

Metabolic responses to adrenocorticotrophic hormone (ACTH) vary
with life-history stage in adult male northern elephant seals.

by

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A thesis submitted to

Sonoma State University

in partial fulfillment of the requirements

for the degree of

MASTERS OF SCIENCE

in

Biology

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3/5/2014

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Abstract

Purpose of study: Strong individual and life-history variation in serum levels of glucocorticoids has been documented in many wildlife species. Less is known about the underlying variation in hypothalamic-pituitary-adrenal axis (HPA) responsiveness and its impacts on metabolism.

Procedure: . To characterize changes due to a simulated acute stress, 18 free-ranging adult male northern elephant seals were challenged with an intramuscular injection of slow release adrenocorticotrophic hormone (ACTH) over 3 sample periods: early in the breeding season, after 70+ days of the breeding fast, and during peak molt. Subjects were blood sampled every 30 minutes for 2 hrs post-injection. Breeding animals were recaptured and sampled at 48 hrs. This was not possible in molting subjects due to animal density and mobility.

Findings: In response to the ACTH injection, cortisol increased 4-6 fold in all groups, and remained elevated at 48 hrs in early breeding subjects. ACTH was also a strong secretagogue for aldosterone, causing a 3-8 fold increase in concentration. Cortisol and aldosterone responses did not vary between groups but were significantly correlated within individuals. Endogenous ACTH was not suppressed by the challenge in any study group. The ACTH challenge resulted in elevations in plasma glucose during late breeding and molting. The ACTH challenge suppressed testosterone and thyroid hormone (T3) at 48 hrs in early breeding males. The ACTH challenge increased plasma non-esterified fatty acids only during molting.

Conclusion: These data suggest that sensitivity of the HPA axis is maintained but the metabolic impacts of cortisol and feedback inhibition of the axis varies with life history stage. Strong impacts of the challenge on testosterone, thyroid hormone and protein sparing suggest the importance of maintaining low cortisol levels during the breeding fast. These data suggest that metabolic adaptations to extended fasting in northern elephant seals include significant alterations in tissue responses to regulatory hormones that mitigate deleterious impacts of acute or moderately sustained stress responses.

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MS Program: Biology
Sonoma State University

Date: 3/5/2014

Acknowledgement

I would like to express my special appreciation and thanks to my advisor Dr. Daniel Crocker, you have been a tremendous mentor for me. I would like to thank you for encouraging my research and for allowing me to grow as a scientist as well as a scholar. I would also like to thank my committee members, Dr. Nicholas Geist and Dr. Dorian Houser for serving as my committee members despite difficulties in distance and scheduling. I also want to thank you for your brilliant comments and suggestions at all stages of my thesis and studies. I would especially like to thank the faculty, staff, and technicians in the Biology Department at Sonoma State University. All of you have been there to support me while I obtained supplies and used equipment.

I would like to express my gratitude to my lab mates. I would like to thank Jeffrey Sharick for his constant help in the field and his knowledge and experience with elephant seals. I would like to thank Derek Somo for his help in the field and in the lab, as well as his great enthusiasm for many things. I also want to thank the whole lab, Jeff, Derek, Hannah Peck, and Sarah Codde for your amazing attitude and great sense of humor. You guys helped make the field and lab work much more enjoyable.

I would also like to thank all of my friends who supported me in writing and striving towards my goal. Your messages of encouragement always helped to bring a smile to my face.

Finally, I want to give special thanks to my family. Words cannot express how grateful I am for all of the sacrifices that you've made on my behalf. Your prayers and support for me was what sustained me thus far.

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Introduction

All vertebrates potentially experience stressors, such as a lack of food, limited space, environmental perturbations, and conflicting interactions with conspecifics and other species. While the generalized endocrine response to stress is well studied, the terminology for describing stress responses as distinct from normal homeostatic regulation is more controversial, with several proposed frameworks. Allostasis is the maintenance of stability through changes in homeostatic set points [1]. As the cost (e.g. energetic expenditure, oxidative damage) of allostasis accumulates, the animal enters allostatic overload, which is a state where detrimental effects on the organism body and behavior are evident [1-3]. Reactive scope first defines predictive and reactive homeostasis, which allow the animal to respond to circadian, seasonal, and unpredictable variations, respectively [4]. As reactive homeostasis persists, wear and tear accumulates as a cost of maintaining physiological conditions in the reactive homeostasis range [4]. This accumulation increases the likelihood of entering homeostatic overload or failure, which potentially compromises short or long-term health [4]. Despite the differences in terminology, sustained stress can produce deleterious impacts, including suppression of immune and reproductive function [5, 6]. Studies on the impacts of anthropogenic stressors to wildlife have begun to recognize that integration of various stressors, such as pollution, disturbance, alterations in food availability and others may have demographic impacts by increasing the allostatic load on individuals and affecting health and reproduction. This realization has led to the development of the field of conservation physiology [3, 7-9].

The primary hormonal response to stress is the activation of the hypothalamic-pituitary-adrenal (HPA) axis. In response to stress, the hypothalamus releases corticotrophin-releasing hormone (CRH) [10], which stimulates the pituitary gland to release adrenocorticotrophic hormone (ACTH) [11]. ACTH then acts upon the adrenal cortex, which releases corticosteroids, such as cortisol and aldosterone [12]. These adrenal hormones have wide-ranging physiological effects on metabolism, reproductive hormones, and immune function [6, 13-16]. They are known to interact with and affect the concentrations or reactivity of thyroid hormones (thyroxine, T4; and triiodothyronine, T3), which themselves regulate many metabolic pathways and strongly influence whole-animal metabolism [17]. Despite how important understanding the integration of endocrine responses to multiple stressors is to ecology and conservation, little is known about how HPA axis function and associated metabolic responses vary with life history stages in most wildlife systems. Understanding natural life-history variation in HPA axis activity and responsiveness is critical to providing a context for interpretation of baseline glucocorticoid levels [18].

Although there have been numerous studies of stress on terrestrial mammals [19, 20], few studies have looked at how the HPA axis integrates stressors in wild marine mammals. Marine mammals are difficult to observe and study due to their pelagic lifestyle. Nevertheless, these animals face a number of anthropogenic stressors, such as pollution, anthropogenic noise, declining prey stocks, and boating/fisheries interactions [21-24]. Current information on the impacts of stressors to the HPA axis in marine mammals is sparse and variable across species [25-27]. Work with captive or rehabilitated Stellar sea lions and harbor seals has addressed relationships between acute

stressors and the function of the HPA axis as reflected in fecal and serum corticosteroids [28-30]. Interestingly, while aldosterone, a key hormone in electrolyte balance, is not viewed as a major stress hormone in terrestrial mammals, it has been linked to the stress response in bottlenose dolphins (*Tursiops truncatus*) and harbor seals (*Phoca vitulina*) and has been hypothesized as a key stress hormone in marine mammals [28, 31, 32]. Levels of cortisol have been related to fasting durations, lactation, molting and breeding behavior in some phocid species [33-37]; however, data on how HPA responsiveness varies with fasting and reproductive state in the wild are limited. The combination of food abstinence and reproductive effort is a natural and predictable stressor in pinnipeds, yet little is known about the physiological response to the reproductive fasts as reflected in hormonal stress markers.

Adult male northern elephant seals (NES) provide an ideal system to investigate the alteration in HPA axis function and its metabolic impacts. Male elephant seals have the highest fasting energy expenditure, the longest fasting duration, and exhibit dramatic increases in reproductive effort relative to body size when compared to other phocids [38]. The three month breeding fast is energetically costly and incorporates terrestrial movement and combative male-male competition to establish access to females. During this time, body mass decreases by approximately 40% in dominant males and 30% in subordinate males [39, 40]. Dominant males experience higher mating success, but at the cost of increased energy expenditure, with successful males having sustained fasting energy expenditures approaching 4 times their standard metabolic rates [38]. Males return to sea after breeding and then later undergo a second month-long haulout, during which they undergo a catastrophic molt of their pelage [41]. This also occurs while fasting and

presumably incurs additional metabolic costs associated with tissue turnover and pelage synthesis.

Elephant seals lack a cortisol response to handling when chemically sedated, which allows measurement of basal values in natural, field conditions [42]. In marked contrast to conspecific females, cortisol remains stable across breeding in males, although highly variable among individuals [43]. This suggests that potential lipolytic benefits of elevations in cortisol [44] are secondary to other impacts on metabolism, including protein sparing. In contrast, aldosterone concentrations increased across the fast [45], which may support the hypothesis that aldosterone is an important factor in the stress response (in this case, prolonged nutrient deprivation).

Here we report changes in adrenal, thyroid and sex hormones, as well as key metabolites, in response to an ACTH challenge in breeding and molting adult male northern elephant seals. We measured acute effects for 2 hrs and then sampled again at 48 hrs to identify impacts of sustained ACTH stimulation. The ACTH challenge was performed to examine the responsiveness of the HPA axis and its effects on metabolism under the combined natural and predictable stressors of prolonged food and water abstinence, breeding activity and combative intrasexual competition, and the physiological constraints of molting. Our objectives were to determine hormonal markers of the stress response and how body condition and life-history stage relate to variability in the responsiveness of the HPA axis, knowledge which is necessary for understanding the potential impact of additional stressors on the seals, particularly those of anthropogenic origin.

Methods

Study site and study animals

All animal handling procedures were approved by the Sonoma State University Institutional Animal Care and Use Committee and work was conducted under National Marine Fisheries Services marine mammal permit #14636. Fourteen adult male northern elephant seals were studied during the breeding and molting season at Año Nuevo State Reserve. The molting period was examined in order to observe alterations in hormones and metabolites outside of the breeding season. Animals were considered adult based on body mass and development of secondary sexual characteristics [39]. If not already present, animals were marked with rear flipper tags (Dalton jumbo Roto-tags, Oxon, England) and hair dye (Lady Clairol, Stamford, CT, USA).

Field procedures

Males were sampled at three stages: in early January (Early Breeding), in late February or early March (Late Breeding), and mid-July (Mid Molt). Six males were sampled during each stage. Due to availability at the study site, 4 males were represented in both the early and late breeding samples. Each male was sedated with an intramuscular injection of $\sim 0.3 \text{ mg kg}^{-1}$ of tiletamine HCl and zolazepam HCl (Telazol, Fort Dodge Animal Health, Fort Dodge, IA). Immobilization was maintained with bolus, intravenous injections of ketamine HCl (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA). Blood samples were obtained from the extradural vein via an 18 gauge spinal needle and collected into chilled serum, sodium heparin, and potassium EDTA blood tubes. Samples were placed on ice until returned to the lab. After initial blood samples (pre)

were collected, mass was visually estimated and the animal was given an intramuscular injection of 0.152 ± 0.003 IU kg⁻¹ corticotrophin LA gel (Westwood Pharmacy, Richmond, VA). ACTH dosages for the study were chosen based on pilot studies on weaned pups. Blood samples were then taken every 30 minutes until 2 hrs post-ACTH injection. Approximately 48 hrs later (51.1 ± 0.4 hrs), animals were again immobilized and blood sampled. We were not able to obtain 48 hr samples during the molt due to male movement and density. Blood samples were centrifuged for 15 min (1,500g at 4°C) and the plasma collected and frozen at -80°C for later analysis. An ultrasound scanner was used to measure blubber thickness and combined with morphometrics to estimate body composition using the truncated cones method [46]. This method has been validated against isotopic measures of body composition and provides estimates of body mass that are within 5% of that obtained via direct measurement [47].

Hormone and metabolite analyses

Hormone concentrations were measured by radioimmunoassay or enzyme immunoassay using commercially available kits: cortisol, aldosterone, tT3, tT4 (Siemens, Washington, D.C., USA), ACTH, and rT3 (Alpco, Salem, NH, USA). All assays have been validated previously for use in elephant seals [48-50] except for rT3 and ACTH. ACTH and rT3 assays demonstrated parallelism of diluted samples to the standard curve and recovery of standard additions > 95%. All samples were analyzed in duplicate with an average coefficient of variation for ACTH of 5.2 % and the rest below 2%. The ACTH assay antibodies did not bind to the synthetic ACTH preparation allowing measurement of endogenous ACTH during the procedure.

Glucose and lactate were measured in duplicate using a YSI 2300 STAT plus auto-analyzer (YSI, Yellow Springs, OH, USA). Non-esterified fatty acids (NEFA; Wako Diagnostics, Richmond, VA, USA), blood urea nitrogen (BUN; Stanbio, Boerne, TX, USA), and beta-hydroxybutyrate (β -HBA; Cayman Chemical Company, Ann Arbor, MI, USA) were analyzed in duplicate using enzymatic colorimetric assays. Sodium and potassium were analyzed using Na⁺ and K⁺ perfectION combination electrodes (Mettler Toledo, Columbus, OH, USA). Cortisol, aldosterone, ACTH, glucose and lactate were assayed for the full time series, while testosterone, tT3, rT3, tT4, BUN, β -HBA, NEFA, K⁺, and Na⁺ were assayed at 3 time points (pre, 120 min, 48 hr).

Data analysis

Linear mixed models with a repeated measures covariance structure and seal as a random effect were used to assess significant changes from baseline across the 120 min time series (JMP Pro 11). This approach was used for cortisol, aldosterone, ACTH, glucose, and lactate. Changes in those hormones and metabolites over the 2 hr post-injection period were also assessed as area under the curve above baseline (AUC), using the trapezoid rule in Prism 5 (Graphpad Software, San Diego, CA). AUC values were compared using linear mixed models to observe how the magnitude of the response varied across different life history stages and to identify any effects of mass, adiposity, and ACTH dose. For the remaining hormones and metabolites, a general linear model (GLM) was used to assess differences between the pre, 120 min (acute), and 48 hr (sustained) time points within sampling periods. Mixed model linear regressions were used to assess relationships between AUC's. R² for mixed models were calculated and regressions were visualized using the slope and intercept parameters from the mixed

model [51]. Post-hoc differences between life history stages and time points were evaluated using Tukey's corrected differences in least square means. All data are expressed \pm standard error of the mean (SEM). Results were considered significant at $p < 0.05$.

Results

Mass, adipose tissue proportions, and ACTH dose are shown in Table 1.

Adiposity and ACTH dose had no significant effect on any of the responses and were removed from the models. Mass had a negative effect on the acute response of cortisol to the ACTH challenge ($F_{4,54.9} = 3.66$, $p = 0.007$), but did not affect any other response variables ($p > 0.05$).

Hormone response to ACTH

Cortisol concentrations increased relative to baseline by 60 min in all study groups (Figure 1A; $F_{4,54.9} = 9.67$, $p < 0.001$). The magnitude of cortisol release (AUC) was greater in EB males than at MM (Figure 2A; Table 2). When comparing acute and sustained responses, cortisol increased from base line at 120 min for all groups ($p < 0.05$) but remained elevated at 48 hrs for EB males (Figure 3A, $F_{2,15.7} = 11.67$, $p = 0.0009$), returning to baseline by 48 hrs in LB males.

Aldosterone increased from baseline in all study groups but the rate of increase varied (Figure 1B, $F_{4,56.3} = 3.08$, $p = 0.02$). The magnitude of the aldosterone response to ACTH (AUC) was significantly higher at LB than at MM and the response at EB was marginally significantly higher than MM (Figure 2C, Table 2). Aldosterone showed an acute response and was elevated at 120 min in all groups, with values returning to

baseline by 48 hrs (Figure 3B, Table 3, EB: $F_{2,15}=12.89$, $p<0.05$; LB: $F_{2,15}=11.12$, $p=0.0011$; MM: $F_{1,10}=40.83$, $p=0.0014$). There was a strong positive association between cortisol and aldosterone AUC (Figure 2E, $R^2=0.32$, $F_{2,15}=6.7$, $p=0.0197$).

Endogenous ACTH concentrations did not vary significantly from baseline across the time series for all the subjects. Testosterone had no acute alteration from baseline at 120 min, but showed a sustained response by being suppressed at 48 hrs in EB males (Figure 3F, $F_{2,15}=3.44$, $p=0.04$). In LB and MM, baseline testosterone was almost undetectable, and there was no response to the ACTH challenge.

Both tT3 and rT3 had varying responses to the challenge, while tT4 remained constant at all of the time points (Figure 3C, 3D, 3E; Table 3). At 48 hours post-ACTH injection, tT3 decreased ($F_{2,15}=5.09$, $p=0.02$) and rT3 increased ($F_{2,15}=28$, $p=0.0001$) in EB males. MM males showed an acute positive thyroid response to ACTH stimulation with tT3 being elevated at 120 min (Figure 3C, Table 3 $F_{1,10}=11.7$, $p=0.02$). There was no impact of ACTH stimulation on tT4 at any time point in any group (Figure 3E).

Metabolite response to ACTH

Based on the time series, there were no consistent changes in glucose or lactate compared to baseline within sample groups (Figure 1C, 1D, $p>0.05$). However, when comparing AUCs, the magnitude of the total response for glucose was significantly higher during MM when compared to early breeding (Figure 2B, Table 2). There was a marginally significant elevation in lactate AUC in EB males compared to MM males (Figure 2D, Table 2). The ACTH challenge had an acute significant effect on both glucose and lactate during MM, with glucose increasing ($F_{1,10}=156.2$, $p<0.0001$, Figure

1C) and lactate decreasing ($F_{1,10}=48$, $p=0.0005$, Figure 1D) at 120 min. The ACTH challenge had a sustained significant effect on glucose and lactate during LB, with glucose increasing ($F_{2,15}=4.13$, $p=0.037$, Table 4) and lactate decreasing ($F_{2,15}=9.24$, $p=0.0024$). When AUC_{glucose} was regressed against AUC_{lactate} there was a strong negative relationship among individuals (Figure 2F, $R^2=0.67$, $F_{2,15}=33.44$, $p<0.0001$).

β -HBA and NEFA concentrations showed no response to the ACTH during breeding, but both significantly increased at 120 min in MM males (Table 4, $F_{1,10}=6.92$, $P=0.046$; $F_{1,10}=14.6$, $DF=5$, $p=0.012$). BUN, K^+ , and Na^+ showed no response to the challenge at any time point during the different stages (Table 4, $p>0.05$).

Discussion

Our data suggest that the HPA axis is highly responsive to ACTH stimulation across life history stages in adult male NES. ACTH dosages causing strong release of cortisol and aldosterone were an order of magnitude lower than that required for similar responses in other pinnipeds [29, 52]. The strong sensitivity of adult male NES to ACTH contrasts the lack of cortisol response evident in response to chemical immobilization [42]. However, indirect impacts of ACTH stimulation on non-adrenal hormones and metabolites differed strongly between sampling groups. ACTH stimulation affected thyroid and sex hormones, as well as the metabolites, glucose, lactate, β -HBA, and NEFA in some, but not all life history stages. This variability in response suggests that tissue responses to cortisol are dependent on reproductive state and the length of the fasting period.

The level of negative feedback in the cortisol response to sustained ACTH stimulation varied with the duration of the breeding fast. The sustained response at 48 hrs in early breeding suggests weak negative feedback regulation of adrenal sensitivity to ACTH relative to that observed late in breeding. Similarly, the total cortisol response to the challenge was higher in early breeding when compared to molting. These changes may reflect the ability to enact adaptive stress responses to sustained combat and agonistic encounters early in breeding when establishment of the dominance hierarchy occurs [47]. Conversely, the reduced response and strong negative feedback evident during molting likely reflects the strong temporal regulation of cortisol during the catastrophic molt [53].

During breeding, the ACTH challenge had no significant effect on ketone, NEFA, or BUN levels. Unlike lactating females, which show a 3-fold increase in NEFA across lactation [35], males maintained consistent NEFA values across the breeding fast [54]. In contrast, we observed a modest but significant increase in NEFA and β -HBA during the molt, probably as a result of increased lipolysis and β -oxidation of fatty acids. Nevertheless, the increase was nominal and the lack of significant ketosis in fasting NES of all life-history stages may reflect a tight coupling of beta-oxidation and tricarboxylic acid cycle activity that limits accumulation of ketogenic precursors [44, 55, 56]. Lower rates of metabolism during the molt may have allowed slightly higher rates of ketogenesis from cortisol-enhanced lipolysis. The lack of typical effects of elevated cortisol on protein catabolism and blood glucose [57, 58] is consistent with a reduced role for cortisol stimulation of gluconeogenesis from amino acids in fasting NES [42]. This

feature may be critical to avoiding the loss of body protein in response to the combined stressors of breeding and fasting.

Elevations in glucose during the molt occurred 120 minutes following the administration of ACTH, consistent with a significant increase in cortisol. However, this was not observed in either of the breeding conditions. Complicating interpretations of the findings, a significant increase in glucose was observed 48 hours after the ACTH injection during the late breeding period, but cortisol had returned to baseline values at this time. The most parsimonious explanation to this finding is that cortisol exhibited gluconeogenic effects during late breeding and during the molt, but the time-course for action varied between the two conditions. It is plausible that elevations in glucose during late breeding might have occurred after the 120-min observation period, but that elevated glucose had not returned to baseline levels by the 48-hour measurement. Prior work in other age classes of elephant seals has demonstrated that the pancreatic release of insulin after a prolonged period of fasting is negligible and that there is an increase in insulin resistance to some tissues [59-61]. It is possible that the same trends in the insulin regulation of glucose exist in the adult males subjected to prolonged fasts. However, the lack of any change in glucose during early breeding is suggestive of a change in the responsiveness to elevations in cortisol with time fasting or changing body condition. Taken across all study subjects, the widely varying individual changes in blood glucose were closely and inversely associated with blood lactate (Figure 2F). Lactate is the primary gluconeogenic substrate in fasting, weaned NES pups [62] and reflects high rates of glucose recycling through the Cori cycle [55]. The lack of alteration in glucose/lactate in males may reflect the tight coupling of pyruvate flux to glucose production in order to

maintain high rates of beta-oxidation without ketosis, as has been proposed for other NES age classes [44, 55, 56].

The strong impacts of ACTH on aldosterone in all groups supports the hypothesis that aldosterone is a hormone related to the adaptive stress response in marine mammals [31, 32]. ACTH is known to increase aldosterone secretion by a renin-angiotensin system (RAS)-independent pathway [63]. Angiotensin II can induce peripheral vasoconstriction [64-66], which may be beneficial to diving marine mammals experiencing acute stressors, but which may also result in an increase in aldosterone production. While increases in aldosterone can be beneficial in a fasting state due to decreased efflux of water and reabsorption of Na^+ , it could also be a detrimental response in a hyperosmotic environment such as the ocean [45, 67, 68]. Thus, it may not be surprising that the aldosterone response showed strong negative feedback in all breeding groups suggesting a reduced response to sustained stress. Aldosterone response to ACTH was highest during late breeding when baseline aldosterone levels are highest. This suggests that pituitary stimulation may contribute independently to the strong upregulation of the RAS system seen across fasting [45]. The elevation of aldosterone had no significant effect on the electrolytes Na^+ and K^+ . However, since elephant seals have a low urinary output [69] and the elevation of aldosterone was not sustained, there was likely insufficient time for elevated aldosterone to affect electrolyte concentrations.

Testosterone, while high early in breeding, showed a significant decrease in response to the ACTH challenge at 48 hours post-injection. The time course of the response suggests that there is potentially a negative impact on sex hormone concentrations when cortisol is maintained at high concentrations. This finding, while not

unique [70-72], shows a potential mechanism by which sustained stress could be detrimental to male reproductive effort. It is widely accepted that testosterone plays a major role in aggression related to breeding and the establishment of territorial boundaries or challenging conspecifics for access to mates [73-76]. If the putative relationship between cortisol and testosterone holds true, then sustained elevation in cortisol early in the season may reduce reproductive effort and success through alterations in testosterone-mediated behaviors.

Similar to testosterone, thyroid hormones were only impacted by sustained ACTH stimulation early in breeding. Most thyroid hormone is released from the thyroid gland as T4, which was not affected by the ACTH challenge. At target tissues, deiodinases (D1 and D2) convert T4 into the more biologically active T3 [77]. A third deiodinase (D3) inactivates T3 but also convert T4 into an inactive form, rT3 [77]. The rT3 binds to T3 receptors without up-regulating gene expression, thus blocking most thyroid hormone action. Many stressors decrease D1 activity, including food limitation, illness, and high cortisol and catecholamine levels [78, 79]. The inverse pattern in tT3 and rT3 seen in the 48 hr sample is consistent with alterations in deiodonase activity. In adult male NES, daily energy expenditure is strongly associated with mating success with alpha males exhibiting higher rates of mass controlled energy expenditure [38]. In addition, the ability of breeding males to elevate tT3 over the breeding fast is associated with high rates of energy expenditure but reduced protein sparing [54]. Thus, elevations in ACTH in response to sustained stressors early in breeding may influence reproductive success by reducing effort via reductions in thyroid hormone.

Conclusion

Adrenal sensitivity to ACTH is maintained across breeding and molting in adult male NES. However, metabolic impacts of ACTH and corticosteroid release varied with life history stage. ACTH was a strong secretagogue for aldosterone in NES at all life history stages. The impacts of the ACTH stimulation on testosterone and thyroid hormones suggest that elevations in cortisol during early breeding could negatively impact reproductive effort and success. ACTH, presumably through elevation of cortisol, caused acute impacts on fatty acid and carbohydrate metabolites during molting that were not evident during breeding, suggesting a down-regulation of adipocyte and hepatic responses to cortisol during extended high-energy breeding fasts. These data suggest that metabolic adaptations to extended fasting include significant alteration in tissue responses to regulatory hormones that minimize deleterious impacts of acute or moderately sustained stress responses. Such information is critical to understanding not only how life history events are physiologically managed within the predictive scope of the stress response, but in interpreting physiological indicators of stress in animals subject to anthropogenic stressors. Without baseline information on the natural variability of stress markers within a species, it can be difficult or impossible to differentiate the relative influence of the compounding effects of natural and predictable stressors with those of human origin.

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Table 1. Mass, adiposity, or ACTH dose given to fasting adult male northern elephants seals. Mass and adiposity were estimated using truncated cones method and ultrasound measurements of blubber depth.

	EB	LB	MM
Mass (kg)	1813 ± 255	1198 ± 170	1435 ± 60
Adiposity (%)	32.6 ± 1.7	22.6 ± 1.4	33 ± 3.3
ACTH Dose (IU kg ⁻¹)	0.147 ± 0.02	0.150 ± 0.01	0.158 ± 0.01

Table 2. Area under the curve (AUC) values for responses to intra-muscular ACTH challenge in fasting adult male northern elephant seals. EB= early breeding, LB= late breeding, MM= mid-molt. Different superscripts denote significant differences between groups (p<0.05) based on post-hoc comparisons of means from the ANOVA.

	EB	LB	MM	ANOVA Stats
Cortisol (ug dL ⁻¹ min)	1439 ± 597 ¹	1192 ± 376 ^{1,2}	820 ± 317 ²	F _{3,14} =6.306, p=0.01*
Aldosterone (ng mL ⁻¹ min)	26.6 ± 1.9 ^{1,2}	35.2 ± 4.7 ¹	15.8 ± 9.9 ²	F _{3,14} =4.703, p=0.03*
ACTH (pg mL ⁻¹ min)	430.7 ± 896.3	11.84 ± 319.6	604.9 ± 511.5	F _{3,14} =0.4102, p=0.86
Glucose (mM min)	-10.45 ± 51.81 ¹	-7.05 ± 62.33 ^{1,2}	61.475 ± 31.65 ²	F _{3,14} =4.683, p=0.03*
Lactate (mM min)	40.03 ± 56.99	4.38 ± 83.34	-49.74 ± 26.40	F _{3,14} =3.472, p=0.06

Table 3. Baseline hormone values and values for time after the injection of ACTH in fasting adult male elephant seals. EB=early breeding, LB= late breeding, MM= mid-molt. * denotes significant differences from baseline ($p < 0.05$) based on post-hoc comparisons of least means squares from the GLM.

	EB			LB			MM	
	0 min	120 min	48 hr	0 min	120 min	48 hrs	0 min	120 min
Cort ($\mu\text{g dL}^{-1}$)	7.3 \pm 1.37	31.1 \pm 11.6*	29.2 \pm 11.5*	7.47 \pm 3.9	29.2 \pm 3.8*	7.1 \pm 2.8	2.9 \pm 0.8	16.8 \pm 5.6*
Aldo (pg dL^{-1})	76 \pm 108	600 \pm 345*	82 \pm 137	334 \pm 347	991 \pm 442*	335 \pm 241	55.6 \pm 56.8	363 \pm 121*
ACTH (pg mL^{-1})	20.3 \pm 7.6	16.4 \pm 6.9	11.2 \pm 5.7	13.5 \pm 4.1	9.4 \pm 3.5	9.0 \pm 3.9	15.6 \pm 3.8	21.1 \pm 6.3
Test (ng dL^{-1})	631 \pm 260	509 \pm 306	251 \pm 153*	39 \pm 62	31 \pm 46	4 \pm 0	7.2 \pm 7.9	12.9 \pm 46.2
TT4 ($\mu\text{g dL}^{-1}$)	3.83 \pm 0.95	3.91 \pm 0.84	3.21 \pm 0.91	3.37 \pm 0.96	3.48 \pm 0.98	3.28 \pm 0.98	3.12 \pm 0.40	3.37 \pm 0.15
TT3 (ng dL^{-1})	57.0 \pm 11.8	60.3 \pm 12.3	40.9 \pm 9.5*	48.9 \pm 9.0	53.6 \pm 13.3	48.8 \pm 17.9	75.1 \pm 7.8	81.2 \pm 10.2
rT3 (ng dL^{-1})	1.22 \pm 0.07	1.25 \pm 0.09	1.9 \pm 0.29*	1.15 \pm 0.34	1.16 \pm 0.3	1.21 \pm 0.23	0.69 \pm 0.23	0.71 \pm 0.25

Table 4. Baseline metabolite values and values for time after the injection of ACTH in fasting adult male elephant seals. EB=early breeding, LB= late breeding, MM= mid-molt. * denotes significant differences from baseline ($p<0.05$) based on post-hoc comparisons of least means squares from the ANOVA.

	EB			LB			MM	
	0 min	120 min	48 hrs	0 min	120 min	48 hrs	0 min	120 min
Glucose (mM)	7.62 ± 0.37	7.78 ± 0.57	7.05 ± 0.88	6.83 ± 0.89	6.79 ± 0.74	7.82 ± 0.36*	5.72 ± 0.64	6.85 ± 0.7*
Lactate (mM)	1.61 ± 0.65	1.82 ± 0.52	2.05 ± 1.1	3.09 ± 0.85	3.29 ± 0.63	1.7 ± 0.58*	4.63 ± 0.64	3.71 ± 0.8*
B-HBA (mM)	0.33 ± 0.09	0.34 ± 0.1	0.42 ± 0.09	0.44 ± 0.06	0.42 ± 0.06	0.42 ± 0.07	0.12 ± 0.02	0.14 ± 0.01*
NEFA (mM)	0.80 ± 0.23	1.02 ± 0.14	0.98 ± 0.21	0.73 ± 0.24	0.90 ± 0.33	0.60 ± 0.30	0.78 ± 0.07	0.95 ± 0.12*
BUN (mM)	14.1 ± 3.1	15.2 ± 3.5	13.5 ± 3.1	16.0 ± 3.2	18.4 ± 3.6	22.5 ± 6.4	39.9 ± 2.5	41.1 ± 2.8
K ⁺ (mM)	4.34 ± 0.40	4.32 ± 0.15	4.26 ± 0.13	3.59 ± 0.26	3.65 ± 0.41	3.64 ± 0.11	4.3 ± 0.26	3.64 ± 0.11
Na ⁺ (mM)	138.7 ± 2.9	138.7 ± 2.3	139.1 ± 1.4	139.3 ± 1.9	139.4 ± 3.1	139 ± 2.1	140.8 ± 2.3	140.1 ± 2.3

Figure 1. Time series for changes in cortisol (A), aldosterone (B), glucose (C) and lactate (D) concentrations in response to intramuscular ACTH injection in adult male NES. Time 0 represents pre-injection. Closed circle represent early breeding, open circle represents late breeding, and closed triangle represents mid-molt

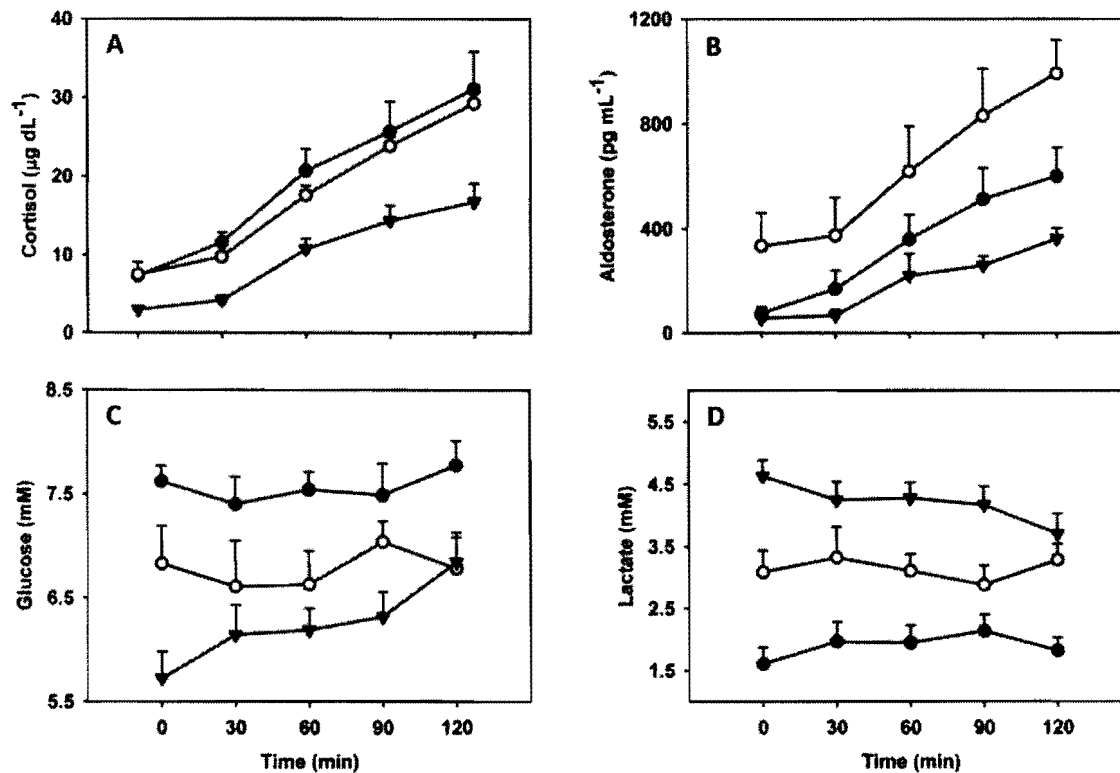


Figure 2. Comparison of area under the curve (AUC) for A: Cortisol, B: Glucose, C: Aldosterone, and D: lactate compared to baseline following ACTH administration in NES. Different numbers denote significant differences between groups ($p < 0.05$) based on post hoc comparisons of least square means from the ANOVA. Baseline is denoted by 0. EB= early breeding, LB= late breeding, MM= mid-molt. E: Relationship between AUC for cortisol and aldosterone in response to an ACTH challenge in male NES. Fitted line is based on the mixed model parameter estimates : $y = 17.6x + 6318$, $R^2 = 0.32$, $F_{1,16} = 6.7$, $p = 0.02$. F: Relationship between AUC for glucose and lactate in response to an ACTH challenge in male NES. Fitted line is based on mixed model parameter estimates : $y = -0.98x + 11.19$, $R^2 = 0.67$, $F_{1,16} = 33.4$, $p = 0.0001$. Closed circle represent early breeding, open circle represents late breeding, and closed triangle represents mid-molt.

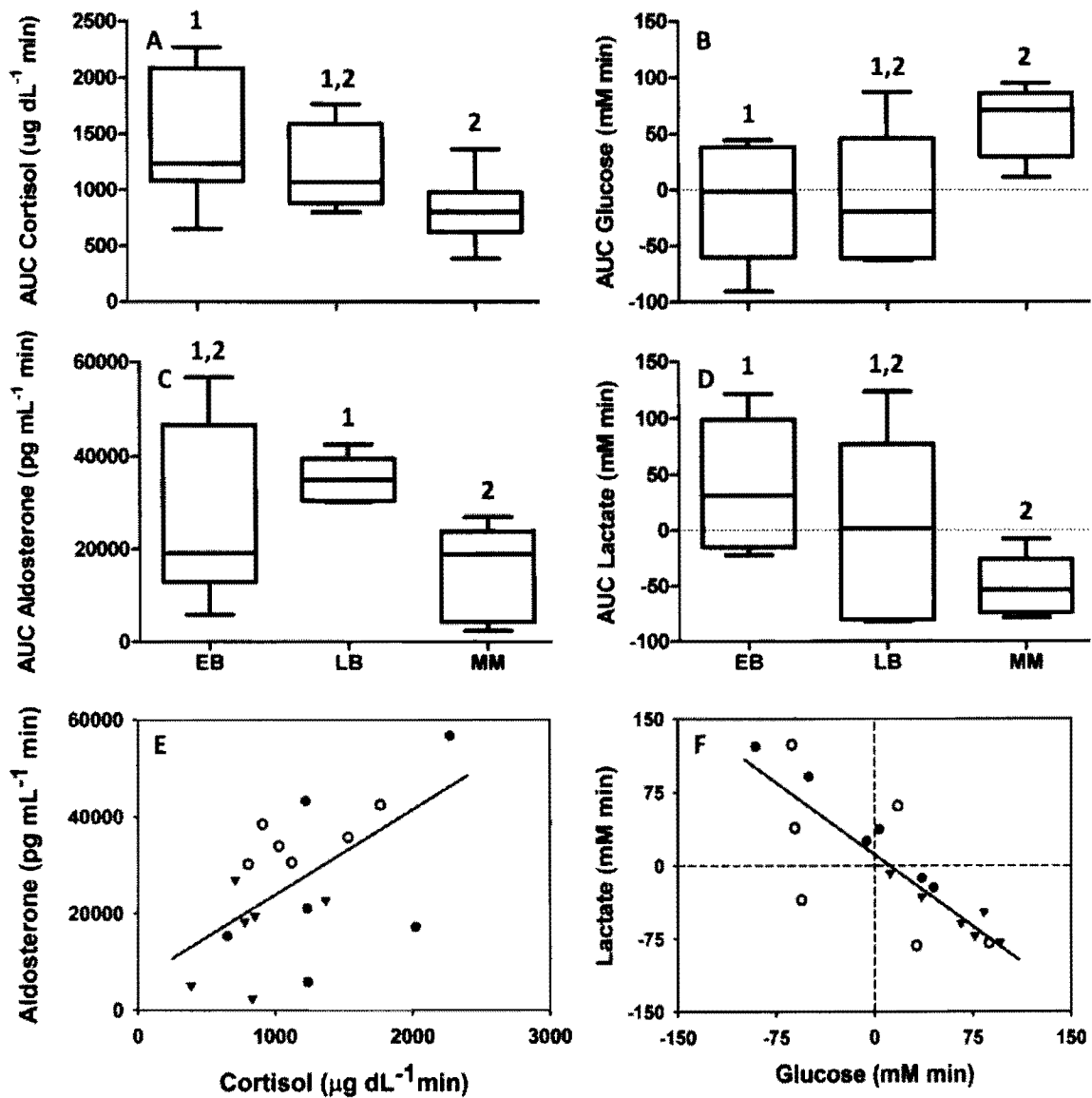


Figure 3. Comparison of pre-ACTH, 2 hr, and 48 hr values of A: Cortisol, B: Aldosterone, C: Total triiodothyronine (tT3) D: reverse triiodothyronine (rT3), E: Total thyroxine, and F: Testosterone in adult male NES in response to an ACTH challenge. * denotes significant difference from pre-ACTH concentrations ($p < 0.05$) based on post hoc comparisons of least square means from the LMM. Black bars represent early breeding, light gray bars represent late breeding, and dark grey bars represent mid-molt.

