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Development of a biomarker panel for identifying stressed marine mammals

Laura Pujade
University of the Pacific, l_pujadebusqueta@u.pacific.edu

DEVELOPMENT OF A BIOMARKER PANEL FOR IDENTIFYING STRESS ON MARINE MAMMALS

by

Laura Pujade Busqueta

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APPROVED BY:

Thesis Advisor: Jane Khudyakov, Ph.D.

Committee Member: Zach Stahlschmidt, Ph.D.

Committee Member: Tara Thiemann, Ph.D.

Department Chair: Eric Thomas, Ph.D.

Dean of Graduate School: Thomas H. Naehr, Ph.D.

DEDICATION

I would like to dedicate this thesis to my extended family and friends, who have supported me throughout my college career, from undergraduate to graduate school. A special dedication to my dad, Xavi, for always believing in me and being my number one fan. To my mom, Gloria, for being the most loving and caring mother one can have. To my brother, Oriol, for being there whenever I need him and for making me feel like the proudest older sister in the world. To my sister, Ester, for being the light in a dark room and for bringing joy in any situation. To my boyfriend, Colton, for listening to my countless complaints, for being one of my biggest pillars, and for always making me laugh.

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Development of a Biomarker Panel for Identifying Stress on Marine Mammals

Abstract

by Laura Pujade Busqueta

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Increasing anthropogenic disturbance in marine ecosystems such as fishing, oil-drilling, and noise pollution can have detrimental effects on the reproduction and survival of apex predators such as marine mammals. Stress activates the hypothalamic-pituitary-adrenal (HPA) axis, resulting in increased circulating glucocorticoid (GCs) hormones, which alter expression of target genes encoding metabolic enzymes and other mediators of stress. Prolonged HPA axis stimulation may increase catabolism of nutrient stores and suppress immune and reproductive functions, impacting the fitness of marine mammals. GCs measurements are used to identify wild animals experiencing stress. However, these measurements may not be sensitive enough to distinguish between an acutely and a chronically stressed individuals. In this study, we present a new approach of assessing stress states in marine mammals, by measuring expression levels of gene markers in blubber. We previously characterized transcriptional and metabolic profiles and identified genes and metabolites that were differentially expressed in response to single and repeated adrenocorticotropic hormone (ACTH) administration in juvenile northern

elephant seals. We then measured expression of these target genes in blubber tissue collected from juvenile northern elephant seals in their natural baseline stress states (n=30), and correlated their gene expression values with cortisol, aldosterone, total triiodothyronine (tT3), reverse triiodothyronine (rT3), and triglyceride levels, and body condition index. We found that blubber genes that were upregulated in response to repeated ACTH administration in the previous study were positively correlated with cortisol and inversely correlated with tT3 in the baseline sample set. These markers included genes that encode a lipid particle protein (PLIN1), an adipogenesis promoting transcription factor (DKK1), an oxidative stress enzyme (GPX3), and a lipid metabolism enzyme (AZGP1). Blubber genes differentially expressed in response to acute ACTH administration in the previous study included an adipokine (ADIPOQ) and a ketogenesis enzyme (HMGCS2), which were upregulated, and an adipogenesis inhibitor, TGFBI, which was downregulated. ADIPOQ and HMGCS2 were positively correlated with cortisol and negatively correlated with tT3 levels, while TGFBI was positively correlated with tT3 and body condition index, and negatively correlated with rT3 in the baseline sample set. These results provide insights into the molecular mediators of the physiological stress response and provide markers that can be used as a part of a potential diagnostic panel for differentiating between acute and prolonged stress states in marine mammals.

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Chapter 1: Introduction

Vertebrates cope with unpredictable and uncontrollable stimuli by mounting an adaptive stress response that causes hormonal, behavioral, and physiological changes [1, 2]. Chronic or sustained stress can lead to an allostatic overload, which occurs when the energy requirements of the stress response impair homeostatic processes [3, 4]. This impairment can greatly affect key physiological functions and impact the survival of the animal [5]. In addition to predictable and unpredictable biological stressors, such as reproduction and fluctuations in prey availability, marine mammals experience additional physiological challenges, such as hypoxia and extended fasting during energy-demanding life history stages, which are uncommon in other mammals [6, 7]. Furthermore, marine mammals are subject to disturbance by human activities in their environment, such as oil drilling, ocean transport, industrialization of coastal lines, ecotourism, commercial fishing and noise pollution. The combination of these natural and anthropogenic stressors has been correlated with marine mammal population declines [8-10]. There are a number of marine mammal species listed as endangered under the U.S. Endangered Species Act including the Hawaiian monk seal, the Amazonian manatee, the dugong, and some populations of humpback, killer, and right whales. Given their status at the top of the food chain, a decrease in population numbers of marine apex predators can greatly impact the stability of the marine ecosystem [11]. An increasing number of studies have attempted to develop stress markers that can be used to identify stressed and unhealthy animals, but the majority of this work has been focused on terrestrial mammalian species [12-14]. However, studies in marine mammals have suggested that there are differences in their stress response from that of terrestrial mammals due to their unique physiological adaptation to an aquatic existence [15]. Unraveling the biological and physiological impacts of both acute and chronic stress in marine mammals is of foremost

interest for understanding how prolonged exposure to environmental and anthropogenic disturbances can alter marine mammals' fitness. Proper evaluation of an animal's stress state can be used to inform conservation management strategies that reduce anthropogenic disturbance and prevent marine mammal population declines [16, 17].

Mammalian Stress Response

The principal mediator of the physiological stress response in vertebrates is the hypothalamic-pituitary-adrenal (HPA) axis, the stimulation of which results in the secretion of glucocorticoids (GCs): cortisol in mammals, and corticosterone in birds, amphibians and reptiles [1, 15, 18]. In mammals, the adaptive response to acute stress induces an increase in secretion of catecholamines (norepinephrine and epinephrine) by the adrenal medulla, stimulated by the sympathetic nervous system, which allow for a rapid response by regulating vital functions (e. g. increased heart rate and respiration). At the same time, the paraventricular nucleus of the hypothalamus releases corticotropin-releasing hormone, which regulates the secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland, which in turn stimulates the synthesis of GCs by the adrenal gland [1, 15, 19, 20].

As the end product of the HPA axis hormone cascade, cortisol is considered the primary mammalian stress hormone and is commonly used as a biomarker of stress [1, 21-23]. In target cells, cortisol binds to intracellular cortisol receptors, which translocate to the nucleus and bind to glucocorticoid-response elements (GREs), affecting transcription of genes implicated in metabolic, immune, and other pathways. Chromatin immunoprecipitation sequencing (ChIPSeq) and microarray analyses of GCs target tissues have identified genes containing GREs, many of which are involved in triglyceride synthesis, lipolysis, insulin signaling, and lipid transport and

storage [24-26]. These findings provide insights into the role of GCs in regulating many physiological functions.

Acute increases in cortisol during stress-induced conditions promote an adaptive response to stress by increasing catabolism of metabolic stores to meet increased energy demands, and suppress energetically expensive functions such as immune responses, reproduction and growth [27]. This mechanism is regulated by negative feedback, which can be impaired due to repeated stress and can lead to adverse physiological effects, such as alterations in behavior, growth inhibition, immune and reproductive suppression and hyperglycemia [19, 28, 29]. Depletion of adipose tissue lipids as a result of increased catabolism in marine mammals can have detrimental consequences, as marine mammals rely on fat stores in blubber not only as an energy source, but for other life history challenges as well (e.g. buoyancy, thermoregulation, hydrodynamics).

Another endocrine pathway altered by stress in mammals is the hypothalamic-pituitary-thyroid (HPT) axis [30, 31]. The hypothalamus releases thyrotropin releasing hormone which causes the anterior pituitary gland to produce and secrete thyroid stimulating hormone (TSH). The thyroid gland produces thyroid hormones (thyroxine, T4 and triiodothyronine, T3) when stimulated by TSH. This mechanism is regulated by negative feedback [32]. Deiodinases can convert T4 into the metabolically active form T3, as well to the inactive form, reverse T3 (rT3) [33]. In target cells, T3 binds to intracellular receptors that activate or repress transcription of target genes. Thyroid hormones regulate homeostasis and many metabolic functions such as lipid, protein and carbohydrate metabolism, as well as development and sexual maturation [32, 34, 35]. Stress causes a decline in circulating T3, the biologically active form of thyroid hormone, and an increase in T4 deiodination to reverse T3, its inactive form, leading to a decrease in metabolic rate [30, 31]. The consequent reduction in metabolism can have

detrimental effects on animals that require especially high energy expenditure during life history challenges such as reproduction and migration [36-38]. Marine mammals are particularly susceptible to stress-induced hormonal changes due to their metabolically challenging life histories, which include prolonged periods of fasting concomitant with reproduction, migration, molting, and repeated bouts of hypoxia [6, 39].

Many stress studies have used baseline cortisol measurements as an indicator of stress in wild animals, with the assumption that stressed animals have elevated baseline GCs [40-43]. However, many animals show seasonal patterns in GC secretion, and baseline levels may be routinely elevated during reproduction, fasting, or other life history stages [44]. For example, northern elephant seals elevate cortisol, the primary driver of lipolysis, during their breeding and molting fasts [45]. In addition, while a rise in circulating levels of GCs is indicative of an activation of the physiological stress response, it does not determine whether the animal is responding to an acute or chronic stressor. An animal exposed to chronic stress can experience a physiological acclimation, or habituation of the HPA axis and may no longer respond to the repeated stressor, making it appear unstressed [27]. Most of the stress studies do not differentiate between acutely and chronically stressed individuals, which can be critical for identifying animals experiencing chronic stress and predicting its impacts on their physiology and fitness [46]. Thus, there is a need for additional markers of stress, such as downstream changes in GC target gene expression, that can provide a better mechanistic understanding of acute and chronic stress and additional diagnostics of stress state. Hormones that bind intracellular receptors, such as corticosteroid and thyroid hormones, produce biological effects by altering expression of target genes, causing long-lasting effects that can be detected even after blood hormone levels return to baseline levels. Furthermore, gene expression measurements are

more sensitive and tissue-specific than measurements of circulating hormones. Therefore, differences between acute and chronic stress states may be more apparent at the levels of target gene expression rather than circulating hormone levels. Previous studies of marine mammals have identified endocrine and metabolic responses and gene expression changes in response to acute stress [23, 47-50], but there is not a clear understanding on the effects of repeated stress on gene expression in GC target tissues in marine mammals and most wild animals.

Northern Elephant Seals as a Marine Mammal Study System

This study was conducted using the northern elephant seal (*Mirounga angustirostris*) as a model marine mammal. Northern elephant seals undergo extreme physiological challenges as part of their life history. They spend most of the year foraging in the mesopelagic zone, and come out on land (haul out) for 1-3 months twice a year, fasting for the entire duration of the haul out while enduring energetically demanding activities such as breeding, lactation, or skin and hair growth in the winter and spring, respectively [51]. Juveniles (1-3 years old) haul out for a month during the fall instead of the winter. Animals generally return to the same site where they were born [7]. The predictability of these haul outs, and relative ease of research handling due to the lack of extant land predators, make this organism an amenable marine mammal model system [47-50, 52, 53]. In addition, well-established anesthesia procedures minimize the impact of handling on baseline GC levels in this species [54], allowing for measurement of true baseline hormone and gene expression levels. Consequently, northern elephant seals are an ideal model system for stress manipulation studies and can serve as a proxy for other marine mammals that are difficult to sample (e.g. fully aquatic cetaceans or critically endangered pinnipeds).

Repeated Stress in Northern Elephant Seals

Previously, our laboratory conducted a repeated stress manipulation experiment was performed during which ACTH was injected once a day, for a period of four days, to juvenile northern elephant seals to simulate exposure to repeated stressors [55, 56]. Blood and blubber samples were collected immediately before ACTH administration and four hours post-ACTH on the first and fourth days of the experiment. Blubber, a modified subcutaneous adipose tissue in marine mammals, was targeted due to its ease of sampling, its importance in marine mammal energy homeostasis, and the role of adipose tissue in response to GCs in other mammals. Hormone, metabolite, and transcriptome analyses were then used to examine differences between baseline and stress-induced hormone and metabolite responses and blubber gene expression levels on the first (acute ACTH response) and last day (repeated ACTH response) days of the experiment.

The repeated ACTH stress response was characterized by suppression of total T3 (tT3) levels, an increase in its inactive form, reverse T3 (rT3), as well as a decrease in circulating triglyceride (TAG) levels, and an increase in levels of cortisol and the osmoregulatory hormone aldosterone [55, 57]. However, the magnitude of the cortisol response to ACTH did not vary between the first and last ACTH administration, highlighting the inability of cortisol levels to distinguish between acute and repeated stress states, suggesting that expression of hormone target genes may therefore be more indicative of stress states than hormone levels alone [55].

Transcriptome analyses of blubber biopsies collected during the experiment identified 31 differentially expressed genes (DEGs) in response to the first ACTH administration, 7 DEGs in response to the fourth ACTH administration, and 34 DEGs over the course of the entire experiment [56].

Objectives

The purpose of this study was to characterize the downstream effects of repeated stress, and to evaluate the potential of gene expression as an indicator of stress that can distinguish between acute and repeated stress in marine mammals. To validate these new potential markers of stress, I collected blood and blubber samples from a separate cohort of juvenile northern elephant seals of variable body condition, and thus, potentially variable baseline stress states. I correlated the expression values of the candidate gene markers with stress hormone measurements, triglyceride levels, and body condition index of the animals, to evaluate variability in baseline stress-related gene expression levels and the potential use of these markers to detect physiological stress in northern elephant seals. Therefore, expression levels of hormone target genes in blubber may provide a new diagnostic tool for conservation management strategies. For instance, these markers, in combination with other physiological metrics, may be able to test if low reproductive rates of some marine mammal populations are related to stress, or to evaluate and manage the physiological impacts of anthropogenic disturbance on vulnerable marine mammal populations.

Chapter 2: Methodology

Study Subjects

All animal handling procedures were conducted under National Marine Fisheries Service permit 19108 and approved by Sonoma State University and University of the Pacific Institutional Animal Care and Use Committees and Department of the Navy Bureau of Medicine and Surgery. A total of 30 juvenile northern elephant seals of varying apparent body condition were selected as study subjects: 17 females, 11 males and two animals of unknown gender. Seals that had not been previously identified for other studies were marked with rear flipper tags (Dalton, Oxon, UK).

Sample Collection and Preservation

Baseline sample collection took place from September to November of 2017 in Año Nuevo State Park, San Mateo County, CA, USA. Seals were chemically immobilized using an intramuscular injection of ~1 mg/kg tiletamine-zolazepam HCl (Telazol, Fort Dodge Animal Health, Fort Dodge, IA, USA), and sedation was maintained with intravenous doses of ketamine (0.25-1 mg/kg) (Fort Dodge Animal Health, Fort Dodge, IA, USA). Adipose tissue samples were collected from the posterior flank of the animal, previously sterilized with ethanol and iodine to avoid infection of the wound, using a 6.0 mm diameter biopsy punch (Miltex, USA). Two blubber biopsies were cut to separate the inner and outer blubber sections. Inner blubber is highly vascularized and is the closest section to musculature, while outer blubber is closer to

skin, and the two layers can be distinguished morphologically. One set was immediately frozen in liquid nitrogen while the other set was incubated in RNA*later*™ Stabilization Solution (~300 mg tissue per 1.5 mL; Invitrogen USA). RNA*later*™ solution was removed from samples after 24 hours of incubation at 4°C. All samples were brought to the laboratory and stored at -80°C. Blood samples were collected from the extradural vessel using an 18 G, 3.25-inch spinal needle and drawn into vacuum collection tubes, which were stored at 4°C immediately after collection. Serum and plasma were isolated by centrifugation at 3,000 x *g* for 15 minutes, kept frozen on dry ice until return to the laboratory, and stored in -80°C until further analysis. After collection of morphometric measurements (standard length, axillary girth), seals were allowed to recover from anesthesia and resume normal activity.

Stress Markers Analyses

Body condition index was determined by calculating the residuals of linear regression of the axillary girth plotted against standard length of each animal [58]. Serum cortisol was measured using a radioimmunoassay (RIA; MP Biomedicals, Burlingame, CA, USA) previously validated for northern elephant seals [48]. Aldosterone was measured using an enzyme-linked immunosorbent assay (ELISA; cat #11-ALDHU-E0, lot # 172040; Alpco, Salem, NH, USA), validated for northern elephant seals in a previous study [55]. Total triiodothyronine (tT3) and reverseT3 (rT3) were measured in duplicate using RIAs (tT3: cat #0613254215, lot # T3K1825; rT3: cat #38-RT3HU-R125, lot # REW125-1827A; MP, Biomedicals, Burlingame, CA); average CVs were 2.2% and 2.4% for tT3 and rT3 assay, respectively. Total T3 and reverse T3 RIAs were previously validated for northern elephant seals [59]. A previous study used lipidomics to show that triglyceride levels in plasma were significantly suppressed by repeated ACTH administration [57]. These results were validated in the same sample set (from Deyarmin et al.)

using a colorimetric triglyceride (TAG) assay (Triglyceride Colorimetric Assay Kit, Cayman Chemical, MI, USA). Circulating levels of TAG were then measured in duplicate in plasma from the baseline sample set using the same assay; average replicate CVs were 3.1 and 2.4% for the repeated ACTH samples and the baseline samples respectively.

Selection of Candidate Genes

Genes of interest were previously identified as differentially expressed in elephant seal blubber in response to repeated ACTH administration [56]. Candidate genes were selected based on three characteristics: significant BlastX hit to a protein with known function in the UniProt SwissProt proteome database, transcript abundance (number of transcripts per million equal to or greater than 20), low variability in expression level between biological replicates for each condition (Table 1). Candidate genes were also selected based on differential expression between first and last ACTH response, to account for differential gene expression in acute and repeated physiological stress.

RNA Isolation

Inner blubber samples were minced with a sterile scalpel on ice (~300 mg of tissue) and added to NextAdvance RNase-free RINO-blue tubes pre-filled with Qiazol (500 µL, Qiagen, USA) and one 3.5 mm UFO bead (Next Advance, USA). The tissue was homogenized in a Bullet Blender Storm 24 (Next Advance, USA; Speed 12, two 2-minute cycles with 1 minute of cooling on ice between blend cycles). Another 0.5 mL of Qiazol was added to each sample after homogenization and incubated for 5 minutes at room temperature with occasional vortexing to allow further lysis of the tissue. Lysed tissue was added to QIAshredder tubes (Qiagen, USA) to filter out insoluble cell debris and reduce viscosity of the tissue lysate. Flow-through was centrifuged again to separate insoluble material and separate lipids from the rest of the

homogenate. To separate RNA from protein content, clean homogenate was added into microcentrifuge tubes pre-filled with 200 µL of chloroform (VWR Life Sciences, USA), vortexed and centrifuged. The aqueous layer containing RNA was extracted and RNA was precipitated with RNase-free 70% ethanol. RNA was isolated using RNeasy Lipid Mini Kit (Qiagen, USA) with a 20-minute on-column DNase I digest step (10 µL per column; Qiagen, USA). RNA was stored at -80°C until further analysis. RNA quantity was determined using Qubit 3.0 Fluorometer Broad-Range RNA Assay (Life Technologies, USA). The two sample handling methods, flash freezing in liquid nitrogen in the field and preservation in RNA*later*™ Stabilization Solution, were compared to evaluate their effects on RNA quality, which was assessed using microcapillary gel electrophoresis (Bioanalyzer, Agilent Technologies, USA) (Fig. 5). No differences in RNA quality were observed between samples preserved in liquid nitrogen and those preserved in RNA*later*™ solution. RNA samples had integrity values (RIN) between 7.0 – 8.6. Therefore, tissues preserved using either method were used interchangeably for RNA isolation.

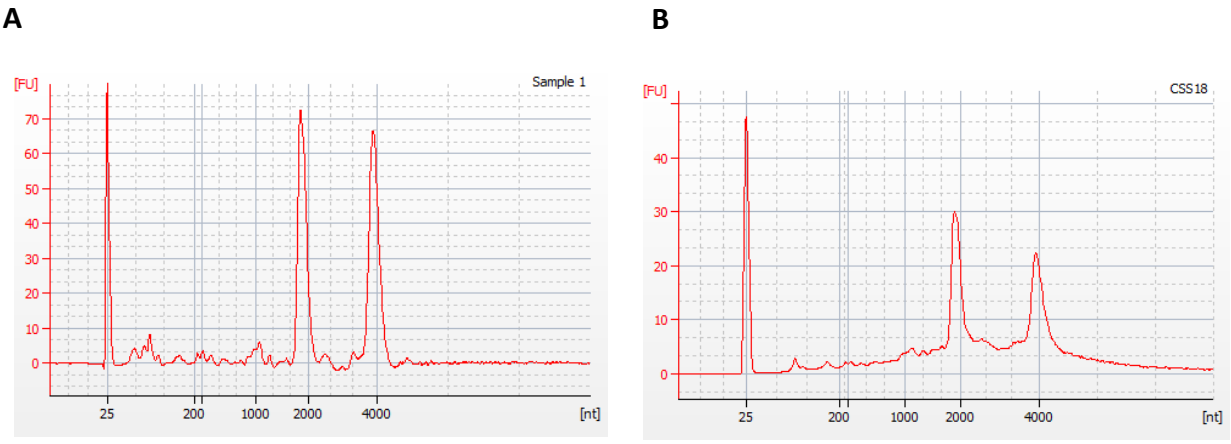


Figure 1: Bioanalyzer electropherograms of RNA isolated from a sample flash frozen in the field with liquid nitrogen, RIN = 8.10 (A), and a sample preserved in RNA*later*™ solution, RIN = 8.60 (B).

Quantitative RT-PCR

Complementary DNA (cDNA) was synthesized from 500 ng of total RNA using SuperScript IV VILO Master Mix with ezDNase digestion (Thermo Fisher, USA). cDNA samples were diluted 1:10 and 2 μL were used in each 20 μL real-time PCR (qPCR) reaction, in triplicate, with PowerUp SYBR-Green Master Mix (Thermo Fisher, USA). qPCR was performed on a QuantStudio 5 Real-Time PCR System instrument (Thermo Fisher, USA) using the following program: 2 min at 50°C, 2 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 60 s at 60°C. All the replicate SDs were < 0.167 Ct, as recommended by MIQE Guidelines [60]. No-template and no-reverse transcriptase controls were included in each run and showed no amplification.

Primers were designed using the NCBI Primer-Blast program and elephant seal transcript sequences generated in the previous study [56]. Gene primers were designed to target the portion of the transcript that encoded a highly conserved sequence within the encoded protein, basen on the NCBI RefSeq database. Primers were selected to have a melting temperature of 60-62°C and a qPCR product size between 75 to 150 bp for all genes. Primer sequence specificity was confirmed by BlastN against the RefSeq database. All primers were used at 400 nM final concentration. Primer efficiency was determined using standard curves with five 1:2 dilutions of cDNA dilution of blubber samples of northern elephant seals from a previous ACTH administration study done by Khudyakov et al. [48], and primer efficiency was calculated as recommended in MIQE Guidelines [60]. Primer efficiency scores were within 90 and 110% (Table 1). Melting curve analysis was used to determine specificity of the primer by the presence of a single peak produced by dissociation of the qPCR amplicon. Amplification of a single product of the expected size was also confirmed using agarose gel electrophoresis (E-gel

2% Agarose, Thermo Fisher, USA) (Fig. 6). Faint double bands at the bottom of the gel (< 100 bp) correspond to unbound primer sequences.

Table 1: Primer sequences and efficiency values of candidate gene markers used for qPCR analysis.

Transcript ID	Gene homolog	Protein name	Primer sequence	Amplification Efficiency (%)
TRINITY_DN592797_c2_g1	PLIN1	Perilipin 1	F:GCGGTCAACAAGGACCCAAC R:GGAAACACTCACAGGTGCCG	94
TRINITY_DN572974_c28_g1	ADIPOQ	Adiponectin	F: CCAATGTTCCCATTCGCTTTAC R: CATTCCTGGGCTGTACTACTTC	93
TRINITY_DN581259_c1_g6	LEP	Leptin	F: ACAGGACCAAAGCCACAGGA R:GCGAGGCCTGAGAAGCACAT	104
TRINITY_DN585354_c4_g5	DKK1	Dickkopf-related protein 1	F: CCAAGATCTGTAAACCTGTCCTC R: CACAGTAACAGCGCTGGAATA	103
TRINITY_DN571555_c4_g2	GPX3	Glutathione peroxidase 3	F: CCGGACGGTGTACCCATCAT R: GGCAGGTCTGATTTACTGCCC	107
TRINITY_DN587426_c4_g1	CIDEA	Cell death activator CIDE-A	F: CGCGTTGCCAATCTAGACGC R: TCCGTATCCACCACGGTTCC	100
TRINITY_DN560507_c9_g1	HMGCS2	Hydroxymethylglutaryl -CoA synthase 2	F:TGATGTTCAGTGACTTCCTGTC R: TGTAGGTTTCTTCCAGCGTTAG	100
TRINITY_DN591927_c4_g2	CDO1	Cysteine dioxygenase 1	F:TGACAAATTCCTGTCCGATAAGT R:TTCTGCCCTCCCTTTCATTAC	97
TRINITY_DN591034_c2_g1	TGFBI	Transforming growth factor beta-induced	F:TCCTGAAGGGGGACAATCGC R: GACCCCTTCCCTGTTGAGCA	97
TRINITY_DN571072_c3_g3	ACSL1	Long-chain-fatty-acid CoA ligase 1	F:GTAGCGATGGTGCTCGGAGA R: ACTTGACACCTGGATGCCCC	97
TRINITY_DN587150_c2_g2	AZGP1	Zinc-alpha-2-glycoprotein 1	F: TCGTTCTCCCAGTCCTCTATTC R:CCTACCTCAATGACCAGGATTTC	94
TRINITY_DN551341_c9_g1	GLRA2	Glycine receptor subunit alpha-2	F: GTCTCCAGACAACACAAGGAG R: CCCATCCCATAACCACTGAAA	107
TRINITY_DN587322_c7_g1	PIAS 4	E3-SUMO-protein ligase PIAS4	F:GCGGACTTAAACACGAACTTGTCA R: GAGCTCTTCTTGGCGTAGCG	106
TRINITY_DN592891_c7_g1	MGST1	Microsomal glutathione S-transferase 1	F: TTAACGACTGAGCCACCCAGG R:GCATCAAGATGAACCACAAGTTGG	100
From Khudyakov et al., 2017	NONO	Non-POU domain-containing octamer-binding protein	F:GAGGAAGGTTTCGGACTGTAAG R:GCGGAGATTGCCAAAGTAGA	95
From Khudyakov et al., 2017	YWHAZ	14-3-3 Protein zeta/delta	F: AGCAGAGAGCAAAGTCTTCTATT R: GACTGATCCACAATCCCTTTCT	100

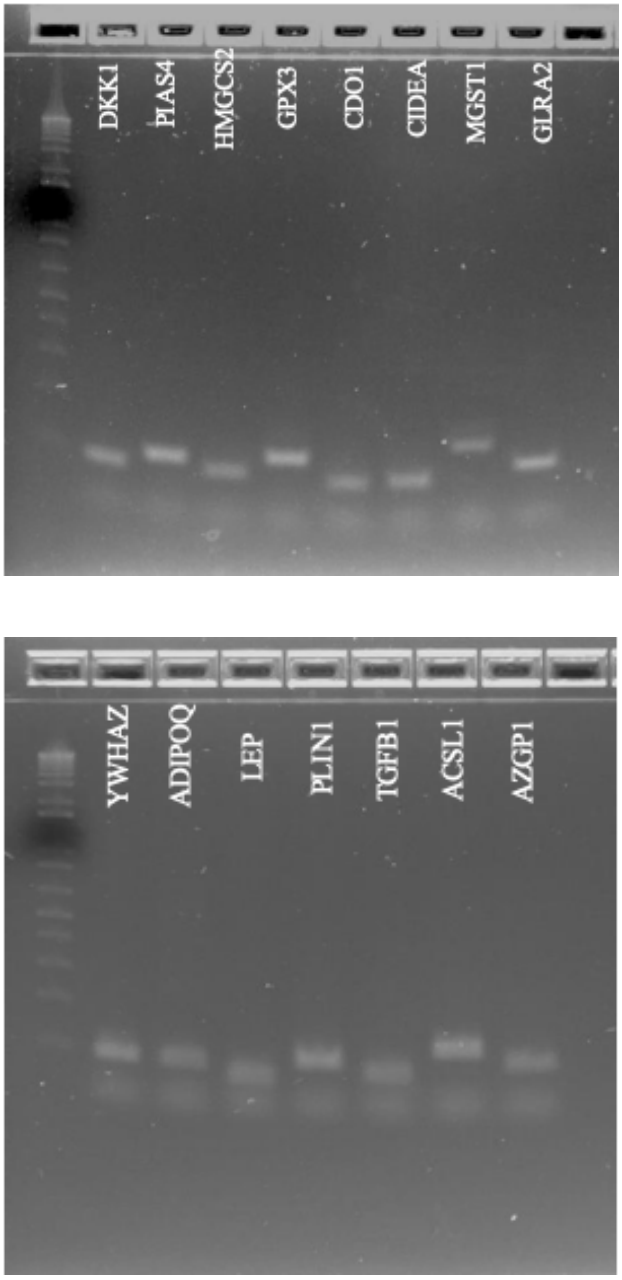


Figure 2: Specificity of primer pairs for RT-qPCR amplification. cDNA from all samples were mixed as the template.

Two genes, YWHAZ and NONO, were evaluated for stability as reference genes as they were previously used for similar gene expression analyses of elephant seal blubber tissue [49]. Expression stability was evaluated using BestKeeper algorithm [61]. YWHAZ was the most

stable gene across all samples (SD [±CP] = 0.47, CV [%CP] = 2.06) and was selected for use as reference gene in this study. Gene expression was determined by the qPCR cycle during which fluorescent amplicons were first detected by the instrument, or cycle threshold (Ct). Relative gene expression values were normalized to the reference gene expression values using the delta Ct method (Equation 1) [62, 63].

$$\Delta Ct = Ct\ (YWHAZ) - Ct\ (GOI)$$

Equation 1: Mathematical equation of the delta Ct method used to normalize qPCR data. Ct: cycle threshold, GOI: gene of interest.

Statistical Analyses

All statistical analyses were conducted using JMP 14 Software (SAS, USA). Linear mixed models, with subject ID as a random effect and sampling time as fixed effect was used to assess differences in triglyceride levels in plasma collected from elephant seals during a repeated ACTH administration experiment [56]. Variables measured in the present study were analyzed for normality and homogeneity of variance. Variables that met normality were correlated with the gene markers using Pearson's correlation analysis. Variables that did not meet normality were correlated with gene markers using Spearman's correlation analysis.

Chapter 3: Results

Baseline blood and blubber samples were collected from 30 juvenile northern elephant seals, of varying apparent body condition, for evaluation of biomarkers identified in a previous repeated ACTH administration experiment [55, 56]. The biomarkers included cortisol, aldosterone, tT3, rT3, TAG, and genes that were identified as significantly altered by repeated ACTH administration in elephant seals.

Body condition index (BCI), which is related to adiposity in elephant seals, was determined using residuals of linear regression of axillary girth plotted against standard length of each study animal (Fig. 1) [58]. Animals with lower adiposity are likely experiencing nutritional stress, another type of physiological stress [64]. Thus, positive residual values indicate healthy animals, whereas negative residual values indicate individuals with low body condition, as animals with larger axillary girth than average for their length have higher adiposity, and vice versa (Table 2). Body condition residuals indicate that 15 individuals had negative BCI, and 15 animals had positive BCI.

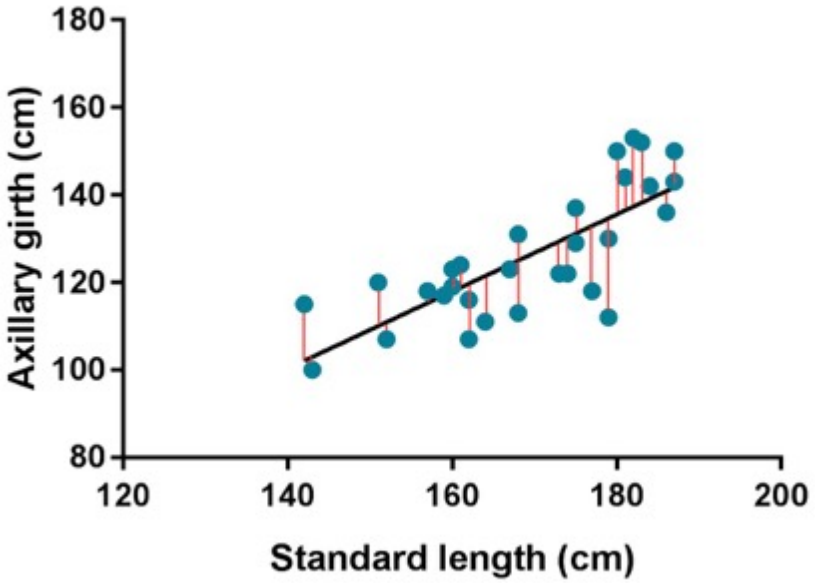


Figure 3: Body condition residuals calculated from the linear regression of axillary girth and standard length for all study subjects (n = 30). Dots represent each individual sampled; red lines indicate body condition residuals.

Table 2: Morphometrics of animals used in this study.

Seal ID	Sex	Axillary girth (cm)	Standard Length (cm)	Body condition residual
CSS 1	F	131	168	6.0
CSS 2	F	136	186	-4.9
CSS 3	M	122	173	-7.4
CSS 4	F	150	187	8.2
CSS 5	F	137	175	5.8
CSS 6	M	153	182	15.7
CSS 7	M	150	180	14.4
CSS 8	F	144	181	7.5
CSS 9	F	142	184	2.9
CSS 10	F	143	187	1.2
CSS 11	F	152	183	13.8
CSS 12	-	100	143	-3.0
CSS 13	M	118	157	2.7
CSS 14	M	122	174	-8.3
CSS 15	M	116	162	-3.7
CSS 16	M	115	142	12.9
CSS 17	F	119	160	1.0
CSS 18	-	111	164	-10.5
CSS 19	M	117	159	-0.1
CSS 20	F	107	152	-3.9
CSS 21	F	130	179	-4.7
CSS 22	F	107	162	-12.7
CSS 23	M	118	177	-14.9
CSS 24	F	112	179	-22.7
CSS 25	F	113	168	-12.0
CSS 26	M	120	151	10.0
CSS 27	F	129	175	-2.2
CSS 28	F	123	160	5.0
CSS 29	F	124	161	5.1
CSS 30	M	123	167	-1.1

Circulating concentrations of cortisol, aldosterone, total T3, reverse T3 and triglycerides (TAG) were measured from the 30 study subjects to assess their potential to discriminate between physiological stress states. Study subjects displayed variability in baseline levels of total serum cortisol (mean ± s.d.: 231.7 ± 155.2 nM, max = 562.4 nM, min = 46.5 nM; Fig. 2A) and aldosterone (mean ± s.d.: 1019.6 ± 780.6 pM, max = 3906.3 pM, min = 317.6 pM; Fig. 2B), and the data were positively skewed (Shapiro-Wilk test for normality, $p = 0.02$ and $p < 0.0001$ for cortisol and aldosterone, respectively). Total T3 (mean ± s.d.: 2.10 ± 0.54 nM, max = 3.04 nM, min = 1.15 nM; Fig. 2C), reverse T3 (mean ± s.d.: 2.84 ± 1.20 nM, max = 5.34 nM, min = 1.18 nM; Fig. 2C), TAG levels (mean ± s.d.: 78.1 ± 34.1 mg/dL, max = 196.9 mg/dL, min = 30.7 mg/dL; Fig. 2D), and body condition index were normally distributed (Shapiro-Wilk test for normality, $p > 0.02$ for all the previously mentioned hormones, metabolite, and BCI).

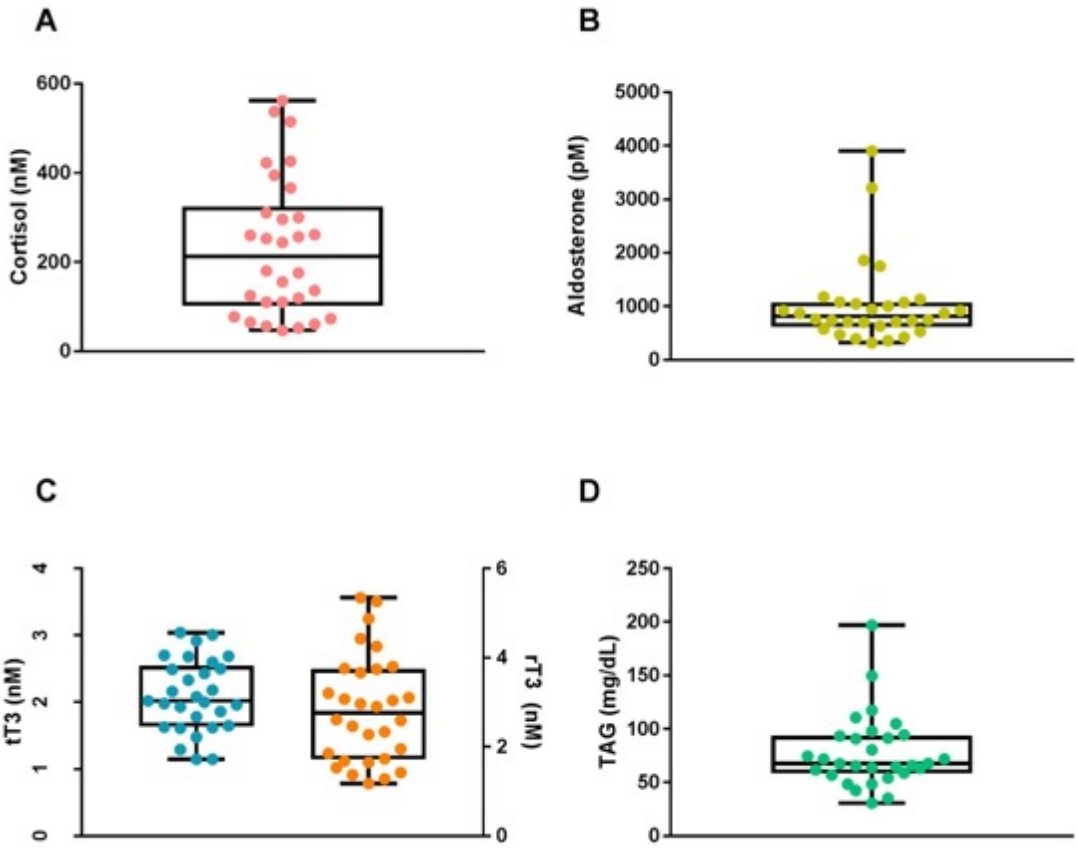


Figure 4: Measurements of cortisol (A), aldosterone (B), total and reverse triiodothyronine (T3, C), and triglycerides (TAG, D) for all study subjects (n=30). Whiskers indicate maximum and minimum concentration values, box indicates first and third quartile, line indicates the median.

In the previous repeated ACTH administration study, a lipidomics approach showed that TAG levels were significantly suppressed by ACTH [65]. To use TAG as a marker in this study, I first validated these data in the same sample set using a targeted colorimetric TAG assay. I used samples that were collected from 4 individuals before each of four ACTH. Injections (administered 24 hours apart), and four and eight hours after the first and fourth ACTH administration. TAG levels significantly decreased between the first day and the last day of ACTH injections (LMM: $F_{7,21} = 4.62$, $p = 0.003$; Tukey's post-hoc test on Day 1 and Day 4

ACTH administration: p = 0.0012; Fig. 3), and were consistent with the lipidomics data. Consequently, TAG levels in plasma were used as a stress marker in the present study.

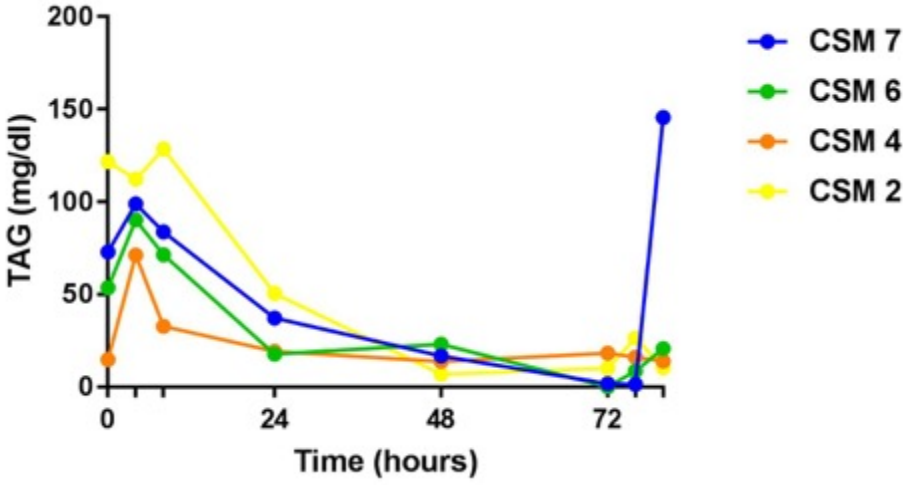


Figure 5: Circulating triglyceride (TAG) levels in plasma samples from 4 elephant seals during a repeated ACTH administration experiment [56]. Each subject is represented by a different color according to the legend. Seals received 4 ACTH injections 24 hours apart and blood samples were collected prior to and 4 and 8 hours after each ACTH injection.

Correlation Between Corticosteroids, Thyroid Hormones, Triglycerides, and BCI

Pairwise correlation analyses were used to examine potential associations between corticosteroid, thyroid hormones, body condition, and triglycerides. Serum cortisol was negatively correlated with total T3 levels (Pearson's correlation, r = -0.41, p = 0.026; Fig. 4A) and body condition index (Pearson's correlation, r = -0.44, p = 0.014; Fig. 4B), and positively correlated with aldosterone concentration (Pearson's correlation, r = 0.50, p = 0.004; Fig. 4C). Reverse T3 was negatively correlated with body condition index of the subjects (Pearson's correlation, r = -0.40, p = 0.031; Fig. 4D) and triglyceride levels in plasma (Pearson's correlation, r = -0.43, p = 0.017; Fig. 4E). There was, however, no significant correlation

between total T3 and reverse T3 ($p > 0.05$). Triglyceride levels were not significantly correlated with any of the corticosteroids, and BCI ($p > 0.05$).

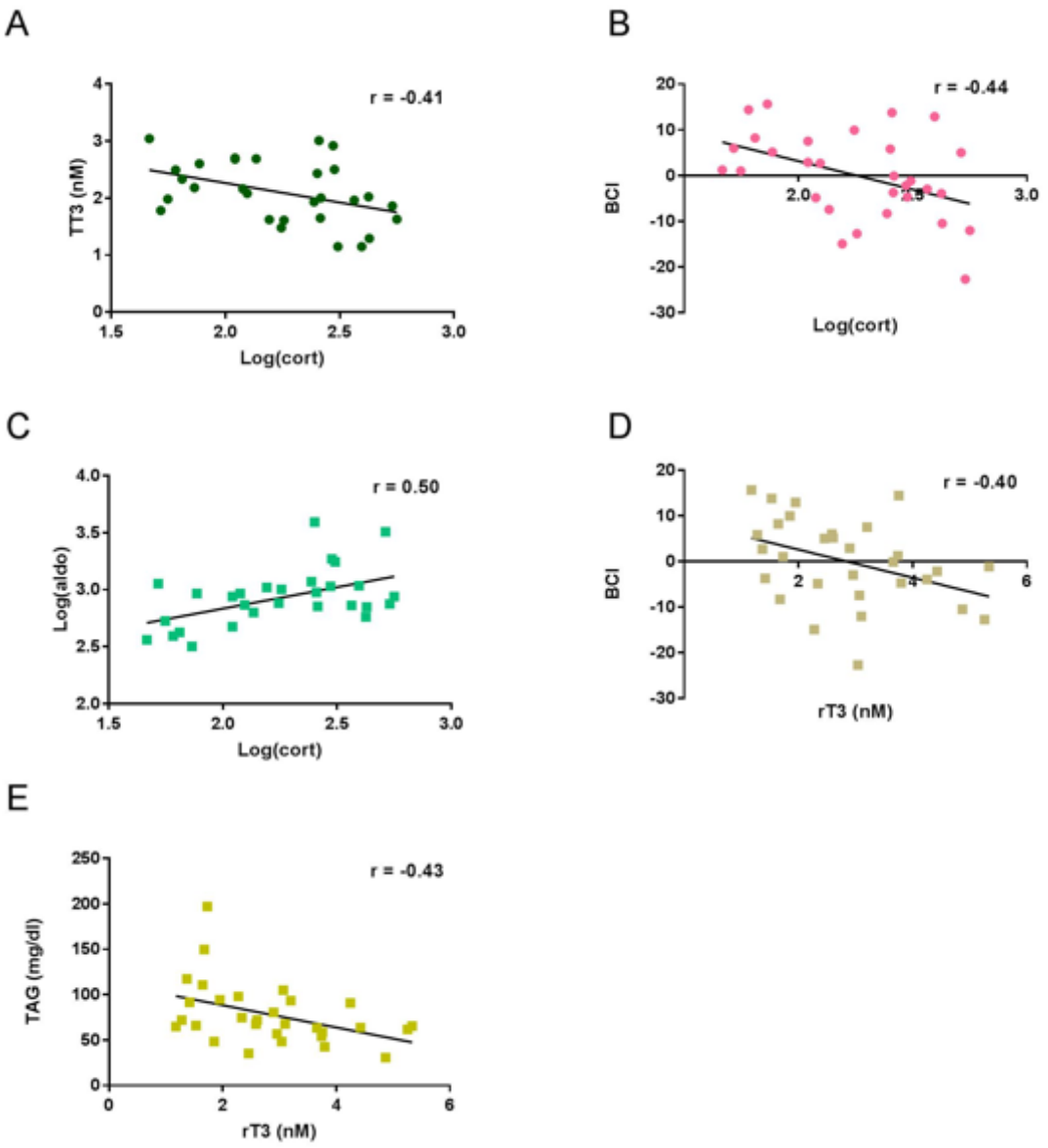


Figure 6: Significant correlations ($p < 0.05$) between hormones, metabolites, and body condition index of the study subjects. Pearson's correlations (r) were detected between (A) total T3 (tT3) and cortisol concentrations, (B) body condition index (BCI) and cortisol concentration, (C) aldosterone and cortisol concentrations, (D) triglycerides (TAG) and reverse T3 (rT3) concentrations, and (E) body condition index and rT3 levels.

Gene Expression Analyses

Candidate genes for qPCR assays were selected from the set of genes differentially expressed in elephant seal blubber in response to single and repeated ACTH administration in a previous study [56]. They had the following characteristics: 1) a significant BlastX hit to a protein with known function in the UniProt SwissProt proteome database, 2) high transcript abundance in the transcriptome (≤ 20 transcript per million), and 3) low variability in expression level between biological replicates for each condition (Table 3).

Table 3: Candidate gene markers selected using a previous study [56]. Genes selected were differentially expressed in response to a single and repeated exogenous ACTH administration (acute response and repeated response, respectively). Two genes were differentially expressed in both responses. ↑ : upregulation; ↓ : downregulation.

Acute ACTH response	↑ Hydroxylmethylglutaryl CoA-synthase 2 (HMGCS2)
	↑ Cysteine dioxygenase 1 (CDO1)
	↑ Adiponectin (ADIPOQ)
	↑ Microsomal glutathione S-transferase 1 (MGST1)
Repeated ACTH response	↑ Perilipin 1 (PLIN1)
	↑ Long-chain-fatty-acid CoA ligase 1 (ACSL1)
	↑ Leptin (LEP)
	↑ Zinc-alpha-2-glycoprotein 1 (AZGP1)
	↑ Cell death activator CIDE-A (CIDEA)
	↑ E3-SUMO-protein ligase PIAS4 (PIAS4)
	↑ Glycine receptor subunit alpha-2 (GLRA2)
	↓ Transforming growth factor beta-induced (TGFBI)
Differentially expressed genes common to both ACTH responses	↑ Glutathione peroxidase 3 (GPX3)
	↑ Dickkopf-related protein 1 (DKK1)

Candidate Gene Expression Level Correlations With Other Stress Markers

We used targeted qPCR assays to measure expression of 14 genes that were differentially expressed in response to HPA axis stimulation in a previous study in blubber tissue of northern elephant seals sampled in their baseline stress state. Normalized gene expression value (delta Ct) was analyzed for potential associations with the other stress markers previously measured. Total serum cortisol concentrations were positively correlated with baseline expression levels of six of the candidate genes. Cortisol was correlated with the adipokine adiponectin (ADIPOQ) (Spearman's correlation, $\rho = 0.52$, $p = 0.004$; Fig. 7A), the lipid droplet protein perilipin 1 (PLIN1), and the lipolysis stimulator zinc-alpha-2-glycoprotein 1 (AZGP1), were positively correlated with cortisol (Spearman's correlation, $\rho = 0.38$, $p = 0.05$ and $\rho = 0.40$, $p = 0.03$; Fig. 7B and 7C, respectively). The adipogenesis promoter, dickkopf-related protein 1 (DKK1), the ketogenesis enzyme, hydroxymethylglutaryl-CoA synthase 2 (HMGCS2), and the antioxidant enzyme, glutathione peroxidase 3 (GPX3) were also positively correlated with cortisol levels (Spearman's correlation, $\rho = 0.53$, $p = 0.0034$, $\rho = 0.46$, $p = 0.01$ and $\rho = 0.64$, $p = 0.0002$; Fig. 7D, 7E and 7F, respectively). There was no correlation between aldosterone concentration and normalized expression levels of any of the target genes ($p > 0.05$).

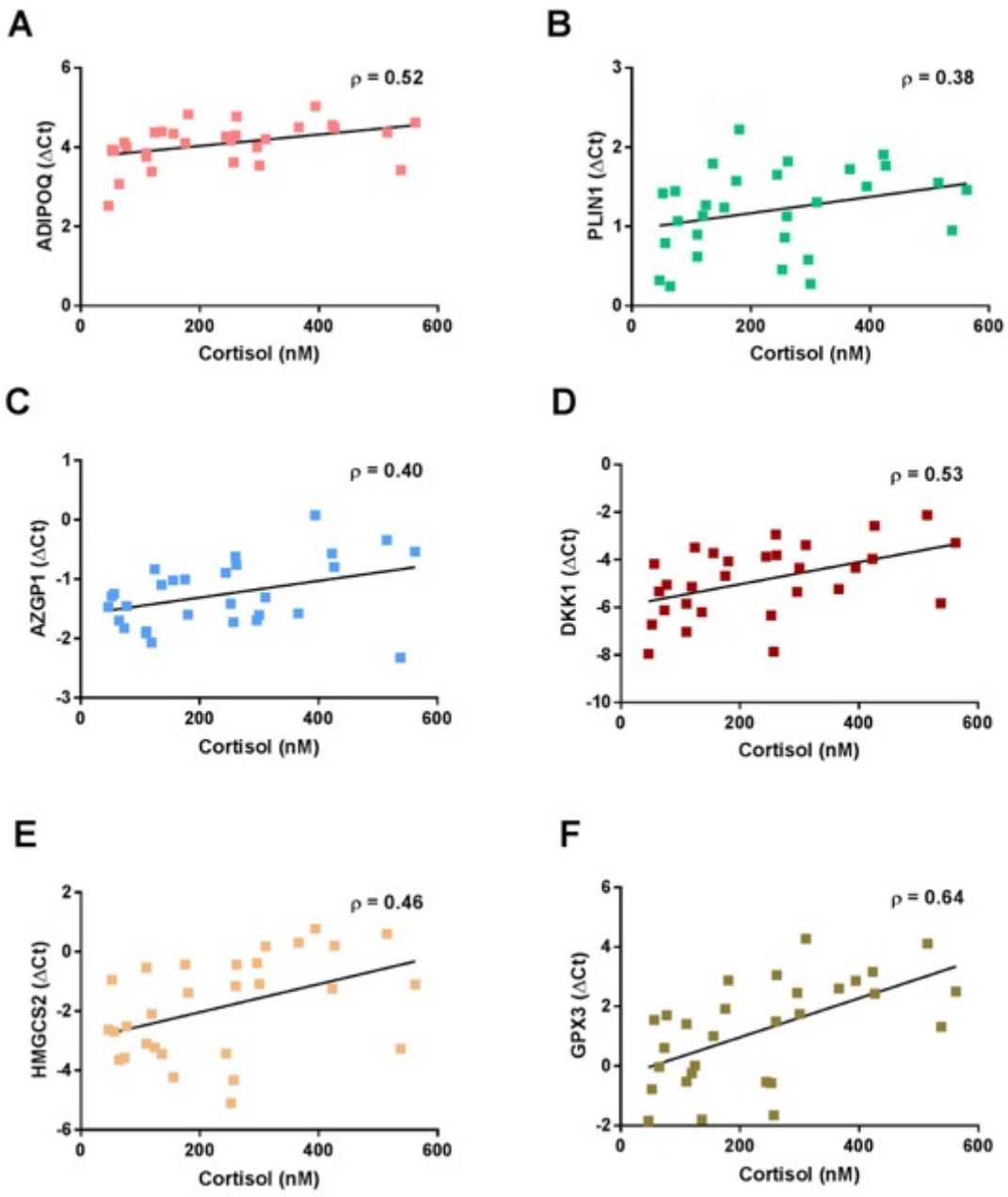


Figure 7: Significant Spearman's correlation (ρ) between blubber gene expression and cortisol levels for all study subjects ($p < 0.05$), Cortisol was positively associated ($p < 0.05$) with normalized expression values (ΔCt) of (A) ADIPOQ, (B) PLIN1, (C) AZGP1, (D) DKK1, (E) HMGCS2, and (F) GPX3.

The same genes that were positively correlated with cortisol were negatively correlated with total T3 concentrations (ADIPOQ: $r = -0.59$, $p = 0.001$; DKK1: $r = -0.72$, $p < 0.0001$;

PLIN1: r = -0.59, p = 0.001; GPX3: r = -0.60, p = 0.0007; AZGP1: r = -0.54 p = 0.003; HMGCS2: r = -0.47, p = 0.012; Fig. 8A-C, 8G-H). Two additional genes showed positive correlation with total T3: fatty acid oxidation enzyme long-chain-fatty-acid CoA ligase 1 (ACSL1) and lipid droplet protein cell death activator CIDE-A (Pearson's correlation; ACSL1: r = -0.40, p = 0.037; CIDEA: r = -0.48, p = 0.01; Fig. 8E and 8F). Transforming growth beta-induced (TGFBI) was the only target gene used in the study that was downregulated in response to repeated ACTH administration [56]. TGFBI was positively correlated with total T3 (Pearson's correlation, r = 0.38 p = 0.049; Fig. 8I).

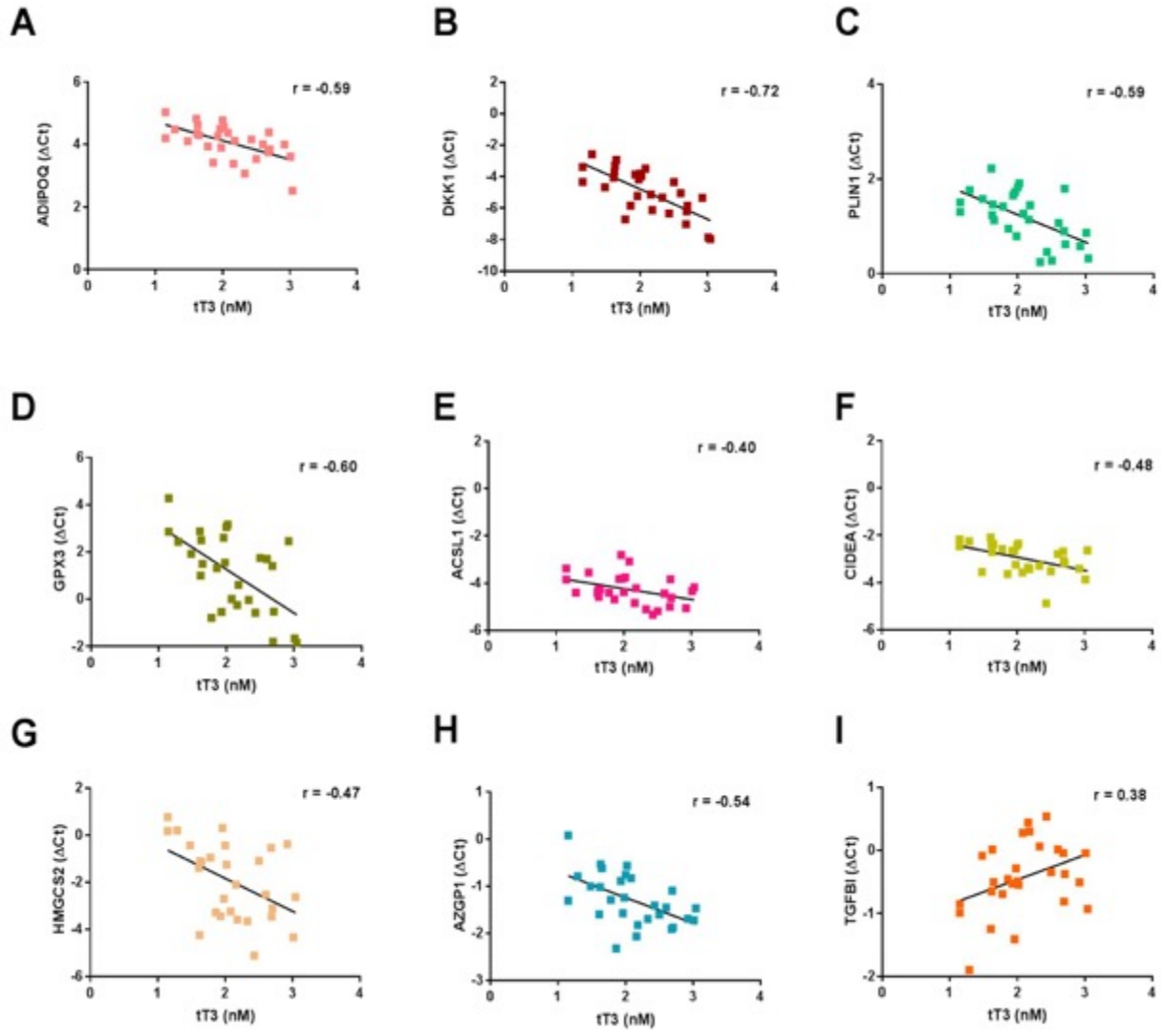


Figure 8: Significant correlation (r) between blubber gene marker expression value and circulating total T3 levels ($p < 0.05$). Total T3 was negatively associated with normalized gene expression values (ΔCt) of (A) ADIPOQ, (B) DKK1, (C) PLIN1, (D) GPX3, (E) ACSL1, (F) CIDEA, (G) HMGCS2, (H) AZGP1, and positively associated with (I) TGFBI.

Reverse T3 was negatively correlated with TGFBI and the adipokine leptin (LEP) (Fig. 9A-B). HMGCS2, GPX3, CIDEA and the adipogenesis marker, cysteine dioxygenase (CDO1) [66], were positively correlated with reverse T3 (Fig. 9C-F).

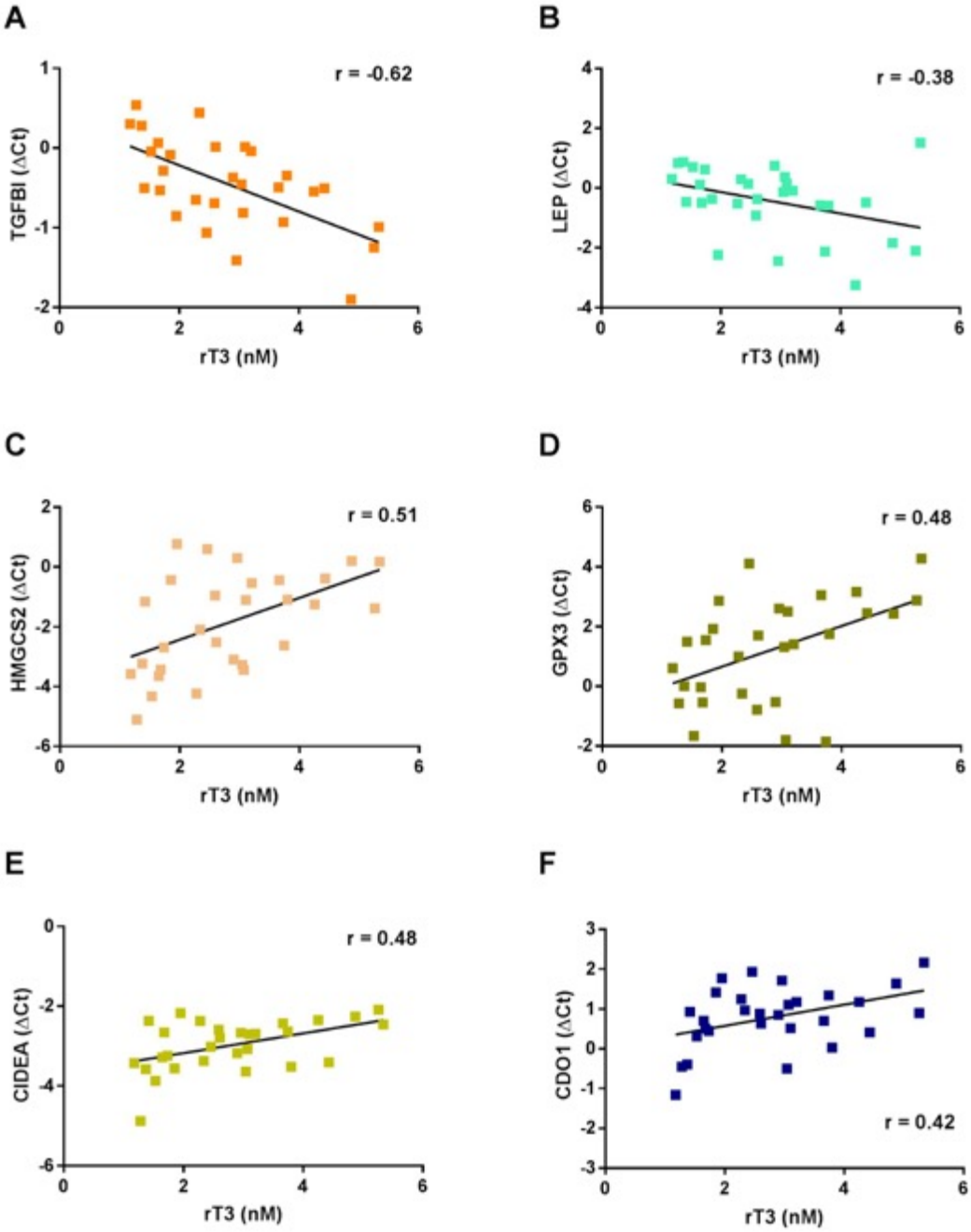


Figure 9: Significant correlation (r) between blubber gene expression value and circulating reverse T3 levels ($p < 0.05$). Reverse T3 was negatively associated ($p < 0.05$) with normalized gene expression values (ΔCt) of (A) TGFBI, (B) LEP, and positively associated with ΔCt of (C) HMGCS2, (D) GPX3, (E) CIDEA, and (F) CDO1.

Circulating TAG levels were negatively correlated with HMGCS2 (Pearson's correlation, $r = -0.38$, $p = 0.043$; Fig. 10). TAG concentration showed no significant correlations with any of the other target genes ($p > 0.05$). Body condition index was positively correlated with TGFBI (Pearson's correlation, $r = 0.37$, $p = 0.047$; Fig. 11) and did not show any significant correlation with any other gene markers ($p > 0.05$).

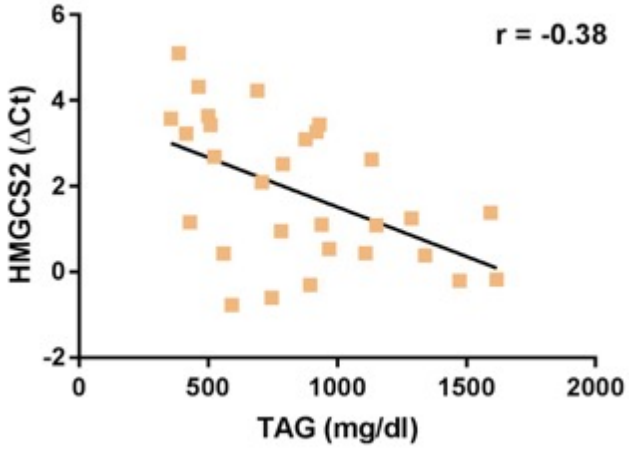


Figure 10: Plasma triglyceride (TAG) levels were negatively associated ($p < 0.05$) with normalized gene expression value (ΔCt) of HMGCS2.

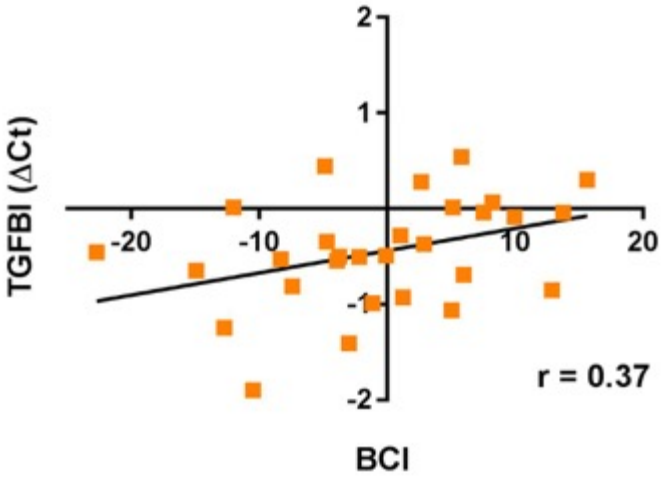


Figure 11: Body condition index was positively associated ($p < 0.05$) with normalized gene expression value (ΔCt) of TGFBI.

Chapter 4: Discussion

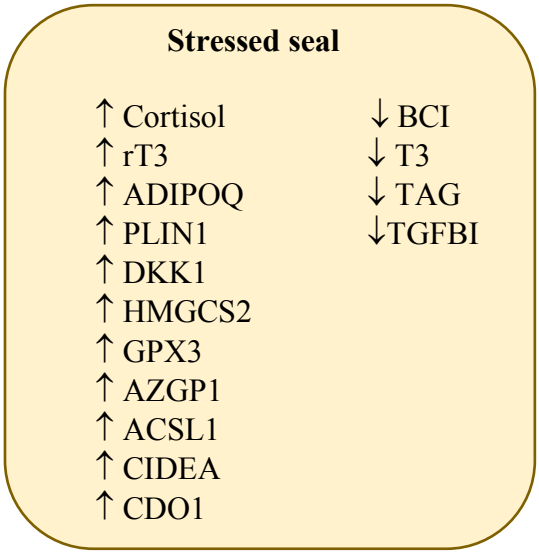
The aim of this study was to validate if variability in baseline stress states can be detected as changes in expression of stress-related genes measured by real-time quantitative polymerase chain reaction (RT-qPCR). I selected 14 candidate genes that were differentially expressed in elephant seal blubber during responses to acute and repeated HPA axis stimulation [56] and measured their expression levels in blubber tissue of 30 juvenile northern elephant seals of varying body conditions, and thus, variable baseline stress states. I used circulating corticosteroid, thyroid hormone, triglyceride levels and body condition index as metrics of the baseline physiological stress state of our study subjects. I then examined whether these metrics were correlated with normalized expression values of the candidate genes. A total of 11 genes, out of the 14 total that were evaluated, were correlated with one or more of the aforementioned metrics (Table 4). Three of the genes that were significantly correlated with hormone levels in this study were also differentially expressed in response to a single ACTH administration in our previous experiment, six genes were differentially expressed in response to repeated ACTH administration, and two genes were differentially expressed in both ACTH responses [56]. These novel molecular markers of tissue responses to HPA axis activation could potentially be used to differentiate a stressed marine mammal from a healthy one, and predict if the animal's stress response was due to an acute or a repeated (chronic) stressor.

Circulating free cortisol and aldosterone levels were used as hormone markers to assess baseline stress state of the study subjects. Values were within the range of baseline corticosteroid concentrations previously reported in juvenile northern elephant seals [47, 48]. Genes that were positively correlated with cortisol were associated with lipid metabolism

(ADIPOQ), lipid droplet formation (PLIN1), lipolysis (AZGP1), adipogenesis (DKK1), ketogenesis (HMGCS2), and oxidative stress (GPX3). All of these genes were upregulated in response to repeated HPA axis activation in a previous experiment, with the exception of ADIPOQ and HMGCS2, which were upregulated in response to a single ACTH administration. DKK1 was upregulated during both stress responses in the transcriptome study [56], as well as in another acute ACTH challenge on northern elephant seals [48]. ADIPOQ and HMGCS2 are associated with fatty acid oxidation [67, 68]. In addition, ADIPOQ has anti-inflammatory properties, and is correlated with increased energy expenditure, important for the stress response [69]. Increased expression of these two genes during an acute stress response can stimulate energy production from fatty acid catabolism to meet increased energy demands, as well as reduce inflammation, which is energetically costly. DKK1 promotes differentiation of pre-adipocytes into mature adipocytes, by Wnt signaling inhibition [70]. Adipogenesis can be beneficial during both stress responses, as it increases the number of mature adipocytes available for energy usage. AZGP1 and phosphorylation of PLIN1 have been associated with an increase in lipid mobilization and consequently, a decrease in adipose tissue in humans [71, 72], which is consistent with the known lipolytic effects of cortisol [73, 74]. Dysregulation of adipose GPX3 has been associated with an accumulation of local oxidative stress markers in obese subjects [75]. Upregulation of this gene during a chronic stress response could prevent damage from the increased production of reactive oxygen species in result of the sustained secretion of glucocorticoids [76]. Furthermore, oxidative genes could be used as markers to determine oxidative status of wild animals to enhance success of conservation and wildlife management [77]. An increase in expression levels of these genes in response to cortisol suggests that these

genes are its potential downstream effectors and may be used as additional molecular markers of stress in combination with hormone, metabolite, and other measurements.

Table 4: Stress-related biomarkers studied in this thesis, that could be used to identify and differentiate a stressed seal from a healthy seal. ↑ : increase in concentration or expression; ↓ : decrease in index value, concentration or expression.



Aldosterone is a mineralocorticoid involved in osmoregulation. Although its role in the stress response has not been extensively studied in terrestrial mammals [78], studies in several marine mammals, such as bottlenose dolphins and harbor seals, have shown increases of this mineralocorticoid in response to stressors, suggesting its potential involvement in the stress response in marine mammals [15, 16, 47, 55, 59]. Cortisol and aldosterone measurements were positively correlated in this study, as it has been previously seen in other northern elephant seal stress studies [47, 59, 79]. However, none of the candidate stress-related genes measured in this study were correlated with aldosterone. Mineralocorticoid receptors (MR) are present in adipose tissue, as well as other non-epithelial tissues in the body [80]. Increased levels of circulating

aldosterone have been correlated with body mass index and insulin sensitivity in obese patients [81], however its role in adipose tissue is not clear. In addition, MR may regulate preadipocytes differentiation to mature adipocytes [82]. Thus, further research on adipose-MR is necessary to understand aldosterone's role in adipose tissue regulation.

HPA axis activation induces a decrease in thyroid stimulating hormone release, which in turn inhibits biologically active triiodothyronine (T3) synthesis. I measured total T3 (tT3) as well as rT3 concentrations of our study subjects, and used these as additional stress markers for the validation of the candidate gene markers. Total T3 and rT3 levels were within the range of previously reported baseline values in juvenile northern elephant seals [47, 59]. While tT3 was negatively correlated with cortisol, rT3 was not associated with either tT3 or cortisol. However, other studies, in this species have shown that rT3 is positively correlated with cortisol [47, 59]. This suppression of tT3 and increase in rT3 has also been observed in other marine mammal species, such as odontocetes [83]. While tT3 regulates metabolism of many tissues, such as brain [84], muscle [85], liver [86], and adipose tissue [87], rT3 suppresses metabolism of these tissues by binding to tT3 receptors and blocking its normal function [33].

The same genes that were positively correlated with cortisol were negatively correlated with tT3, with the addition of a fatty acid metabolism gene, ACSL1 [88], and a lipid droplet protein, CIDEA [89]. ACSL1 is involved in anabolic and catabolic pathways of triglycerides and fatty acids [90]; for example, ACSL1 expression in adipocytes is related to TAG synthesis [91]. CIDEA expression regulates lipid metabolism by stimulating the formation of lipid droplets in adipose tissue [92-94]. Both genes were upregulated in blubber tissue of northern elephant seals during the response to repeated ACTH administration [56]. Expression of these genes during prolonged or repeated exposure to stress could be an adaptation of marine

mammals, as both genes are involved in replenishment of energy stores in fat tissue, crucial for marine mammals undergoing highly metabolic activities while fasting.

In addition, tT3 was positively correlated with TGFBI, a gene that was downregulated in response to repeated ACTH administration in juvenile northern elephant seals [56]. TGFBI was also positively correlated with body condition index and negatively correlated with rT3 levels. TGFB expression is associated with adipogenesis and inflammation in mice and humans [95, 96]. Expression of TGFBI is induced by TGFB, and has been linked to suppression of human preadipocyte differentiation [97]. Elevated tT3 is associated with fasting in northern elephant seals [98]. Furthermore, body condition in marine mammals is associated with increased adiposity [64]. In this study we show a negative correlation between body condition index and rT3 concentration. Based on these results, we suggest that increased expression of TGFBI is correlated with adiposity, and thus the absence of nutritional stress in marine mammals. However, further investigation is required to clarify the role of TGFBI in adipose tissue.

Reverse T3 was positively correlated with some of the genes that were negatively correlated with tT3; HMGCS2, CIDEA, and GPX3. In addition, other genes positively associated with rT3 were a gene involved in adipogenesis, CDO1, and an adipokine, LEP. CDO1 was upregulated during an acute stress response to ACTH, whereas LEP was upregulated in response to repeated ACTH administration on our previous study [56]. CDO1 catalyzes the oxidation of cysteine to cysteine sulfinic acid during the synthesis of taurine [99], an amino acid important in the metabolism of fats in the liver [100]. Also, expression of this gene increases during differentiation of preadipocytes to mature adipocytes, which suggests that CDO1 may regulate adipogenesis [66, 101]. LEP is an adipokine involved in control of energy homeostasis, as well as modulating immune responses [102]. Expression of these genes during the stress

response might be advantageous, as they act to manage energy storage and regulate inflammatory signals.

Lipidomics analysis of samples from our previous study showed that triglycerides (TAG) levels of individuals were significantly decreased in response to repeated ACTH administration [56, 57]. Furthermore, previous research on northern elephant seals has shown that non-esterified free fatty acids, product of triglyceride catabolism, increase with increasing cortisol in the system [47]. These measurements are consistent with the known lipolytic functions of cortisol and the increases in free fatty acids observed in multiple studies on northern elephant seals in response to ACTH [48, 49, 57, 59]. We used this metabolite as one of our markers of stress. To our surprise, triglyceride levels were only associated with rT3, and were not correlated with cortisol. However, we previously found that both rT3 and cortisol concentrations increased significantly in response to repeated ACTH administration [55], while TAG decreased. Nonetheless, triglyceride levels were negatively correlated with HMGCS2, a gene involved in ketogenesis, the formation of ketone bodies from fatty acids [68, 103]. In addition, HMGCS2 is also involved in beta-oxidation in the liver [68], a metabolic process that might be happening in adipose tissue as well. This negative correlation makes sense in the context of acute HPA axis activation, during which cortisol stimulates fatty acids mobilization from triglycerides, which are then catabolized by beta-oxidation.

Glucocorticoids and thyroid hormone measurements alone can be unreliable markers of stress states due to diurnal, seasonal, and life history variation in their baseline levels [46, 55, 59]. In this study, I introduce additional markers of stress state in marine mammals. Few wild animal studies that have used gene expression markers to evaluate stress states. However, transcriptome analyses of the cortisol target tissues blubber and muscle in elephant seals have

detected changes in gene expression 24 hours after a single HPA axis activation, even after cortisol levels returned to baseline[48, 49]. Therefore, gene activity may be a sensitive indicator of recent stress exposure that cannot be detected at the hormone level. Because tissue responses to GC are driven by changes in expression of target genes, identifying these downstream effectors of GCs can elucidate the functional consequences of stress, as well as potentially distinguish between acute and repeated stress responses. These data and those from our previous ACTH administration experiment suggest that HMGCS2 and ADIPOQ are upregulated in response to a single, or acute stressor in northern elephant seals, whereas, PLIN1, ACSL1, AZGP1, CIDEA, and TGFBI, are differentially regulated in response to repeated, or chronic stressors. In addition, GPX3 and DKK1, are genes involved in both stress responses. All of the previously mentioned genes were correlated with cortisol, as well as tT3. Our study suggests that high cortisol, a negative BCI, decreased levels of circulating tT3, and upregulation of PLIN1, GPX3, ADIPOQ, HMGCS2, AZGP1, and DKK1, could predict a stressed versus a non-stressed marine mammal.

Further research needs to be done to be able to utilize these biomarkers of stress on other marine mammal species, especially those that are endangered, such as the Hawaiian monk seal, or whale populations with low reproductive success. An increase in anthropogenic disturbance in the marine ecosystem can impact the fitness of marine mammal populations by inducing physiological stress that compounds the extreme physiological challenges these animals experience during the frequent hypoxia bouts, and the prolonged periods of fasting as part of their life histories. The ability to distinguish a stressed marine mammal from a healthy one, can assist marine ecosystem conservation strategies, and be used to justify closure of an area or

restriction of human activities in the area, such as reducing boat traffic, fishing, narrow shipping lanes, or decreasing sonar tests.

References

1. Sapolsky RM, Romero LM, and Munck AU, *How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preperative actions.* Endocrine Review, 2000. **21**: p. 55-89.
2. McEwen, BS, *Protective and damaging effects of stress mediators.* Seminars in Medicine of the Beth Israel Deaconess Medical Center, 1998.
3. McEwen, BS, *Stress, adaptation, and disease. Allostasis and allostatic load.* Ann N Y Acad Sci, 1998. **840**: p. 33-44.
4. McEwen BS and Wingfield JC, *The concept of allostasis in biology and biomedicine.* Hormones and Behavior, 2003. **43**(1): p. 2-15.
5. Romero LM, Dickens MJ, and Cyr NE, *The Reactive Scope Model - a new model integrating homeostasis, allostasis, and stress.* Horm Behav, 2009. **55**(3): p. 375-89.
6. Champagne CD, et al., *Fasting Physiology of the Pinnipeds: The Challenges of Fasting While Maintaining High Energy Expenditure and Nutrient Delivery for Lactation*, in *Comparative Physiology of Fasting, Starvation, and Food Limitation*. 2012. p. 309-336.
7. Adams C, Adams P, *Elephant Seals: A Comprehensive Guide*. 1999: Central Coast Press. 60.
8. Maxwell SM, Hazen EL, Bograd SJ, Halpern BS, Breed GA, Nickel B, Teutschel NM,Crowder LB, Benson S, Dutton PH, Bailey H, Kappes MA, Kuhn CA, Weise MJ, Mate B, Shaffer MA, Hassrick JL, Henry RW, Irvine L, McDonald BI, Robinson PW, Block BA, Costa DP, *Cumulative human impacts on marine predators.* Nature Commincations, 2013.

9. Atkinson S, Demaster D P, Calkins DG, *Anthropogenic causes of the western Steller sea lion Eumetopias jubatus population decline and their threat to recovery.* Mammal Review, 2008. **38**(1): p. 1-18.

10. Springer AM, Estes JA, van Vliet GB, Williams TM, Doak DF, Danner EM, Forney KA, Pfister B, *Sequential megafaunal collapse in the North Pacific Ocean: An ongoing legacy of industrial whaling?* Proceedings of the National Academy of Sciences 2003. **100**(21).

11. RS, Steneck, *Apex predators and trophic cascades in large marine ecosystems: learning from serendipity.* Proc Natl Acad Sci U S A, 2012. **109**(21): p. 7953-4.

12. Stevenson, Robert D., et al., *EcoPhysiology and Conservation: The Contribution of Endocrinology and Immunology– Introduction to the Symposium1.* Integrative and Comparative Biology, 2005. **45**(1): p. 1-3.

13. Milton, Sarah L., *Arctic ground squirrels handle stress with finesse.* Journal of Experimental Biology, 2005. **208**(19): p. v-v.

14. Hernández-Arciga, Ulalume, et al., *Baseline and post-stress seasonal changes in immunocompetence and redox state maintenance in the fishing bat Myotis vivesi.* PLOS ONE, 2018. **13**(1): p. e0190047.

15. Atkinson S, et al., *Stress physiology in marine mammals: how well do they fit the terrestrial model?* J Comp Physiol B, 2015. **185**(5): p. 463-86.

16. Cooke SJ and O'Connor CM, *Making conservation physiology relevant to policy makers and conservation practitioners.* Conservation Letters, 2010. **3**(3): p. 159-166.

17. Dantzer B, et al., *Measures of physiological stress: a transparent or opaque window into the status, management and conservation of species?* Conserv Physiol, 2014. **2**(1): p. cou023.

18. Kyrou, I. and C. Tsigos, *Stress hormones: physiological stress and regulation of metabolism.* Curr Opin Pharmacol, 2009. **9**(6): p. 787-93.

19. Herman JP, et al., *Neural regulation of the stress response: glucocorticoid feedback mechanisms.* Brazilian Journal of Medical and Biological Research, 2012. **45**(4): p. 292-298.

20. Dallman MF, et al., *Stress, feedback and facilitation in the hypothalamo-pituitary-adrenal axis.* J Neuroendocrinol, 1992. **4**(5): p. 517-26.

21. Lee, Do Yup, Eosu Kim, and Man Ho Choi, *Technical and clinical aspects of cortisol as a biochemical marker of chronic stress.* BMB Reports, 2015. **48**(4): p. 209-216.

22. Houser DS, Yeates LC, and Crocker DE, *Cold stress induces an adrenocortical response in bottlenose dolphins (Tursiops truncatus).* J Zoo Wildl Med, 2011. **42**(4): p. 565-71.

23. Champagne CD, et al., *Comprehensive endocrine response to acute stress in the bottlenose dolphin from serum, blubber, and feces.* Gen Comp Endocrinol, 2018. **266**: p. 178-193.

24. Yu CY, et al., *Genome-wide analysis of glucocorticoid receptor binding regions in adipocytes reveal gene network involved in triglyceride homeostasis.* PLoS One, 2010. **5**(12): p. e15188.

25. Kuo, T., et al., *Genome-wide analysis of glucocorticoid receptor-binding sites in myotubes identifies gene networks modulating insulin signaling.* Proc Natl Acad Sci U S A, 2012. **109**(28): p. 11160-5.

26. Phuc Le, P., et al., *Glucocorticoid receptor-dependent gene regulatory networks.* PLoS Genet, 2005. **1**(2): p. e16.

27. Romero, LM, *Physiological stress in ecology: lessons from biomedical research.* Trends Ecol Evol, 2004. **19**(5): p. 249-55.

28. Oppong E and Cato AC, *Effects of Glucocorticoids in the Immune System.* Adv Exp Med Biol, 2015. **872**: p. 217-33.

29. Crossin GT, et al., *Glucocorticoid manipulations in free-living animals: considerations of dose delivery, life-history context and reproductive state.* Functional Ecology, 2016. **30**(1): p. 116-125.

30. Helmreich DL, et al., *Relation between the Hypothalamic-Pituitary-Thyroid (HPT) Axis and the Hypothalamic-Pituitary-Adrenal (HPA) Axis during Repeated Stress.* Neuroendocrinology, 2005. **81**(3): p. 183-192.

31. Helmreich DL and Tylee D, *Thyroid hormone regulation by stress and behavioral differences in adult male rats.* Horm Behav, 2011. **60**(3): p. 284-91.

32. Brent, GA, *Mechanisms of thyroid hormone action.* J Clin Invest, 2012. **122**(9): p. 3035-43.

33. Bianco AC and Kim BW, *Deiodinases: implications of the local control of thyroid hormone action.* J Clin Invest, 2006. **116**(10): p. 2571-9.

34. Williams, GR, *Neurodevelopmental and neurophysiological actions of thyroid hormone.* J Neuroendocrinol, 2008. **20**(6): p. 784-94.

35. Cheng, Sheue-Yann, Jack L. Leonard, and Paul J. Davis, *Molecular aspects of thyroid hormone actions.* Endocrine reviews, 2010. **31**(2): p. 139-170.

36. Tomasi, T. E., Hellgren E. C., Tucker T. J., *Thyroid Hormone Concentrations in Black Bears (Ursus americanus): Hibernation and Pregnancy Effects.* General and Comparative Endocrinology, 1998. **109**: p. 192-199

.

37. JG, Eales, *The Influence of Nutritional State on Thyroid Function in Various Vertebrates.* American Zoology, 1988. **28**: p. 351-362.

38. Oki, C. and S. Atkinson, *Diurnal patterns of cortisol and thyroid hormones in the Harbor seal (Phoca vitulina) during summer and winter seasons.* Gen Comp Endocrinol, 2004. **136**(2): p. 289-97.

39. Rommel, John E. Reynolds III and Sentiel A., *Biology of marine mammals*. 1999, Washington: Smithsonian Institution Press. 578.

40. Muller, Martin N. and Richard W. Wrangham, *Dominance, cortisol and stress in wild chimpanzees (Pan troglodytes schweinfurthii).* Behavioral Ecology and Sociobiology, 2004. **55**(4): p. 332-340.

41. Beaulieu-McCoy NE, et al., *Initial validation of blubber cortisol and progesterone as indicators of stress response and maturity in an otariid; the California sea lion (Zalophus californianus).* Gen Comp Endocrinol, 2017. **252**: p. 1-11.

42. Carlitz EH, et al., *Measuring Hair Cortisol Concentrations to Assess the Effect of Anthropogenic Impacts on Wild Chimpanzees (Pan troglodytes).* PLoS One, 2016. **11**(4): p. e0151870.

43. Rangel-Negrín, A., et al., *Stress in Yucatan spider monkeys: effects of environmental conditions on fecal cortisol levels in wild and captive populations.* Animal Conservation, 2009. **12**(5): p. 496-502.

44. Crocker, D. E., et al., *Adiposity and fat metabolism in lactating and fasting northern elephant seals.* Adv Nutr, 2014. **5**(1): p. 57-64.

45. Fowler MA, Champagne CD, and Crocker DE, *Adiposity and fat metabolism during combined fasting and lactation in elephant seals.* J Exp Biol, 2018. **221**(Pt Suppl 1).

46. Dickens MJ and Romero LM, *A consensus endocrine profile for chronically stressed wild animals does not exist.* Gen Comp Endocrinol, 2013. **191**: p. 177-89.

47. Champagne C, et al., *Adrenal sensitivity to stress is maintained despite variation in baseline glucocorticoids in moulting seals.* Conserv Physiol, 2015. **3**(1): p. cov004.

48. Khudyakov JI, et al., *Blubber transcriptome response to acute stress axis activation involves transient changes in adipogenesis and lipolysis in a fasting-adapted marine mammal.* Sci Rep, 2017. **7**.

49. Khudyakov JI, et al., *Muscle transcriptome response to ACTH administration in a free-ranging marine mammal.* Physiol Genomics, 2015. **47**(8): p. 318-30.

50. Khudyakov, J. I., et al., *Transcriptome analysis of northern elephant seal (Mirounga angustirostris) muscle tissue provides a novel molecular resource and physiological insights.* BMC Genomics, 2015. **16**: p. 64.

51. Le Boeuf B., Laws R. M., *Elephant Seals: An Introduction to the Genus.* University of California Press, 1994.

52. Crocker DE, Williams JD, Costa DP, Le Boeuf BJ, *Maternal trais and Reproductive effort in Northern Elephant Seals.* Ecology, 2001. **82**(12): p. 3541-3555.

53. Fowler MA, Champagne CD, Crocker DE,, *Adiposity and fat metabolism in lactating and fasting northern elephant seals.* Adv Nutr, 2014. **5**(1): p. 57-64.

54. Champagne CD, et al., *The effects of handling and anesthetic agents on the stress response and carbohydrate metabolism in northern elephant seals.* PLoS One, 2012. **7**(5): p. e38442.

55. McCormley M, Champagne CD, Deyarmin JS, Stephan AS, Crocker DE, Houser DS and Khudyakov JI, *Repeated adrenocorticotropic hormone administration alters adrenal and thyroid hormones in free-ranging elephant seals* Conservation Physiology, 2018. **6**.

56. Deyarmin JS, McCormley MC, Champagne CD, Stephan AP, Pujade Busqueta L, Crocker DE, Houser DS, Khudyakov JI, *Distinct blubber transcriptome responses to single and repeated ACTH challenges in a marine mammal.* Scientific Reports 2019. **In press**.

57. Champagne CD, Khudyakov JI, McCormley MC, Deyarmin JS, Houser DS, Crocker DE *Metabolic response to acute and repeated stress in the northern elephant seal.* 2019. **Society of Integrative and Comparative Biology Annual Meeting**.

58. Viblanc, Vincent A., et al., *Body Girth as an Alternative to Body Mass for Establishing Condition Indexes in Field Studies: A Validation in the King Penguin.* Physiological and Biochemical Zoology, 2012. **85**(5): p. 533-542.

59. Ensminger DC, et al., *Metabolic responses to adrenocorticotropic hormone (ACTH) vary with life-history stage in adult male northern elephant seals.* Gen Comp Endocrinol, 2014. **204**: p. 150-7.

60. Bustin SA, et al., *The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments.* Clin Chem, 2009. **55**(4): p. 611-22.

61. Pfaffl MW, Tichopad A, Prgomet C and Neuvians TP, *Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations.* Biotechnology Letters, 2004. **26**: p. 509-515.

62. Huggett J, et al., *Real-time RT-PCR normalisation; strategies and considerations.* Genes Immun, 2005. **6**(4): p. 279-84.

63. Schmittgen, Thomas D. and Kenneth J. Livak, *Analyzing real-time PCR data by the comparative CT method.* Nature Protocols, 2008. **3**(6): p. 1101-1108.

64. Le Boeuf BJ and Crocker DE, *Ocean climate and seal condition.* BMC Biol, 2005. **3**: p. 9.

65. Fowler MA, et al., *Adipose Triglyceride Lipase, Not Hormone-Sensitive Lipase, Is the Primary Lipolytic Enzyme in Fasting Elephant Seals (Mirounga angustirostris).* Physiol Biochem Zool, 2015. **88**(3): p. 284-94.

66. Deng P, et al., *Cysteine dioxygenase type 1 promotes adipogenesis via interaction with peroxisome proliferator-activated receptor gamma.* Biochem Biophys Res Commun, 2015. **458**(1): p. 123-7.

67. Yamauchi T, et al., *Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase.* Nature Medicine, 2002. **8**: p. 1288.

68. Vila-Brau, A., et al., *Human HMGCS2 regulates mitochondrial fatty acid oxidation and FGF21 expression in HepG2 cell line.* J Biol Chem, 2011. **286**(23): p. 20423-30.

69. Kwon, H. and J. E. Pessin, *Adipokines mediate inflammation and insulin resistance.* Front Endocrinol (Lausanne), 2013. **4**: p. 71.

70. Christodoulides C, et al., *The Wnt antagonist Dickkopf-1 and its receptors are coordinately regulated during early human adipogenesis.* J Cell Sci, 2006. **119**(Pt 12): p. 2613-2620.

71. Hirai K, et al., *Biological evaluation of a lipid-mobilizing factor isolated from the urine of cancer patients.* Cancer Res, 1998. **58**(11): p. 2359-65.

72. Yamaguchi T, et al., *CGI-58 facilitates lipolysis on lipid droplets but is not involved in the vesiculation of lipid droplets caused by hormonal stimulation.* J Lipid Res, 2007. **48**(5): p. 1078-89.

73. Djurhuus CB, et al., *Effects of cortisol on lipolysis and regional interstitial glycerol levels in humans.* American Journal of Physiology-Endocrinology and Metabolism, 2002. **283**(1): p. E172-E177.

74. Djurhuus CB, Gravholt CH, Nielsen S, Pedersen SB, Møller N, Schmitz O, *Additive effects of cortisol and growth hormone on regional and systemic lipolysis in humans.* American Journal Physiology Endocrinology Metabolism, 2004. **286**: p. 488-494.

75. Lee YS, et al., *Dysregulation of adipose glutathione peroxidase 3 in obesity contributes to local and systemic oxidative stress.* Mol Endocrinol, 2008. **22**(9): p. 2176-89.

76. Costantini D, Marasco V, and Moller AP, *A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates.* J Comp Physiol B, 2011. **181**(4): p. 447-56.

77. Beaulieu M, Costantini D, *Biomarkers of oxidative status: missing tools in conservation physiology.* Conservation Physiology, 2014. **2**.

78. Kubzansky LD and Adler GK, *Aldosterone: a forgotten mediator of the relationship between psychological stress and heart disease.* Neurosci Biobehav Rev, 2010. **34**(1): p. 80-6.

79. Jelincic JA, et al., *Variation in adrenal and thyroid hormones with life-history stage in juvenile northern elephant seals (Mirounga angustirostris).* Gen Comp Endocrinol, 2017. **252**: p. 111-118.

80. Jia G, Aroor AR, and Sowers JR, *The role of mineralocorticoid receptor signaling in the cross-talk between adipose tissue and the vascular wall.* Cardiovasc Res, 2017. **113**(9): p. 1055-1063.

81. Dinh Cat AN, et al., *Adipocytes, aldosterone and obesity-related hypertension.* J Mol Endocrinol, 2016. **57**(1): p. F7-F21.

82. Caprio M, et al., *Pivotal role of the mineralocorticoid receptor in corticosteroid-induced adipogenesis.* The FASEB Journal, 2007. **21**(9): p. 2185-2194.

83. St. Aubin DJ, Geraci JR, *Capture and Handling Stress Suppresses Circulating Levels of Thyroxine (T4) and Triiodothyronine (T3) in Beluga Whales Delphinapterus leucas.* Physiological Zoology, 1988. **61**(2): p. 170-175.

84. J, Bernal, *Action of thyroid hormone in brain.* Journal of Endocrinological Investigation, 2002. **25**(3): p. 268-288.

85. Simonides WS, van Hardeveld C, *Thyroid hormones as a determinant of metabolic and contractile phenotype of skeletal muscle.* Thyroid, 2008. **18**: p. 205-216.

86. Sinha RA, Singh BK, and Yen PM, *Direct effects of thyroid hormones on hepatic lipid metabolism.* Nature reviews. Endocrinology, 2018. **14**(5): p. 259-269.

87. MJ, Obregon, *Adipose tissues and thyroid hormones.* Front Physiol, 2014. **5**: p. 479.

88. Souza SC, et al., *Modulation of hormone-sensitive lipase and protein kinase A-mediated lipolysis by perilipin A in an adenoviral reconstituted system.* J Biol Chem, 2002. **277**(10): p. 8267-72.

89. Puri V, Ranjit S, Konda S, Nicoloro SMC, Straubhaar J, Chawla A, Chouinard M, Lin C, Burkart A, Corvera S, Perugini RA, and Czech MP, *Cidea is associated with lipid droplets and insulin sensitivity in humans.* Proc Natl Acad Sci USA, 2008. **105**(22).

90. Coleman RA, Lewin TM, and Muoio DM, *Physiological and nutritional regulation of enzymes of triacylglycerol synthesis.* Annu Rev Nutr, 2000. **20**: p. 77-103.

91. Coleman RA, et al., *Do long-chain acyl-CoA synthetases regulate fatty acid entry into synthetic versus degradative pathways?* J Nutr, 2002. **132**(8): p. 2123-6.

92. Wu L, et al., *Cidea controls lipid droplet fusion and lipid storage in brown and white adipose tissue.* Sci China Life Sci, 2014. **57**(1): p. 107-16.

93. Xu L, Zhou L, Li P, *CIDE proteins and lipid metabolism.* Arterioscler. Vasc. Biol., 2012(32): p. 1094-1098.

94. Abreu-Vieira G, et al., *Cidea improves the metabolic profile through expansion of adipose tissue.* Nat Commun, 2015. **6**: p. 7433.

95. Fain JN, Tichansky DS, and Madan AK, *Transforming growth factor beta1 release by human adipose tissue is enhanced in obesity.* Metabolism, 2005. **54**(11): p. 1546-51.

96. Samad F, et al., *Tumor necrosis factor α is a key component in the obesity-linked elevation of plasminogen activator inhibitor 1.* Proceedings of the National Academy of Sciences, 1999. **96**(12): p. 6902-6907.

97. Hirata K, et al., *Role of leukotriene B(4) receptor signaling in human preadipocyte differentiation.* Biochem Biophys Res Commun, 2012. **429**(3-4): p. 197-203.

98. Crocker DE, et al., *Hormone and metabolite changes associated with extended breeding fasts in male northern elephant seals (Mirounga angustirostris).* Comp Biochem Physiol A Mol Integr Physiol, 2012. **161**(4): p. 388-94.

99. Stipanuk MH, et al., *Cysteine dioxygenase: a robust system for regulation of cellular cysteine levels.* Amino Acids, 2009. **37**(1): p. 55-63.

100. Yan CC, Bravo E, Cantafora A, *Effect of Taurine Levels on Liver Metabolism: An In Vivo Study in the Rat.* Experimental Biology and Medicine, 1993. **202**(1): p. 88-96.

101. Shaker M, et al., *Differential expression of cysteine dioxygenase 1 in complex karyotype liposarcomas.* Biomarkers Cancer, 2014. **6**: p. 1-10.

102. Francisco V, et al., *Obesity, Fat Mass and Immune System: Role for Leptin.* Frontiers in physiology, 2018. **9**: p. 640-640.

103. FG, Hegardt, *Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase: a control enzyme in ketogenesis.* Biochem J, 1999. **338**: p. 569-582.