ALL ANIMALS EXPERIENCE STRESS at some point in their life histories, which elicits a conserved physiological response that enables organisms to focus their resources on coping with immediate challenges (53). The vertebrate stress response is mediated by activation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in secretion of glucocorticoid (GC) hormones. GCs bind to intracellular receptors [glucocorticoid receptors (GR), and mineralocorticoid receptors (MR)] in target tissues such as liver, adipose, and muscle. Once bound with their ligand, these receptors alter the expression of genes that regulate numerous functions including metabolism and growth (51). These transcriptional changes ultimately result in liberation of energy stores and suppression of energetically costly processes such as reproduction and inflammation. Finely tuned negative feedback mechanisms terminate the stress response after the challenge has passed (51). Repeated or sustained stressors, however, can result in chronic activation of the stress axis and may deplete physiological resources and impair an organism’s ability to respond appropriately to subsequent stressors (52). This can significantly affect the health of human and animal populations alike and presents significant public health and wildlife conservation challenges.

Much of our current understanding of vertebrate stress physiology derives from biomedical research in humans and laboratory animals in a pathological context (51). Animals in nature, however, may not experience or respond to stressors in the same manner as laboratory species (4). Many organisms are routinely exposed to stressors in their environments, such as predation, food shortages, intraspecific conflicts, and reproductive challenges, among others. If a free-living animal is to survive and reproduce in an unpredictable environment, its reactivity to acute perturbations must be modulated to avoid long-term, deleterious consequences (4). However, little is known about how such responses are regulated at cellular levels in nonlaboratory species, which have only been profiled in a handful of studies (33, 37). This hinders our ability to understand the molecular mechanisms underlying development and pathology of maladaptive, chronic stress in animals, a key research focus in emerging fields such as conservation physiology (10).

Phocid seals such as the northern elephant seal (Mirounga angustirostris) present an ideal free-living vertebrate system for stress studies in an environmental context. As part of their natural life history, elephant seals routinely experience extreme physiological challenges associated with prolonged fasting coupled with energetically demanding life history pressures of breeding, molting, and neonatal development (6, 11, 12, 59). Due to the amenability of this species to research handling and endocrine manipulation (7), much is known about elephant seals’ metabolic adaptations (6, 11). Circulating cortisol concentrations increase markedly during fasting in this species (8, 45) but do not result in the typical adverse consequences, such as increased protein catabolism (13, 23) or immunosuppression (46) seen in laboratory animals under similar circumstances. At the same time, fasting seals retain the capacity to elevate adrenocortical hormone levels in response to experimental HPA axis stimulation (9, 17). This suggests that fasting-adapted animals may possess characteristics that mitigate deleterious effects of elevated GCs while maintaining HPA axis sensitivity to acute perturbation.

Khudyakov JI, Champagne CD, Preeyanon L, Ortiz RM, Crocker DE. Muscle transcriptome response to ACTH administration in a free-ranging marine mammal. Physiol Genomics 47: 318–330, 2015. First published June 2, 2015; doi:10.1152/physiolgenomics.00030.2015.—While much of our understanding of stress physiology is derived from biomedical studies, little is known about the downstream molecular consequences of adaptive stress responses in free-living animals. We examined molecular effectors of the stress hormones cortisol and aldosterone in the northern elephant seal, a free-ranging study system in which extreme physiological challenges and cortisol fluctuations are a routine part of life history. We stimulated the neuroendocrine stress axis by administering exogenous adrenocorticotropic hormone (ACTH) and examined the resultant effects by measuring corticosteroid hormones, metabolites, and gene expression before, during, and following administration. ACTH induced an elevation in cortisol, aldosterone, glucose, and fatty acids within 2 h, with complete recovery observed within 24 h of administration. The global transcriptional response of elephant seal muscle to ACTH was evaluated by transcriptomics and involved upregulation of a highly coordinated network of conserved glucocorticoid (GC) target genes predicted to promote metabolic substrate availability without causing deleterious effects seen in laboratory animals. Transcriptional recovery from ACTH was characterized by downregulation of GC target genes and restoration of cell proliferation, metabolism, and tissue maintenance pathways within 24 h. Differentially expressed genes included several adipokines not previously described in muscle, reflecting unique metabolic physiology in fasting-adapted animals. This study represents one of the first transcriptome analyses of cellular responses to hypothalamic-pituitary-adrenal axis stimulation in a free-living marine mammal and suggests that compensatory, tissue-sparing mechanisms may enable marine mammals to maintain cortisol and aldosterone sensitivity while avoiding deleterious long-term consequences of stress.

ACTH; corticosteroid; transcriptome; muscle; seal

Address for reprint requests and other correspondence: J. I. Khudyakov, 1801 E. Cotati Ave., Rohnert Park, CA 94928 (e-mail: khudyako@sonoma.edu).
In response to HPA axis manipulation, downstream GC effectors (such as genes that suppress protein synthesis, glucose uptake, inflammation, and cell survival) can be identified by profiling global gene expression changes in target tissues using transcriptomics. Endogenous GC secretion can be stimulated by administering exogenous adrenocorticotropic hormone (exACTH), mimicking the downstream effects of HPA axis activation in response to stressors (9, 17, 18, 40). This manipulation, when performed under dissociative anesthesia in northern elephant seals, avoids artifacts of capture and handling stress (7). Administration of slow-release exACTH in juvenile and adult elephant seals has been shown to significantly elevate cortisol and aldosterone for 24–48 h. The mineralocorticoid aldosterone is released in response to a variety of stressors, including handling, physical, and thermoregulatory stress in marine mammals and is likely a key component of the stress response in this group (9, 17, 18, 24, 56–58).

We evaluated downstream responses to exACTH administration in juvenile elephant seals during a brief annual haul-out when they are not molting, breeding, or fasting extensively to remove confounds of life history stress (27). We describe the acute endocrine, metabolic, and transcriptional response and subsequent recovery of northern elephant seals following exACTH administration. Gene expression changes were profiled in skeletal muscle, a metabolically active GC target tissue that is easily accessible in marine mammals (29). We previously sequenced and assembled a reference transcriptome obtained from muscle samples collected during the exACTH experiment and conducted preliminary differential expression analysis (28). In this study, we report a detailed analysis of changes in gene expression between 1) baseline and acute response and 2) acute response and recovery conditions and relate them to other physiological measurements (hormones, metabolites) of the response to exACTH. We found that the cellular response of muscle to exACTH administration involved transient upregulation of a highly conserved and coordinated gene network that is similar mass (124.7 ± 8.4 kg) and apparent body condition were selected. Samples from three female animals were used for RNA-Seq (28) and quantitative real-time PCR (QPCR) validation and hormone and metabolite analyses. Samples from an additional two females and two males were used for hormone, metabolite, and QPCR analyses.

Pilot experiments were conducted to determine appropriate sampling times that would capture maximal acute response to endocrine manipulation in this age class of seals. Corticosteroid values were higher 2 h after exACTH administration than at earlier sampling times, consistent with similar experiments in molting juveniles that showed a maximal corticosteroid response within 2–2.5 h (9). We reasoned that 2 h would provide sufficient time to observe changes in gene expression in response to manipulation. Recovery was evaluated by decline of corticosteroids to baseline levels, which occurred within 24 h after exACTH administration and was maintained at 48 h. Field sampling between 2 and 24 h postinjection was not feasible. Therefore, markers were evaluated in three conditions: prior to exACTH administration (“baseline”), 2 h after exACTH administration (“acute response”), and ∼24 h after exACTH (“recovery”).

ACTH Administration and Sample Collection

Study animals were approached while sleeping at the rookery and were immobilized as previously described (27, 28). Briefly, seals were initially sedated with 1 mg/kg intramuscular injection of tiletamine-zolazepam (Telazol; Fort Dodge Laboratories, Fort Dodge, IA). Sedation was maintained with periodic intravenous doses of ketamine and diazepam (Fort Dodge Laboratories). This sedation procedure does not cause an increase in circulating cortisol concentration and thus appears to alleviate any stress response due to animal handling in elephant seals (7). Baseline blood samples were obtained via an 18 G 3.25-inch needle from the extradural vein (3) within 22.1 ± 7.4 min of initial Telazol injection. All blood samples were collected into chilled serum and EDTA-treated plasma tubes and stored on ice until return to the laboratory. After subcutaneous injection with 1 ml lidocaine, samples of the left external abdominal oblique muscle were collected via a 6.0 mm diameter biopsy punch (Miltex, York, PA) and immediately frozen in liquid nitrogen.

After baseline sample collection, each animal received an intramuscular injection of 0.23 ± 0.02 U/kg corticotropin LA gel (ex-ACTH; Wedgewood Pharmacy, Richmond, VA) on the left side, ~3 cm anterior to the initial biopsy site. This exACTH preparation is a synthetically produced bioactive peptide containing the first 24 amino acids of ACTH (aa 1–24) in a proprietary viscous gel matrix to provide extended release after intramuscular injection (Wedgewood Pharmacy). Sedation was maintained for 2 h, and a second set of blood and tissue samples (on the right, contralateral side of the animal) were collected 2 h after exACTH administration to capture the acute response. Animals were weighed (MSI tension dynamometer, Seattle, WA), individually marked with rear flipper tags (Dalton Jumbo Roto-tags, Oxon, UK) and black hair dye (Lady Clairol, Stamford, CT) and released to resume normal activity. Study subjects were resighted and immobilized again the following day, 22.3 ± 2.0 h after initial exACTH injection. A set of recovery blood and tissue samples was collected within 16.8 ± 4.4 min of Telazol injection as described above. Muscle tissue was obtained from the right side of each animal at least 3 cm away from the acute biopsy site. We were unable to relocate and collect recovery samples for one of the seven study animals.

Hormone and Metabolite Assays

Blood samples were centrifuged at 5,000 rpm for 15 min at 4°C. Isolated plasma and serum were stored at −80°C. Hormone concentrations were measured by radioimmunoassay or enzyme immunoassay with commercially available kits previously validated for use in elephant seals: cortisol, aldosterone (Siemens, Washington, DC), and insulin (EMD Millipore, St. Charles, MO) (17). All samples were analyzed in duplicate with intra-assay coefficient of variation ≤5%.

Glucose and lactate were measured using YSI 2300 STAT plus autoanalyzer (YSI, Yellow Springs, OH). Free fatty acids (FFA;
Cayng1, Ann Arbor, MI) and blood urea nitrogen (BUN; Stanbio, Boerne, TX) were analyzed with enzymatic fluorometric and colorimetric assays, respectively. All samples were analyzed in duplicate with intra-assay coefficient of variation ≤ 5%.

RNA Isolation

Tissue samples were stored at –80°C until extraction as previously described (28). In brief, 75–165 mg of muscle tissue was minced with a scalpel in a sterile glass dish on ice, transferred to a glass tissue grinder (Kimble-Chase Kontes Duall, Vineland, NJ), and homogenized with 1 ml of TRIzol Reagent (Ambion, Life Technologies, Grand Island, NY). RNA was extracted using chloroform, precipitated with isopropanol, and resuspended in 100 μl RNase-free water according to Ambion protocol. RNA samples were then applied to RNeasy columns and sample cleanup was conducted using RNeasy mini kit with a 30-min on-column DNase I digest (Qiagen, Germantown, MD) according to Qiagen protocol. RNA concentration was quantified on a Qubit fluorometer (Life Technologies, Grand Island, NY).

Transcriptome Analysis

RNA-Seq, reference transcriptome assembly and annotation, and preliminary differential expression analysis were described in a previous study (28). Briefly, paired-end 100 base-pair sequencing was conducted on the Illumina HiSeq 2500 platform. The reference transcriptome was assembled de novo using Trinity assembler (19). Transcripts were mapped to the assembly using bowtie read aligner (32). Transcript abundance was calculated using RSEM (35), and differentially expressed transcripts were identified using EBSeq (34).

Here, we assessed all differentially expressed transcripts and explored potential relationships with other physiological metrics (i.e., hormone and metabolite concentrations). We used the DAVID functional annotation tool (25) to identify KEGG and BioCarta metabolic pathways enriched in each differentially expressed gene.

Table 1. Primers used for QPCR validation of differentially expressed genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tr ID</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>E</th>
<th>Mean FC</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccdp</td>
<td>tr398922</td>
<td>F: ATGCAGTCCAAGGGCCATGACTG</td>
<td>1.97</td>
<td>4.32 ± 1.84</td>
<td>2h0h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGGTGCTGCTTGGAAAGGG</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ddit4</td>
<td>tr404230</td>
<td>F: TGGTAATGCCCTTCCCTGTTG</td>
<td>1.85</td>
<td>2.76 ± 1.36</td>
<td>2h0h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AACGGAACAAACGCTTGAAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tob2</td>
<td>tr660220</td>
<td>F: TGACATGCTTATGCTTGGG</td>
<td>1.71</td>
<td>1.98 ± 0.77</td>
<td>2h0h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGGCCCTCTGACTTTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klf15</td>
<td>tr28582</td>
<td>F: TGGGAAATGGTCGTTGGAG</td>
<td>1.72</td>
<td>1.53 ± 0.50</td>
<td>2h0h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGGTGCTGGAACCGGATAGCG</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Zfp36</td>
<td>tr644672</td>
<td>F: TCCAAGCGCATCTCTGTGTT</td>
<td>1.62</td>
<td>1.25 ± 0.29</td>
<td>2h0h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGGACGAGCCAGGAGATTTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medag</td>
<td>tr95009</td>
<td>F: TGGACCAAGGGTATCTGGT</td>
<td>1.90</td>
<td>3.68 ± 2.31</td>
<td>2h4/2h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AAATGTGCTGCTACGTG</td>
<td></td>
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<tr>
<td>Rbp4</td>
<td>tr233357</td>
<td>F: TCTTCCACGAGTCCACTG</td>
<td>1.80</td>
<td>3.10 ± 1.26</td>
<td>2h4/2h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ACTGGATCTTATGTGGG</td>
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<td>Trim62</td>
<td>tr461529</td>
<td>F: ATTTCCTGTCTGTGCTCAGT</td>
<td>1.75</td>
<td>0.92 ± 0.48</td>
<td>2h4/2h</td>
</tr>
<tr>
<td></td>
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<td>R: CAGGCGAACGTTGCTTGAAC</td>
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<td></td>
</tr>
<tr>
<td>Asb15</td>
<td>tr419155</td>
<td>F: AGAGGCCGAGAAATTTCACTG</td>
<td>1.71</td>
<td>0.73 ± 0.20</td>
<td>2h4/2h</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>EF2</td>
<td>tr388769</td>
<td>F: TGCCGTTATTTTTATG</td>
<td>1.67</td>
<td>0.95 ± 0.40</td>
<td>2h4/2h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCGCTGCTGCTTGGTGGC</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ccng1</td>
<td>tr141230</td>
<td>F: TGGACAGATATGGACTGGG</td>
<td>1.66</td>
<td>0.99 ± 0.36</td>
<td>2h0h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ATGGTCTGATACGGTTGGC</td>
<td></td>
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<td></td>
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<tr>
<td>Gapdh*</td>
<td>tr223210</td>
<td>F: GGAAATCTGCGCCGTGATGG</td>
<td>1.78</td>
<td>1.07 ± 0.18</td>
<td>2h0h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCGCTGCTGACGCTTGGG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

QPCR, quantitative PCR; Tr ID, transcript ID; E, primer efficiency; Mean FC, mean fold change in gene expression between conditions noted in “Comparison” (n = 6). *Gapdh primer sequences were obtained from Ref. 39. F, forward; R, reverse; NC, no change in expression.
Data analyses were conducted using JMP 11 (SAS, Cary, NC). Changes in hormone and metabolite concentrations in response to exACTH were analyzed using linear mixed models with time as an ordinal fixed effect and animal ID as a random effect. Response variables were log-transformed as necessary to meet distribution and variance assumptions. Post hoc comparisons among repeated samples were conducted using Tukey’s honest significant difference test. The relationship between log2 fold-change in gene expression values obtained by RNA-Seq and those obtained by QPCR was evaluated by Pearson correlation analysis.

RESULTS

Endocrine and Metabolic Response to exACTH

We evaluated the response of juvenile elephant seals to exACTH by comparing hormone (cortisol, aldosterone) and metabolite (glucose, lactate, FFA, BUN) measurements between three experimental conditions: 1) baseline, prior to exACTH administration, 2) acute response, 2 h after exACTH administration, and 3) recovery from exACTH, 24 h after administration. Baseline values of hormones in study animals were within the range reported in this species (17, 27) and did not appear to vary by sex. ExACTH administration induced a robust spike in cortisol and aldosterone within 2 h. Cortisol increased 11-fold ($F_{2,11} = 125.67, P < 0.0001$; Fig. 1A), while aldosterone increased 3.9-fold ($F_{2,11} = 27.55, P < 0.001$; Fig. 1B). Both hormones recovered to baseline values within 24 h. Insulin levels were not affected by exACTH ($F_{2,11} = 1.75, P = 0.22$).

Baseline metabolites were consistent with others reported in this species (22). Glucose was not altered at 2 h ($P > 0.05$) but was slightly elevated, by 12%, from baseline values 24 h after exACTH administration ($F_{2,11} = 6.76, P < 0.05$; Fig. 2A). Lactate decreased 32% within 2 h and remained suppressed at 24 h ($F_{2,11} = 8.09, P < 0.05$; Fig. 2B). FFA increased 37% within 2 h of exACTH administration but returned to baseline within 24 h ($F_{2,11} = 4.76, P < 0.05$; Fig. 2C). BUN was not altered at 2 h ($P > 0.05$) but decreased 30% from baseline values after 24 h ($F_{2,10} = 45.02, P < 0.0001$; Fig. 2D). Therefore, the peripheral response of juvenile elephant seals to exACTH administration involved an increase of circulating...
cortisol, aldosterone, glucose, and FFA with a concomitant decrease of lactate and BUN.

**Acute Transcriptome Response to exACTH**

Global gene expression changes in elephant seal muscle sampled during the exACTH administration were previously quantified by RNA-Seq (28). In this study, we conducted extensive annotation and analysis of all differentially expressed transcripts. Expression levels were compared between muscle samples collected at baseline and acute response conditions in three female juvenile seals. We identified 48 transcripts that were differentially expressed at FDR < 0.05 and false discovery rate (FDR) < 0.05 and expressed by at least 1 FPKM (fragments per kilobase of transcript per million mapped reads, Supplementary File 1).¹ Thirty-seven transcripts were upregulated at least 1.1-fold, of which 14 were upregulated by twofold or greater. These included several isoforms of the same genes (Cebpd, two isoforms; Tob2, three isoforms; Pdk4, three isoforms; Rab3gap1, two isoforms). Only 11 transcripts were suppressed during the acute response, of which four were downregulated by at least twofold. Two downregulated transcripts were isoforms of the same gene (Zpf36L1). Three significantly upregulated transcripts were not annotated due to lack of gene models in any mammalian system. Expression levels of five upregulated (Cebpd, Ddit4, Tob2, Klf15, Zfp36) and two unaltered (EF2, Cng1) transcripts were verified by QPCR in the same and three additional samples. Fold-change values quantified by the two methods were significantly correlated (Pearson r = 0.88, P < 0.001; Fig. 4, Table 1).

We mapped mouse homologs of differentially expressed seal genes to signaling and metabolic pathways using DAVID functional annotation tool (25). Few pathways were significantly enriched at P < 0.1 relative to the entire mouse proteome due to small size of the query dataset. The only KEGG pathway overrepresented in the differentially expressed gene set was Type II diabetes mellitus, while the sole enriched Biocarta pathway was ATM signaling pathway (Table 2). To predict functional interactions between cortisol, aldosterone, and differentially expressed genes identified in our dataset, we conducted network analysis using the hormone receptors GR and MR and 32 differentially expressed genes as input using Genemania network prediction tool (60). Figure 3A depicts connections between human homologs of these 34 genes based on existing coexpression data, with a maximum of 10 related genes and attributes displayed. Most genes (nodes) altered during the acute response to elevated corticosteroids are likely to be functionally related based on coexpression data (edges), with each node connected to a minimum of three edges in the network (Fig. 3A).

Table 2. KEGG and Biocarta pathways overrepresented during the acute response to exACTH and recovery from exACTH

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute response to exACTH</td>
<td>ATM signaling†</td>
<td>Gadd45α</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IkBα</td>
</tr>
<tr>
<td></td>
<td>Type II diabetes mellitus*</td>
<td>Abcc8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kcnj11</td>
</tr>
<tr>
<td>Recovery from exACTH</td>
<td>NF-κB activation by nontypeable H. influenzae†</td>
<td>IKKα</td>
</tr>
<tr>
<td></td>
<td>Signal transduction through IL1R†</td>
<td>Mkk6</td>
</tr>
<tr>
<td></td>
<td>TNF/stress related signaling†</td>
<td>IκBα</td>
</tr>
<tr>
<td></td>
<td>Keratinocyte differentiation†</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Toll-like receptor signaling*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MAPK signaling*</td>
<td>IκKα</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dusp10</td>
</tr>
<tr>
<td></td>
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<td>Gadd45α</td>
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<td>Mkk6</td>
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<tr>
<td></td>
<td></td>
<td>Pdgfrβ</td>
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<td>p53 signaling*</td>
<td>Gadd45α</td>
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</tr>
<tr>
<td></td>
<td>Igbp3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sesn1</td>
<td></td>
</tr>
<tr>
<td>Small cell lung cancer*</td>
<td>IκKα</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>Lama3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IκBα</td>
<td></td>
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<tr>
<td>Prostate cancer*</td>
<td>IκKα</td>
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</tr>
<tr>
<td></td>
<td>IκBα</td>
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</tr>
<tr>
<td></td>
<td>Pdgfrβ</td>
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</tr>
<tr>
<td>dsRNA-induced gene expression†</td>
<td>IκKα</td>
<td>0.079</td>
</tr>
<tr>
<td></td>
<td>IκBα</td>
<td></td>
</tr>
</tbody>
</table>

*KEGG; †Biocarta.

¹ The online version of this article contains supplemental material.
inhibiting insulin signaling and initiating a proteolytic signaling cascade in elephant seal muscle.

**Cell proliferation and death.** Excessive use of GCs is linked to musculoskeletal pathologies resulting from increased apoptosis and reduced cell proliferation (15). Four proliferation repressors (Tob2, Anks1a, Gadd45α, Klj9) were upregulated during the acute response to exACTH in elephant seal muscle. However, three additional proliferation repressors (Btg2, Nrarp, Zfp3611) were downregulated, with Nrarp being the most strongly suppressed gene during the acute response (Table 3). Additionally, two apoptosis inhibitors (Mcl1, S1pr1) were upregulated, suggesting that elephant seal muscle tissue may be protected from deleterious effects of GCs by inhibiting apoptotic signaling but not cell proliferation (Table 3).

**Immune signaling.** GCs play a well-described role in immunosuppression and are commonly used to treat inflammation (53). Two inflammation inhibitors [Zfp36 (tristetraprolin), IκBα] were upregulated during acute response to exACTH, while another (Nrros) was the second-most highly downregulated gene at 2 h (Table 3). Therefore, the effect of exACTH administration on immune signaling in muscle was mainly repressive.

**Tissue function.** Seven genes involved in generalized cell and tissue functions were upregulated during the acute response to exACTH (Table 3). These included tissue development (fibin and remodeling (CTGF, Pi15) factors, cytoskeletal proteins (Diaph1, Sun2), a sarcomere assembly regulator [MuRF2 (Trim55)], and an autophagy mediator (Rab3gap1). Downregulated genes included myoglobin, angiogenesis-promoting growth factor receptor (PDGFRβ), and neuronal guidance ephrin ligand (Efn1a). This suggests that the acute response of muscle to exACTH administration includes maintenance of some tissue functions but suppression of genes involved in other, energy-intensive processes.

**Transcriptional Changes during Recovery from exACTH**

To identify transcriptional events that may underlie tissue recovery from exACTH injection, we compared gene expression profiles between acute response and recovery conditions. We found 122 differentially expressed genes ($P < 0.05$, FDR < 0.05) that were expressed by at least 1 FPKM during recovery (Supplementary File 2). Of these, 50 were upregulated and 72 were downregulated by at least 1.1-fold. One upregulated and seven downregulated transcripts were not annotated. Expression levels of two upregulated (Medag, Rbp4), two downregulated (Trim62, Asb15), and two unaltered (EF2, Ccng1) transcripts were verified by QPCR and were highly correlated with RNA-Seq data (Pearson $r = 0.88$, $P < 0.001$; Fig. 4, Table 1).

Five KEGG pathways were overrepresented during recovery at $P < 0.1$, which included MAPK, p53, Toll-like receptor (TLR), and two cancer signaling pathways (Table 2). Enriched Biocarta pathways included NF-κB activation, signal transduction through IL-1R, TNF/stress related signaling, keratinocyte differentiation, and double-stranded RNA-induced gene expression. Differentially expressed genes were grouped into functional categories based on literature search as described above (Table 4). Network prediction, conducted using 65 differentially expressed genes, showed that network topology during recovery was more complex than that seen during acute response (Fig. 3B). Each node was connected by a minimum of
seven edges to other nodes in the network, suggesting that transcriptional changes accompanying recovery from exACTH are highly coordinated.

Metabolism. Expression changes in metabolic factors during recovery from exACTH suggest that finely tuned mechanisms may restore metabolic homeostasis (Table 4). Downregulated metabolic genes included muscle growth inhibitors (Klf15, FoxO3, myostatin), an anabolic factor (Asb15), an atrogenic factor (Trim62), and ubiquitin ligase (Asb9). We found that three inhibitors of insulin signaling (Rbp4, Igfbp3, Tenc1) were upregulated during recovery, while an insulin resistance factor (Bbs12), two mTOR inhibitors (Deptor, Sesn1), a pyruvate kinase (Pkm), and an oxidative metabolism repressor (Ncor1) were downregulated (Table 4). Two adipokines (Medag, Gpat3) and two mitochondrial enzymes (Prodh, Cyb5r1) were also differentially expressed during recovery. Therefore, metabolic recovery from exACTH included suppression of proteolytic factors and derepression of some genes involved in anabolism and substrate oxidation.

Cell proliferation/differentiation/death. Cell cycle-related genes altered during recovery included both inducers and inhibitors of proliferation (Table 4). Factors that stimulate myocyte proliferation and suppress differentiation (Id3, Thbx2, Yap1) were strongly upregulated; Id3 was the most highly upregulated transcript during recovery. Other upregulated genes are known to stimulate proliferation and survival in other cell types (Zfp36l2, podocalyxin, L1td1, Axl). Three proliferation repressors (Ier5, Ndrg1, Npdc1) were upregulated concomitant with downregulation of three others (Mlf1, Tobb2, Gadd45α). Other downregulated genes include those involved in differentiation and cell fate specification (Zfp277, Jmjd2c, Table 3. Genes differentially expressed during acute response to exACTH

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EBSeq, *P < 0.05, false discovery rate (FDR) < 0.05. *Primary glucocorticoid receptor targets; †glucocorticoid-associated genes.
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**Cell proliferation/differentiation/death**

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**Immune response**

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**Tissue function**

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<td>cadherin EGF LAG seven-pass G-type receptor 1</td>
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Dpf3), myocyte differentiation (Six4, melusin), and osteogenesis (Alk2; Table 4). We also found evidence for suppression of p38 MAPK signaling, as a p38 activator (MKK6) was downregulated while an opposing phosphatase (Dusp10) was upregulated. Therefore, muscle tissue may recover following an exACTH challenge by stimulating proliferation and survival and suppressing differentiation of various progenitor cell types.

**Immune signaling.** Few immune signaling factors were differentially expressed during recovery from exACTH, which included a cytokine (IL-16), immune signaling receptors (Ackr1, Mrcl, CD302), and a complement protein regulator (Cfi; Table 4). Paradoxically, both an inhibitor (IkBoα) and an activator (IKKα) of NF-κB signaling were downregulated during recovery; the latter was the second-most highly downregulated gene in the transcriptome. Recovery from exACTH may include complex fine-tuning of NF-κB activity and recovery of some immune signaling components.

**Tissue function.** We found 20 general tissue and cell maintenance-related genes differentially expressed during recovery, of which 14 were upregulated and six were downregulated (Table 4). These included extracellular matrix remodeling factors (Lox, Temp2, Mgea5, Lama3, Pi15), cell adhesion proteins (Pcdh1, Celsr), cytoskeletal proteins (Twf1, Tnik, Cdc42ep3, Tppp), solute channels (Scni1b, Scn2b, Nherf2), and angiogenesis-related genes (Tek, Efnb2, Hspa12b, Heg1, Wars, PDGFRβ). Stimulation of vital tissue maintenance and repair processes may enable recovery of muscle tissue from exACTH.

**DISCUSSION**

We used transcriptome analysis to identify changes in gene expression during an acute response to exACTH administration in free-ranging elephant seals. ExACTH significantly elevated cortisol, aldosterone, and metabolites and upregulated a highly specific, conserved, and coordinated set of genes in muscle tissue. These did not, however, include GR targets associated with maladaptive GC effects seen in laboratory systems. Recovery from exACTH occurred within 24 h of administration as measured by recovery of corticosteroid hormones to baseline levels, downregulation of GR target genes, and derepression of positive regulators of tissue metabolism, growth, and remodeling. Metabolic factors differentially expressed during recovery include adipokines not previously described in muscle, reflecting species-specific adaptations to lipid-based fasting metabolism. We found that muscle response to HPA axis stimulation in free-living marine mammals is similar to that seen in laboratory animals but is context-dependent and highly selective to avoid deleterious effects of elevation of GC hormones that may occur as a natural part of their life histories.

**Endocrine and Metabolic Response to exACTH**

Administration of exACTH in this study group substantially increased circulating cortisol concentrations within 2 h, from 154 ± 28 nM to 1,687 ± 592 nM; these values overlapped with the maximal cortisol response elicited by capture and physical restraint (without sedation) in weaned pups [1,101 ± 232 nM (7)] that may mimic psychological and physiological responses to natural events (e.g., predation or conspecific aggression). ExACTH also caused significant elevation of aldosterone, a mineralocorticoid largely known for its osmoregulatory function in the context of the renin-angiotensin-aldosterone system. A number of studies, however, have now shown that aldosterone is elevated in response to stress in both pinnipeds (9, 17, 18) and cetaceans (24), suggesting that it may play a role in stress responses in these animals. The corticosteroid response to exACTH was terminated within 24 h of administration.

The response to exACTH administration and subsequent corticosteroid release had a significant effect on gene expression and substrates involved in carbohydrate, lipid, and protein metabolism. The apparent stimulatory effects of acute response on gluconeogenesis [increased glucose and reduced lactate, the preferred gluconeogenic substrate in this species (10, 57)] and lipolysis (increased FFA) in elephant seals are consistent with a typical stress response, despite the already high rates of both pathways in this species. However, we found no effect of exACTH administration on BUN levels after 2 h, consistent with very low rates of protein catabolism in elephant seals that enable effective sparing of lean tissue during fasting (13, 23). BUN concentrations decreased 24 h after exACTH administration, which may suggest a catabolism-limiting negative feedback mechanism in fasting-adapted animals.

**Acute Transcriptome Response to exACTH**

Metabolic changes elicited by exACTH administration result from glucocorticoid (through GR and MR), mineralocorticoid (through MR), and ACTH (through melanocortin 2 receptors, MC2R) effects on gene expression in target tissues (16, 51). Therefore, the gene expression changes observed in our study are likely the result of tissue responses to all three hormones. However, since the roles of aldosterone and ACTH in muscle physiology are poorly understood, we mainly focus our discussion on known GR effects on muscle gene expression. Muscle is a major GC target that is susceptible to catabolism promoted by elevated cortisol in humans and laboratory animals (29). We hypothesized that muscle tissue, critical for continued organismal function throughout fasting, would be protected from GC-induced degradation and that protective mechanisms would be apparent at the transcriptome level.

We found that 48 and 122 transcripts were differentially expressed during acute response to and recovery from exACTH, respectively. We included transcripts altered by less than twofold in our analysis because conservative fold-change thresholds often disregard changes that may be biologically meaningful (41, 50). For instance, while Sorbs1 was upregulated by only 1.18-fold during acute response, it is a well-described muscle GR target that is involved in tissue stress responses (29).

Acute response to exACTH was characterized by upregulation of a number of primary GR target genes previously identified in mouse myocytes (30). These factors are known to promote catabolism and inhibit insulin signaling, tissue glucose uptake, and oxidative metabolism, contributing to the increase in circulating glucose in response to exACTH. The proteolytic effect of GCs is mediated by master catabolism regulators (FoxO3, Klf15) that activate secondary transcriptional networks of E3 ubiquitin ligases (atrogenes), which target myofibrillar protein for degradation (54). The three most highly upregulated genes during acute response to exACTH were master regulators of proteolysis (Cebpd, Klf15, Ddit4). Ddit4 antagonizes protein synthesis by repressing anabolic mTOR signaling, while Cebpd and Klf15 directly activate atrogenes (54). The gene expression data indicating increased
proteolysis conflict with static metabolite measures of BUN concentration that failed to detect changes in protein mobilization. This may suggest that the timing of sampling captured changes in gene expression before physiological measures of protein mobilization, such as BUN concentrations, were apparent. As no other key proteolytic factors (e.g., FoxO) or downstream atrogens (e.g., myostatin) were also upregulated, the 2 h sampling point likely captured an early transcriptional response to exACTH. In the absence of upregulation of atrogens, protein catabolism may not be initiated, and therefore changes in circulating BUN would not yet be detectable. It would be informative to determine when secondary effectors of proteolysis are activated during the response to exACTH. Surprisingly, we found that the atrogen inhibitor follistatin was significantly upregulated in seal muscle during the acute response to exACTH. Follistatin is usually repressed by GR in other systems (61), and further investigation is necessary to determine if it plays a role in protein sparing during corticosteroid elevation in seals.

Additional muscle-sparing mechanisms may include resistance to GC-mediated apoptosis and maintenance of tissue homeostasis. While GC exposure has been shown to induce apoptosis in laboratory systems (15), we found that two apoptosis inhibitors were upregulated in elephant seal muscle during the acute response to exACTH. Some proliferation inhibitors were upregulated, while others (Nrarp, Zfp36l1) were downregulated, reflecting the heterogeneity of cell types that comprise muscle tissue. Nrarp and Zfp36l1 are repressors of Notch signaling, which drives satellite cell proliferation during muscle repair and confers resistance to GC-mediated apoptosis (42). Their downregulation may reflect a negative feedback mechanism for preserving muscle tissue during corticosteroid elevation.

Consistent with the anti-inflammatory effect of GCs seen in other systems, we found that three immunosuppressants (Zfp36, IκBα, Nrros) were differentially expressed in seal muscle during acute response to exACTH. Zfp36 promotes mRNA degradation of proinflammatory cytokines (55), while IκBα inhibits inflammatory signaling by NF-κB (21). Nrros (Lrrc33, Table 3) is a potent immunosuppressant that inhibits TLR-mediated inflammatory signaling and limits ROS generation by phagocytes during inflammation (44). Its role in muscle tissue is currently unknown.

Tissue homeostasis during acute exACTH response may be maintained by upregulation of factors necessary to maintain basal tissue function (cytoskeletal and extracellular matrix remodeling, development, growth and survival) and downregulation of those involved in energetically costly processes (an-

![Fig. 5. Transcriptional changes in response to exACTH administration are mediated by corticosteroids and affect processes such as catabolism, cell proliferation, apoptosis, and inflammation (solid arrows). Selected genes differentially expressed (red, upregulated; blue, downregulated) during acute response to exACTH (A) and recovery from exACTH (B) and their putative effects on these processes (dashed arrows) are shown.](http://physiolgenomics.physiology.org/)}
giogenesis, innervation, myoglobin production). The second most highly upregulated gene was fibin (31), a GC-responsive factor that may be involved in muscle development. Therefore, the transcriptional response of elephant seal muscle to acute HPA axis perturbation is highly specific and involves upregulation of gene targets that may promote limited catabolism and basal tissue function while suppressing energy-intensive insulin signaling, cell proliferation, inflammation, and tissue development processes (Fig. 5A).

Transcriptional Changes during Recovery from exACTH

Changes in the transcriptional landscape between acute response and recovery states suggest that restoration of tissue metabolism and growth occur within 24 h of exACTH administration. This includes downregulation of GR targets induced earlier (Klf15, Gadd45α, Tob2, Pit15), secondary catabolic factors (FoxO3, myostatin), and mTOR inhibitors, and upregulation of genes involved in glucose uptake and utilization. Downregulation of both primary and secondary proteolytic network genes during the ∼22 h of recovery from exACTH administration may be sufficient to drive the observed decrease in BUN levels, potentially protecting protein stores. Sampling muscle tissue more frequently or earlier than 24 h after exACTH administration may reveal additional negative feedback mechanisms that potentially suppress catabolism and reduce circulating BUN following HPA axis stimulation. Paradoxically, suppression of insulin, an anabolic hormone, was maintained during recovery as evidenced by several upregulated insulin signaling inhibitors. Circulating insulin levels in study animals were low and unaffected by exACTH, consistent with the hypoinsulinemia and peripheral insulin resistance described in this species (22). It appears that despite low baseline insulin secretion, suppression of insulin sensitivity in target tissues is critical during adrenocortical responses in a fasting mammal. Mechanisms by which fasting animals maintain protein stores despite suppressed insulin signaling are currently unknown. Four adipokines (Rbp4, Medag, Gpat3, Bbs12) were differentially expressed in elephant seal muscle during recovery from exACTH administration. Their role in muscle metabolism, with the exception of Rbp4, is not well defined but may parallel nutrient uptake roles seen in adipose (5, 38, 62) and reflect reliance on lipid oxidation in fasting species. Further studies of muscle-expressed adipokines may yield insight on mechanisms used to conserve energy stores during fasting and acute adrenocortical responses. We also found two mitochondrial enzymes (Prodh, Cyb5r1) differentially expressed during recovery from exACTH. Prodh catalyzes proline catabolism in response to nutrient stress and may play a role in protection from oxidative damage (43), while Cyb5r1 is involved in fatty acid elongation and cholesterol biosynthesis. Negative feedback on GR signaling within muscle tissue was evidenced by downregulation of a nuclear receptor coactivator [PGC-1α (20)] at 2 h and downregulation of a negative regulator of GR activity [Ncor1 (49)] at 24 h.

Recovery from HPA axis perturbation may involve stimulation of cell proliferation and inhibition of differentiation, potentially to replenish the myocyte progenitor pool negatively affected by elevated GCs. Differentially expressed genes included chromatin remodeling factors (Zfp277, Jmjd2c, Dpf3), which may play a role in epigenetic regulation of cell stress responses and should be further explored. We found evidence that p38 signaling, which may regulate muscle development, proliferation, metabolism, and inflammation (14), was suppressed at 24 h. Recovery of some components of the immune response was evidenced by upregulation of factors associated with immune signaling, although an activator of NF-κB was the most highly downregulated gene at this time. The role of NF-κB in muscle responses to elevated corticosteroids is likely complex and warrants further investigation. Tissue maintenance genes altered during recovery from exACTH included extracellular matrix remodeling and cytoskeletal factors, solute channels, and angiogenesis regulators. Therefore, elephant seals may restore muscle homeostasis after an HPA axis challenge with finely tuned and highly coordinated alterations in metabolic, proliferative, immune, and tissue repair pathways (Fig. 5B).

Conclusions

To our knowledge, this is one of the first descriptions of a global transcriptome response to HPA axis stimulation in a free-living marine mammal profiled by RNA-Seq. We found that genes differentially expressed in response to exACTH and elevated corticosteroids include highly conserved muscle GR targets in addition to factors not previously described in this context in laboratory species. These may represent novel biomarkers of acute adrenocortical responses in free-ranging marine mammals, which may be used to evaluate stress states in species subject to frequent environmental disturbance (26). We suggest that animals regularly exposed to corticosteroid fluctuations may use buffering mechanisms to protect vital tissues from their adverse effects while retaining the capacity to mount adaptive stress responses, which are likely tightly regulated to avoid long-term consequences deleterious to an animal’s fitness. Examination of exACTH-induced gene expression in other tissues such as adipose, the main energy depot in marine mammals and one that may be more directly affected by aldosterone (36) and ACTH (2), will provide valuable insights on coordination of energy usage during organismal stress responses. In addition, comparison of molecular responses to brief vs. sustained exposure to elevated corticosteroids may elucidate mechanisms involved in the pathological progression from acute to chronic stress, critical to conservation physiology. In the meantime, this study represents a critical step toward a gene-level understanding of marine mammal responses to HPA axis stimulation in the context of natural environments.

ACKNOWLEDGMENTS

The authors thank J. Sharick, D. Somo, D. Esninger, H. Peck, and R. Berger for assistance with field procedures and sample collection.

GRANTS

This work was conducted under National Marine Fisheries Service permit 14636, supported by National Institutes of Health Grant HL-091767 to R. M. Ortiz and D. E. Crocker and Office of Naval Research Grant N000141110434 to D. E. Crocker, and approved by the Sonoma State University Institutional Animal Care and Use Committee.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS


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