure binding capacity in the elephant seal sample bank.

### ***Task 2 – Impact of cortisol on thyroid function.***

Thyroid hormones (thyroxine, T4; and triiodothyronine, T3) regulate many metabolic pathways, upregulating mitochondrial proliferation, facilitating erythropoiesis and strongly influencing whole-animal metabolic rate. Variation in thyroid hormones directly impact energy expenditure as well as reproductive behavior and effort in elephant seals. Most thyroid hormone is released from the thyroid gland as T4. At target tissues, deiodinases (D1 and D2) convert T4 into the more biologically active T3. A third deiodinase (D3) inactivates T3 but may also convert T4 into an inactive form—reverse T3 (rT3). The rT3 binds to T3 receptors without upregulating gene expression, thus blocking most thyroid hormone action. Many stressors decrease D1 activity, including food limitation, illness, and high cortisol and catecholamine levels. The stress-induced decrease in D1 activity causes increased rT3, which likely leads to reductions in metabolic rate by blocking T3 receptors. The influence of thyroid hormones on the stress response is, therefore, significantly influenced by rT3 concentration. Preliminary analysis suggests strong association of cortisol and thyroid levels in elephant seals (Figures 2 and 3). We have validated an RIA for rT3 in elephant seals (Alpco Immunoassays, Salem, NH) and found high levels of rT3. We propose to measure rT3 in the entire elephant seal blood sample bank to better understand the interaction of cortisol with thyroid hormones. High levels of rT3 in bottlenose dolphins (ONR N000141110436) suggest these findings may have broad implications for marine mammal species.

### ***Task 3 – Impact of aldosterone variability on osmolality***

Work on the Parent Project and a parallel project on bottlenose dolphins has shown the importance of aldosterone as a stress hormone in marine mammals. Aldosterone covaries with cortisol in many groups (Figure 4) and ACTH challenges in the Parent Project have shown that ACTH is a more potent secretagogue for aldosterone than cortisol. Aldosterone is an important osmoregulatory hormone regulating salt and water balance in mammals and its function is particularly important in marine

mammals (Ortiz et al. 2006). We propose to use standard laboratory techniques to measure  $\text{Na}^+$ ,  $\text{K}^+$  and osmolality in the entire collection of elephant seal blood samples to better understand the interaction of aldosterone with salt and water balance. Similar findings in dolphins suggest this stress hormone may be a key difference to stress responses in terrestrial mammals and of critical importance given the hyperosmotic environment of marine mammals.

***Task 4 - Impact of stress hormone variability on sex hormones***

In terms of biologically significant impacts of stress responses in marine mammals, one key factor is the interaction of stress hormones with reproductive systems. Cortisol may directly impact the gonadal axis of many species (reviewed in Moore and Evans 1999) and alter or suppress reproductive behavior. Work on the Parent Project has shown that cortisol levels vary with foraging success and allow prediction of natality in female northern elephant seals, but the underlying mechanisms are not understood. We propose to measure several key sex hormones (progesterone, estradiol and prolactin in females, testosterone in males, and dehydroxyepiandrosterone (DHEA) in both sexes) for the entire collection of elephant seal samples and compare these data to the baseline variation in stress hormones. We have validated assays for all of the hormones (DPC, Seimens, Los Angeles, CA; Kelso et al. 2012).

***Task 5 - Impact of stress hormone variability in immune function.***

The most likely mechanism by which stress impacts may have biologically significant effects on marine mammals is through impacts on the immune system, which alter susceptibility to disease and health. We propose to measure several key markers of immune status and systemic inflammation in elephant seals. Interleukin (IL)-6 and IL-1b are produced at the site of inflammation and plays a key role in the acute phase response as defined by a variety of clinical and biological features. IL-6 is the chief stimulator of the production of acute-phase proteins in response to injury, inflammation or illness (Gauldie et al. 1987). IL-1b is an important mediator of the inflammatory response and directly regulates production of haptoglobin. Tumor necrosis factor (TNF)  $\alpha$  is a cytokine that regulates acute phase proteins and is primarily involved in the regulation of immune cells. C-reactive protein (CRP) is an acute phase protein that serves as a marker for systemic inflammation. Immunoglobulin E (IgE) is a mammalian immunoglobulin that regulates immunity to parasitic infections and plays a role in allergic reactions. IgG is the main antibody isotype produced in response to infections. IgM is the first antibody to appear in response to initial exposure to an antigen. We have validated assays for IL-1b, IL-6, TNF $\alpha$ , CRP, IgE, IgG, IgM and haptoglobin in elephant seals (Cayman Chemical, Ann Arbor, MI; DPC/Seimens, Los Angeles, CA; Thermo-scientific, Rockford, IL). We propose to measure these markers in the entire pool of elephant seal blood samples to better understand the interaction of stress with the immune response in marine mammals.

**WORK COMPLETED**

***Task 1 – Corticosteroid Binding Globulin (CBG).***

We validated and used a RIA to measure CBG in 80 adult male, 40 adult female and 40 juvenile samples. We are coordinating with Rudy Boonstra to complete measurements of binding affinity and total binding capacity in elephant seals as well as to provide samples for many other pinniped species. That effort is just beginning.

***Task 2 – Cortisol impacts on thyroid function.***

We have completed measurements of rT3 in the entire elephant seal sample bank. The proposed work for this task is completed.

**Task 3 – Impact of aldosterone variability on osmolality**

We have developed a autoanalyzer based system for precisely measuring Na<sup>+</sup>, K<sup>+</sup>, and total osmolality in serum samples. We are in the process of making these measurements in the elephant seal sample bank. Based on initial measurements we are targeting samples taken from animals that have just returned from foraging. We are trying to understand whether variation in baseline aldosterone levels impacts electrolyte levels in animals that have been ingesting salt in their food and incidentally ingesting seawater during foraging.

**Task 4 - Impact of stress hormone variability on sex hormones**

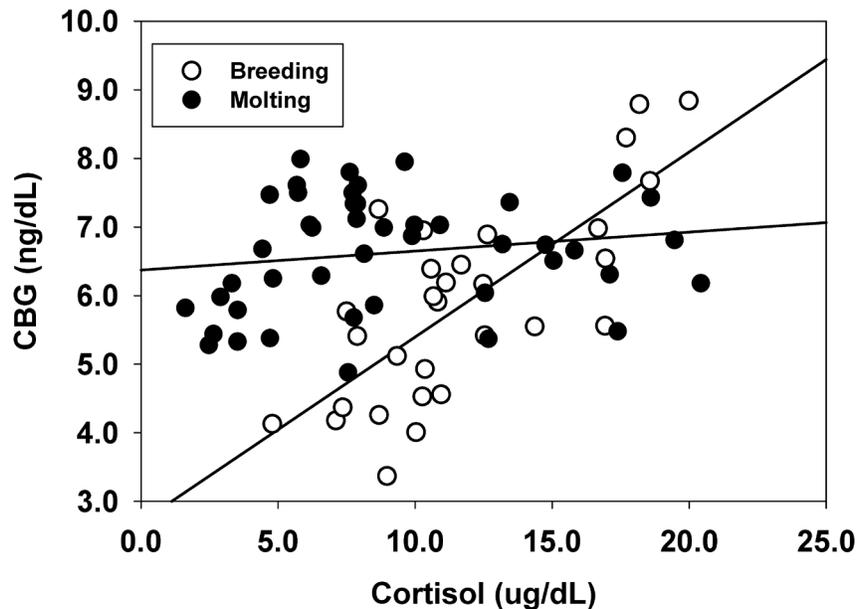
We have focused our initial efforts on reproductive impacts in females. We have validated assays for progesterone, estradiol, DHEA, and FSH. We have completed progesterone measurements in 647 adult female samples. We have completed estradiol measurements in 240 adult female samples. We have completed DHEA measurements in 240 adult female samples. We have developed and validated assays using canine antibodies for FSH, inhibin, activitin and relaxin. These proteins regulate gonadal function and serve as markers for reproductive status.

**Task 5 - Impact of stress hormone variability in immune function.**

We have focused our initial efforts on immune impacts in adult animals. We have measured Il-6, Il-1b, TNFa, haptoglobin, IgG, IgM, and IgE in 197 adult female samples and 115 adult male samples.

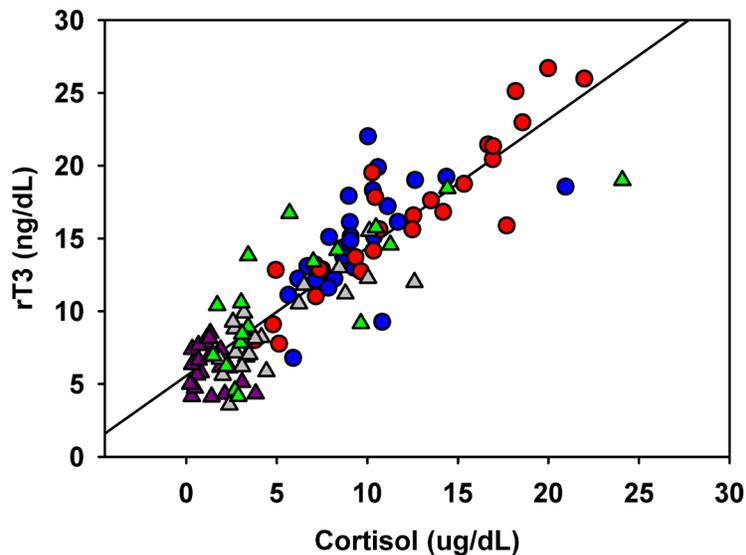
**RESULTS**

Task 1 - Preliminary analysis shows that CBG tracks changes in cortisol during breeding but not during the molt. This suggests cortisol bioavailability is regulated according to life history stage and the CBG is a key determinant of cortisol impacts on physiology.



**Figure 1. Changes in CBG levels with cortisol in breeding and molting adult male elephant seals**

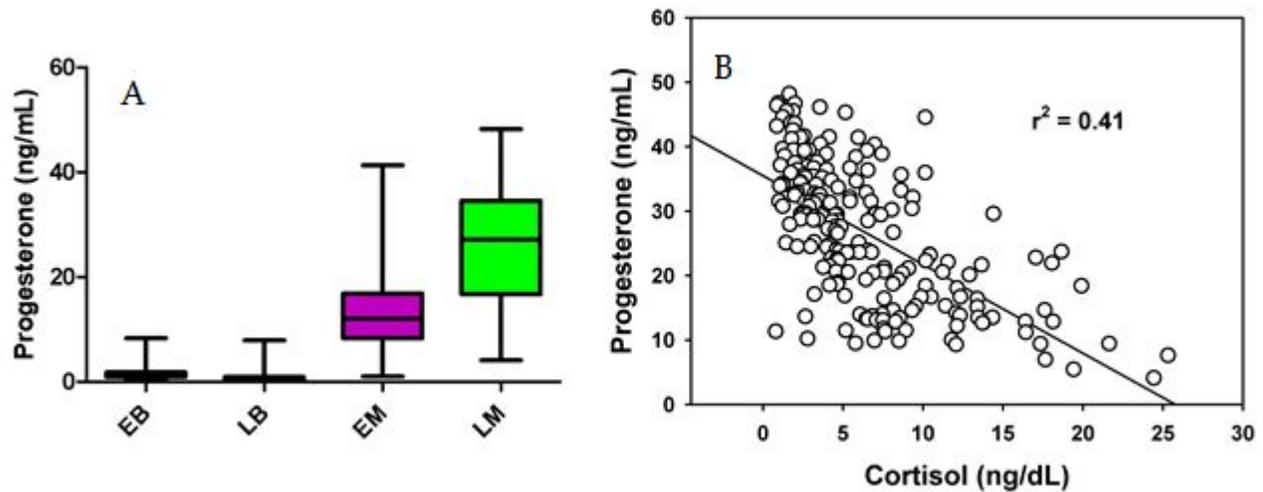
**Task 2** - Preliminary analysis suggests unusually high rT3 levels in elephant seals and a strong positive relationship between cortisol and rT3 levels. This effect is present in all age classes and life history stages except in lactating females. These data suggest that the potential for metabolic suppression in response to chronic stress is unusually strong in elephant seals. Parallel findings in the NMMF dolphin studies suggest this may be a feature of marine mammals.



**Figure 2.** Relationship of rT3 to cortisol in breeding and molting male adult elephant seals.

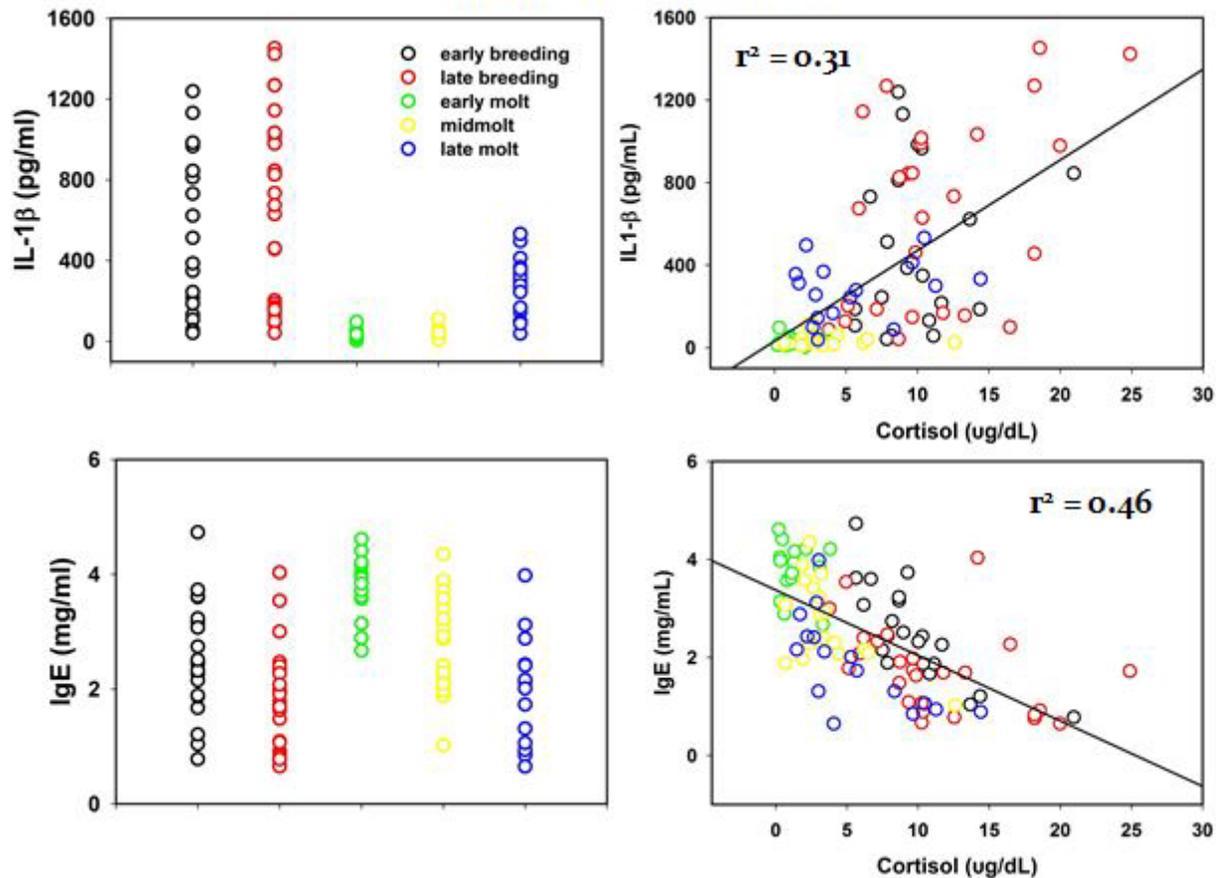
**Task 3** – The autoanalyzer system for ion measurement was compared to measurements of serum  $\text{Na}^+$  and  $\text{K}^+$  in 98 samples run by IDEXX veterinary diagnostic lab and showed excellent agreement ( $r = 0.92$ ). Samples from adult females just returning from foraging at sea showed that serum aldosterone has a strong association with serum  $\text{Na}^+$  concentrations and total osmolality. No associations with serum  $\text{K}^+$  concentrations were evident. These data suggest potential impacts on electrolyte

**Task 4** – Analysis of the entire adult female sample bank shows that progesterone levels are low during breeding but increase dramatically during the molt. Values are highest at the end of the molt when implantation is thought to occur, suggesting an important role for progesterone in creating the uterine conditions necessary for implantation. Despite this dramatic increase there was wide individual variation in progesterone at this time. This variation was significantly negatively related to cortisol levels suggesting that suppression of progesterone by cortisol may be the mechanism underlying cortisol impacts on natality seen in the previous project.



**Figure 3. A: Changes in serum progesterone during the breeding and molt haul-outs in female elephant seals. EB = Early breeding, LB = late breeding, EM = early molt, LM = late molt. B: Relationship of serum progesterone to serum cortisol in late molt females.**

**Task 5** – Adult female results are published in Functional Ecology and showed reduced immune response to parasite infection with cortisol elevation and impacts of body reserves on immune response. Data from breeding and molting adult males showed strong positive associations of baseline cortisol levels with innate immune responses and negative associations with adaptive immune responses. There was a transition from adaptive to innate immune responses with increasing dominance rank. Together with female results these data suggest an uncoupling of immune suppression for inflammatory cytokines and acute phase proteins during breeding but a more typical suppression of adaptive immune response by cortisol.



**Figure 4. Variation in an inflammatory cytokine (IL-1 $\beta$ ) and an immunoglobulin (total IgE) with life history stage in adult male northern elephant seals. IgE is a marker for immune response to helminth infections. Cortisol was positively associated with innate immune markers and negatively associated with adaptive immune markers.**

## IMPACT/APPLICATIONS

The ability to estimate the effects to marine mammals and marine mammal populations resulting from anthropogenic sound exposure directly affects the United States Navy's ability to comply with Federal regulations regarding environmental impacts. It is critical that as information on stress related hormones are acquired as part of acoustic impact studies, that the Navy understand the biological impacts of measured variation. The additional characterization of hormones and physiological responses under widely varying baseline and simulated stress conditions, as described in the current proposal, provides a mechanism by which to better detect the presence and magnitude of the physiological responses of marine mammals exposed to anthropogenic sound. In accordance with National Research Council recommendations (2005), this work, together with the Parent Project, will establish baseline and activated levels for putative stress markers in marine mammals. This proposal makes the most effective implementation of ONR's investment strategy for evaluating stress in marine mammals by minimizing costs and maximizing the return on investment using the unique database generated by the Parent Project. Specifically, understanding the mechanisms by which stress impacts physiology will allow better determination of whether or not stress has biologically significant impacts on 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.

Stress Hormones and their regulation in a captive dolphin population

PI: Cory Champagne

This project examines roles of CBG and rT3 in the sister study on the Navy captive bottlenose dolphin population.

Molecular indicators of chronic stress in a model pinniped - the northern elephant seal.

PI: Cory Champagne

This project examines gene expression response to acute and chronic elevation of cortisol in elephant seals.

## PUBLICATIONS

Peck, H.E., D.P. Costa, D.E. Crocker. Body reserves influence allocation to immune responses in capital breeding female northern elephant seals. *Functional Ecology* (in press).

Khudyakov J.I., C.D. Champagne, L. Preeyanon, R.M. Ortiz, D.E. Crocker. 2015. Muscle transcriptome response to ACTH administration in a free-ranging marine mammal. *Physiological Genomics*. 47:318-330.

Atkinson, S., D.E. Crocker, D.S. Houser, K.L. Mashburn. 2015. Stress physiology in marine mammals: How well do they fit the terrestrial model? *Journal of Comparative Physiology B*. 5:463-486.

Champagne, C.D., M.S. Tift, D.S. Houser, D.E. Crocker. 2015. Adrenal sensitivity to stress is maintained despite variation in baseline glucocorticoids in molting seals. *Conservation Physiology*. 3(4):1-11. doi:10.1093/conphys/cov004.

Khudyakov J.I., L. Preeyanon, C.D. Champagne, R.M. Ortiz, D.E. Crocker. 2015. Transcriptome analysis of northern elephant seal (*Mirounga angustirostris*) muscle tissue provides a novel molecular resource and physiological insights. *BMC Genomics*. 16:64.

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

Manuscripts in preparation:

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

Crocker, D.E. 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 adult northern elephant seals. (Targeted for submission to *General Comparative Endocrinology*).

- Crocker, D.E., H.E. Peck, R.A. Yoast, C. Reichmuth, C. Casey, D.S. Houser. Dominance rank and cortisol levels influence the magnitude of innate and adaptive immune responses in breeding adult male northern elephant seals. (Targeted for submission to Functional Ecology)
- Jelincic, J., M.S. Tift, D.S. Houser, D.E. Crocker. Life-history stage and sex influence adrenal hormones in juvenile northern elephant seals.
- J.F. Cockrem, D.E. Crocker. Timing and magnitude of blubber cortisol change in response to ACTH challenge in northern elephant seals.