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RESEARCH ARTICLE | *Obesity, Diabetes and Energy Homeostasis*

Expression of obesity-related adipokine genes during fasting in a naturally obese marine mammal

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Khudyakov JI, Abdollahi E, Ngo A, Sandhu G, Stephan A, Costa DP, Crocker DE. Expression of obesity-related adipokine genes during fasting in a naturally obese marine mammal. *Am J Physiol Regul Integr Comp Physiol* 317: R521–R529, 2019. First published August 7, 2019; doi:10.1152/ajpregu.00182.2019.—Northern elephant seals (*Mirounga angustirostris*) are exceptional among fasting-adapted animals in coupling prolonged fasting with energetically costly activities, relying on oxidation of fat stores accrued during foraging to power metabolic demands of reproduction and molting. We hypothesized that high rates of energy expenditure, insulin resistance, and immune responses to colonial breeding in fasting seals are mediated by adipokines, or signaling molecules secreted by adipose tissue that are associated with obesity and inflammation in humans. We measured mRNA expression of 10 adipokine genes in blubber tissue of adult female elephant seals sampled early and late during their lactation and molting fasts and correlated gene expression with adiposity and circulating levels of corticosteroid and immune markers. Expression of adiponectin (*ADIPOQ*) and its receptor *ADIPOR2*, leptin receptor (*LEPR*), resistin (*RETN*), retinol binding protein 4 (*RBP4*), and visfatin/nicotinamide phosphoribosyltransferase (*NAMPT*) was increased, whereas that of fat mass and obesity-associated protein (*FTO*) was decreased in late-fasted compared with early-fasted groups. Abundance of adipokine transcripts that increased in late fasting was negatively associated with body mass and positively associated with cortisol, suggesting that they may mediate local metabolic effects of cortisol in blubber during fasting. Expression of several adipokines was correlated with the immune markers IL-6, haptoglobin, IgM, and IgE, suggesting a potential role in modulating immune responses to colonial breeding and molting. Since many of these adipokines have not been measured in other wild animals, this study provides preliminary insights into their local regulation in fat tissue and targeted assays for future studies.

adipokine; blubber; fasting; metabolism; seal

INTRODUCTION

Many animals routinely experience periods of fattening and fasting associated with fluctuations in food availability and life history challenges, such as migration and reproduction. While rapid changes in body mass are associated with adverse health outcomes in humans (28), they are adaptive in other species and are regulated by complex endocrine, metabolic, and behavioral mechanisms. In most animals, fasting is characterized by a reduction in physical and metabolic activity and energy expenditure (39). However, several taxa of marine mammals are exceptional among fasting-adapted animals in coupling prolonged fasting with highly energetically demanding activities, such as lactation, breeding, molting, and migration (6). For example, adult northern elephant seals (*Mirounga angustirostris*) undergo biannual fasting periods that last up to four months for the duration of their life history (4). During the breeding fast, adult males defend breeding harems and mate with females, while adult females give birth and nurse their young for an average of four weeks (23). During the molting fast, elephant seals replace the entirety of their epidermis and hair within the span of one month (46). These terrestrial fasting periods are sustained by oxidation of fat stores accrued in blubber during two pelagic foraging trips and are associated with high rates of glucose recycling and low rates of protein oxidation (7, 37). Fasting elephant seals display low insulin production, insulin resistance, and elevated cortisol, which are characteristics of metabolic syndrome, albeit without any apparent pathologies seen in humans with metabolic disease (20).

Metabolic disease in humans is correlated with altered production of adipokines, or adipose tissue-derived signaling molecules (13). Adipokines, of which over 600 have been identified to date, act both locally and globally to regulate adipogenesis, tissue glucose uptake, carbohydrate and lipid metabolism, insulin secretion, gluconeogenesis, appetite and satiety, energy expenditure, and immune function (13). Adipokines that have been associated with insulin sensitivity and weight loss in humans and laboratory rodents include leptin, adiponectin, apelin, and visfatin/nicotinamide phosphoribosyltransferase, whereas those associated with insulin resistance and obesity include resistin, retinol binding protein 4, fat mass and obesity-associated protein, angiopoietin-like proteins, and chemerin, among many others (34). Many adipokines have also been associated with inflammation in adipose and cardiovascular tissues, which are the hallmarks of metabolic syndrome (32). The significance of adipokines in human pathology has prompted comparative examination of their roles in normal metabolic physiology in other animals, especially in those adapted to fasting.

Plasma levels and tissue-specific expression of well-known adipokines, such as leptin and adiponectin, have been measured in a number of hibernating and migratory species, including marine mammals. In hibernating mammals such as mink (27), marmots (15), and bears (36), plasma leptin and

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adiponectin increased during autumn hyperphagia, a period of rapid fat deposition, and decreased during winter hibernation, in parallel with body fat and insulin levels. In migratory bird species such as sparrows, plasma adiponectin and visfatin decreased during migration, a period of high energy expenditure (41). In pinnipeds (seals and sea lions), plasma leptin levels either decreased or did not change during fasting, and were not associated with fat mass (2, 8, 30, 44). Plasma adiponectin levels and adiponectin mRNA expression in blubber decreased over suckling and between suckling and early fasting in gray seal pups but did not change in lactating females over fasting (3). Together, these data suggest that decreased adipokine production may promote insulin resistance and high rates of lipid oxidation during periods of food deprivation in fasting-adapted animals. However, adipokine responses to fasting vary by species, life history stage, sex, and tissue type, and much remains unknown. For example, adipokines have never been measured in adult female elephant seals during their lactation fast, when they produce one of the most energy-dense milks in nature (up to 55% fat) using solely their lipid stores in blubber (16, 35). This energy-demanding fasting period has also been associated with innate and adaptive immune responses to breeding in a dense colonial environment (33). It is possible that the adipokines that have been associated with inflammation in other mammals (32) may also modulate immune function, and thus life history trade-offs, in adult female elephant seals.

The goal of this study was to examine adipokine gene expression dynamics during fasting associated with lactation and molting in adult female elephant seals. We hypothesized that changes in adipokine production may regulate metabolic and immune responses to fasting, and that these changes are more likely to be detected at the level of blubber mRNA expression than circulating protein concentration. Using a reference blubber transcriptome generated previously for this species (22), we identified and targeted 10 highly expressed adipokine-associated genes: leptin (*LEP*), leptin receptor (*LEPR*), adiponectin (*ADIPOQ*), adiponectin receptor 2 (*ADIPOR2*), resistin (*RETN*), retinol binding protein 4 (*RBP4*), visfatin/nicotinamide phosphoribosyltransferase (*NAMPT*), fat mass and obesity-associated protein (*FTO*), angiopoietin-like protein 4 (*ANGPTL4*), and chemerin (*RARRES2*). We used real-time PCR to measure expression of these genes in blubber sampled from adult female elephant seals early and late in lactation and molting, and correlated gene expression with levels of circulating corticosteroid hormones and immune markers previously measured in the same animals (33). We hypothesized that *1*) expression of genes associated with insulin resistance is upregulated, whereas expression of genes associated with insulin sensitivity and energy expenditure is downregulated during fasting, *2*) adipokine gene expression is correlated with adiposity and corticosteroid hormones, and *3*) expression of immunomodulatory adipokines is correlated with circulating immune marker levels, especially during breeding in adult female elephant seals.

MATERIALS AND METHODS

Study animals. Adult female northern elephant seals were sampled at Año Nuevo State Reserve (San Mateo County, CA). The cross-sectional sampling design is shown in Fig. 1. Four independent cohorts of females were sampled at the beginning and end of their fasting periods associated with lactation (Jan.–Feb. 2013) and molting (Apr.–Jun. 2013). Animals were marked with hair dye (Lady Clairol) within several days of arrival at the rookery to facilitate identification. Parturition dates were established by daily observations. Early and late molt samples ($n = 5$ each) were obtained from animals with ~0% and 100% visibly molted pelage, respectively. Early and late lactation samples ($n = 6$ each) were collected five days and three weeks postpartum, respectively. All animal handling procedures were approved by Sonoma State University and University of California, Santa Cruz Institutional Animal Care and Use Committees and were conducted under National Marine Fisheries Service marine mammal permit 14636.

Sample collection. Study animals were chemically immobilized by intramuscular injection of Telazol (tiletamine/zolazepam HCl, Fort Dodge Animal Health, Fort Dodge, IA) at a dosage of ~1 mg/kg and maintained with ~100 mg bolus intravenous injections of ketamine as previously described (17). Blood samples were collected via an 18-gauge 3.5-inch needle from the extradural vein and stored on ice until return to the laboratory. Blubber samples were collected from the posterior flank of each animal using a 6.0-mm diameter biopsy punch (Miltex, Plainsboro, NJ), flash frozen in liquid nitrogen, and stored at −80°C. Blood samples were centrifuged at 1,500 *g* for 15 min at 4°C, and isolated serum and plasma were stored at −80°C until processing. Body composition (% adiposity) was determined by the truncated cones method using morphometric and ultrasound measurements of blubber thickness (18). Mass was measured using a tripod, canvas sling, and scale (±1 kg, Measurement Systems International, Kent, WA). Mass and body composition were not obtained for one animal.

Hormone assays. Cortisol and aldosterone were assayed in duplicate using commercially available radioimmunoassay kits (Siemens, Munich, Germany), which were previously validated for use in elephant seals (21, 31). Intra-assay and inter-assay coefficients of variation (CV) for all hormones analyzed were <5%.

RNA isolation. RNA isolation was conducted as previously described (22). Briefly, the inner blubber portion (closest to musculature) was separated from frozen blubber biopsies and minced with a sterile scalpel on ice. Minced tissue was added to Qiazol (~100 mg tissue/1.0 ml; Qiagen, Germantown, MD), and homogenized using

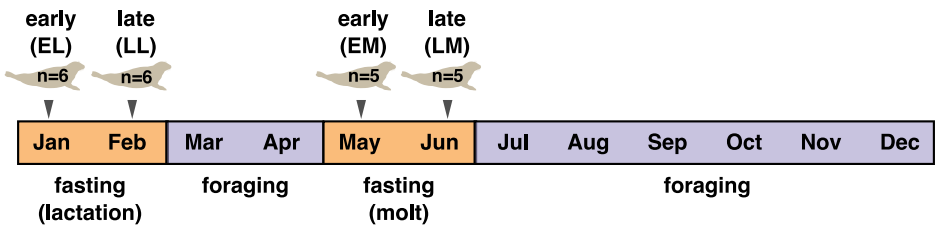

Fig. 1. Overview of cross-sectional sampling design. Four independent cohorts of adult female elephant seals were sampled for the study: six during early lactation (EL), six during late lactation (LL), five during early molt (EM), and five during late molt (LM). Early groups were sampled within ~1 wk of returning to the rookery from a foraging trip, whereas late groups were sampled after ~3–4 wk of fasting. Seal image (color modified, from https://pixabay.com) was obtained under CC0 1.0 Universal, CC0 1.0 Public Domain Dedication.

5-mm steel beads in a TissueLyser II instrument (2 cycles of 2 min at 20 mHz, Qiagen), followed by disruption with a 21-gauge needle. Total RNA was extracted using Lipid RNeasy Tissue Kit (Qiagen) following the manufacturer's protocol with a 20-min on-column DNase I digest (Qiagen). Eluates were concentrated by sodium acetate precipitation. RNA concentration was estimated using Qubit 3.0 fluorometer (RNA Broad Range Assay, Life Technologies, Carlsbad, CA) and RNA integrity was evaluated using 2100 Bioanalyzer (RNA 6000 Pico Kit, Agilent Technologies, Santa Clara, CA). The mean ± SD RNA integrity number (RIN) for RNA samples was 7.7 ± 0.6.

RT-qPCR. Complementary DNA (cDNA) was synthesized using 1 μg total RNA and iScript gDNA Clear cDNA Synthesis Kit according to the manufacturer's protocol (Bio-Rad, Hercules, CA). cDNA was diluted 1:10, and 2 μl were used per 20-μl qPCR reaction with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Santa Clara, CA). Reactions were run in triplicate according to manufacturer's protocol on the QuantStudio 5 RT-PCR System (Thermo Fisher Scientific). The amplification program was as follows: 2 min at 50°C, 2 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All primers were used at 400 nM final concentration. The mean intra-assay and interassay CV for all primers were 0.2% and 1.1%, respectively. *YWHAZ* was used as the reference gene based on expression stability previously confirmed in elephant seal blubber (22). Normalized gene expression ratios (delta Ct, or dCt) were obtained by subtracting the mean threshold cycle (Ct) value for the gene of interest (GOI) from the mean Ct value for the reference gene (ref; $dCt = Ct_{ref} - Ct_{GOI}$).

Primer design and validation. Adipokine targets for qPCR were selected based on *1*) the presence of an elephant seal homolog of the adipokine gene in an elephant seal blubber transcriptome [annotated by BLASTX against UniProt database; e-value threshold of 0.001 (22)] and *2*) its expression at levels that could be measured by RT-qPCR (transcripts per million, or TPM > 10). Primers were designed using the PrimerQuest Tool (Integrated DNA Technologies, Choralville, IA) and the northern elephant seal blubber transcriptome assembly as reference (22). Primer specificity was evaluated by BLAST and confirmed by lack of amplification in reactions with minus-RT and no-template controls. Amplification of a single product was evaluated by melt curve analysis and confirmed by gel electrophoresis and Sanger sequencing of qPCR products. Primer efficiency was calculated using standard curves of serially diluted pooled cDNA (1:5, 1:10, 1:20, 1:40, 1:80, and 1:160). Efficiencies of all primers were > 99% and R^2 values for all standard curves were > 0.99 (Table 1).

Statistical data analysis. Normalized gene expression ratios, expression fold changes, and primer efficiencies were calculated using Microsoft Excel 2017. Statistical analyses were conducted using R version 3.6.0 in RStudio v1.1.453. One-way ANOVA was conducted using the car package. Levene's and Shapiro-Wilk tests were used to determine whether variables met equal variance and normality assumptions, respectively. Post hoc pairwise comparisons were conducted using Student's *t* tests with Benjamini and Hochberg correction for multiple hypothesis testing (false discovery rate (FDR) = 0.05). Nonparametric tests (Kruskal-Wallis χ^2 test, followed by pairwise Wilcoxon rank sum tests with Benjamini and Hochberg correction, FDR = 0.05) were used for variables that did not meet ANOVA assumptions. Spearman correlation (r_S) was used to examine associations between adipokine genes and other variables, because most markers did not have a normal distribution. Principal component analysis was deemed inappropriate for this study because despite significant intercorrelation between adipokine genes (Bartlett's test, $P < 0.0001$), the sample size was inadequate (Kaiser-Meyer-Olkin test overall measure of sampling adequacy = 0.5), and there was some multicollinearity (determinant = 0.00035) (14). Therefore, multiple correlation between body mass, adipokine gene expression values, and corticosteroid hormone levels was conducted with Benjamini and Hochberg correction for multiple hypothesis testing (FDR = 0.05). Correlograms were created using R packages Hmisc and corrplot. Exploratory pairwise correlation was used to examine putative associations between adipokine genes and immune marker levels with a more stringent significance threshold of $P < 0.01$.

RESULTS

We compared hormone and gene expression levels in four independent cohorts of adult female northern elephant seals: early lactation, 5 days after parturition and within an average of 10 days from arrival at the rookery from a ~6-mo postmolt foraging trip ($n = 6$); late lactation, after ~4 wk of lactation ($n = 6$); early molt, within several days of arrival at the rookery from a ~2-mo postbreeding foraging trip ($n = 5$); and late molt, after ~3–4 wk of molting ($n = 5$; Fig. 1). The comparisons between groups were as follows: late lactation/early lactation (lactation fast), late molt/early molt (molting fast), early lactation/late molt (postmolt foraging trip), early molt/late lactation (postbreeding foraging trip), early lactation/early molt, and late lactation/late molt.

Total body mass was significantly different between fasting groups ($F_{3,17} = 12.15$, $P = 0.00017$, Fig. 2*A*) and was, on average, 26.5% lower in late lactation (319 ± 37 kg) than early lactation (434 ± 40 kg; $P = 0.00082$) and 22.8% lower in late molt (322 ± 35 kg) compared with early molt (417 ± 53 kg;

Table 1. *Primer sequences and amplification efficiencies of qPCR assays used in the study. All sequences are written in the 5′ to 3′ direction*

Gene	Forward Primer	Reverse Primer	Efficiency, %
LEP	ACAGGACCAAAGCCACAGGA	GCGAGGCCTGAGAAGCACAT	104.5
LEPR	GGTATCATAGGAGCAGCCTTTAC	TCTCTCTGCAAGTGGCAATC	104.9
ADIPOQ	CACTGTCCCCAATGTTCCCA	CCCAGGAATGTTGCAGTGGA	105.8
ADIPOR2	GGGTGTTCCAGAGCTCCACA	GCTAGCCCAGAGGCTGATCT	100.9
RBP4	CAGCTTCCGAGTCAAGAAGAAC	GCCATTCTCATCCACAGAGAAC	99.1
RETN	GTCACCGCCTGCACTTG	GCTCCGGTCCAGTCCAT	105.3
FTO	ACTCGGTGGGTGGAACTAAA	ACCAAGGAGACTGCTACTTCAT	108.6
NAMPT	AGAAGGTGAAATACGAGGAAACA	TACACCTCTTTGGCTTCTTGG	108.7
RARRES2	GGCCTTCAAGAAGACCAGTATG	CTCGTCTGCTGGAGCTTAAAT	100.3
ANGPTL4	CAAGAGCTGTTTGACGATGGAG	CATCTGAGGTCATCTTGCAGTTC	100.5
YWHAZ	AGCAGAGAGCAAAGTCTTCTATT	GACTGATCCACAATCCCTTTCT	100.3

LEP, leptin; *LEPR*, leptin receptor; *ADIPOQ*, adiponectin; *ADIPOR2*, adiponectin receptor 2; *RBP4*, retinol-binding protein 4; *RETN*, resistin; *FTO*, fat mass and obesity-associated protein; *NAMPT*, visfatin/nicotinamide phosphoribosyltransferase; *RARRES2*, chemerin/retinoic acid receptor responder 2; *ANGPTL4*, angiopoietin-like protein 4; *YWHAZ*, reference gene based on expression stability previously confirmed in elephant seal blubber (22).

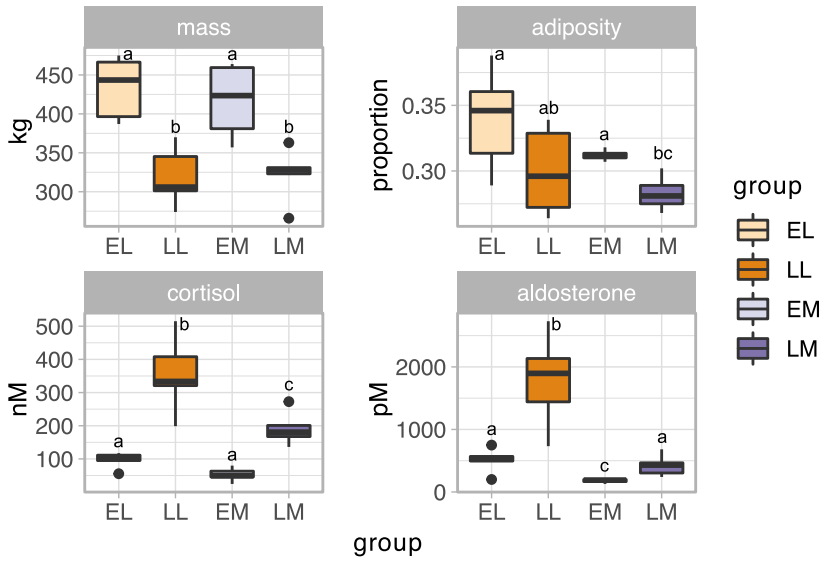

Fig. 2. Total body mass, adiposity (proportion of fat mass), and blood cortisol and aldosterone levels measured in four independent cohorts of adult female elephant seals sampled for the study. Different letters denote significant differences between groups (ANOVA followed by Student's t test with Benjamini Hochberg correction, $P < 0.05$). EL, early lactation ($n = 6$); LL, late lactation ($n = 6$); EM, early lactation ($n = 5$); LM, late lactation ($n = 5$).

$P = 0.0044$). Conversely, mass was, on average, 34.8% higher in females returning from than those departing for the postmolt foraging trip ($P = 0.00088$) and 30.7% higher in those returning from than those departing for the postbreeding foraging trip ($P = 0.0033$). Adiposity (proportion fat mass) was different between fasting groups ($\chi^2 = 8.61$, $df = 3$, $P = 0.035$; Fig. 2*B*); it was, on average, 9.3% lower in late molt (28.3 ± 1.3%) compared with early molt (31.2 ± 0.5%; $P = 0.052$) and 20.1% higher in animals returning from (34.0 ± 3.7%) than those departing for the postmolt foraging trip, which approached significance ($P = 0.052$). Adiposity did not differ between early and late (30.0 ± 3.3%) lactation groups ($P = 0.15$) and between females departing for and those returning from the postbreeding foraging trip ($P = 0.58$). Neither mass nor adiposity were different between early lactation and early molt (mass: $P = 0.63$; adiposity: $P = 0.58$) and late lactation and late molt (mass: $P = 0.90$; adiposity: $P = 0.58$).

Corticosteroid levels were significantly different between groups (cortisol: $F_{3,18} = 25.32$, $P < 0.0001$; aldosterone: $\chi^2 = 17.28$, $df = 3$, $P = 0.00062$; Fig. 2, *C* and *D*). Cortisol was 3.63-fold higher in late lactation (355.07 ± 107.62 nM) compared with early lactation (97.93 ± 22.52 nM; $P < 0.0001$) and 3.65-fold higher in late molt (191.57 ± 51.08 nM) than early molt (52.51 ± 20.57 nM; $P = 0.0042$). Aldosterone was 3.51-fold higher in late lactation (1793.17 ± 694.74 pM) than early lactation (510.85 ± 178.70 pM; $P = 0.0087$) and 2.39-fold higher in late molt (424.23 ± 170.92 pM) than early molt (177.36 ± 30.93 pM; $P = 0.010$). Cortisol levels were 1.96-fold lower in animals returning from the postmolt foraging trip ($P = 0.031$) and 6.76-fold lower in those returning from the postbreeding foraging trip ($P < 0.0001$). Aldosterone did not change during the postmolt foraging trip ($P = 0.33$) but was 10.11-fold lower in females returning from the postbreeding foraging trip ($P = 0.0087$). Cortisol was 1.85-fold higher in late lactation compared with late molt ($P = 0.00096$). Aldosterone was 2.88-fold higher in early lactation compared with early molt ($P = 0.010$) and 4.23-fold higher in late lactation compared with late molt ($P = 0.0087$).

Seven genes differed in normalized expression levels (dCt) between sampling groups (Fig. 3). These included leptin receptor (*LEPR*; $F_{3,18} = 5.07$, $P = 0.010$), adiponectin (*ADIPOQ*; $F_{3,18} = 7.78$, $P = 0.0015$), adiponectin receptor 2 (*ADIPOR2*; $F_{3,18} = 3.47$, $P = 0.038$), retinol-binding protein 4 (*RBP4*; $F_{3,18} = 6.07$, $P = 0.0049$), resistin (*RETN*; $F_{3,18} = 5.83$, $P = 0.0058$), fat mass and obesity-associated protein (*FTO*; $F_{3,18} = 7.13$, $P = 0.0023$), and visfatin/nicotinamide phosphoribosyltransferase (*NAMPT*; $F_{3,18} = 3.83$, $P = 0.028$). Three genes did not differ in expression between groups: leptin (*LEP*; $P = 0.093$), chemerin/retinoic acid receptor responder 2 (*RARRES2*; $P = 0.27$), and angiopoietin-related protein 4 (*ANGPTL4*; $P = 0.55$).

LEPR expression was 3.32-fold higher ($P = 0.013$) and *ADIPOQ* expression was 2.66-fold higher ($P = 0.029$) in late lactation compared with early lactation. In contrast, *FTO* expression was 1.46-fold lower in late compared with early lactation ($P = 0.039$). *ADIPOQ* expression was 4.38-fold higher ($P = 0.0046$) and *RBP4* expression was 3.99-fold higher ($P = 0.0051$) in late molt compared with early molt. Genes that had lower expression in females returning from the postmolt foraging trip included *ADIPOQ* (−5.02-fold, $P = 0.0028$), *RBP4* (−2.46-fold, $P = 0.028$), and *NAMPT* (−1.47-fold, $P = 0.025$). Genes that had lower expression in females returning from the postbreeding foraging trip included *LEPR* (−2.92-fold, $P = 0.021$), *RETN* (−5.79-fold, $P = 0.005$), *RBP4* (−2.60-fold, $P = 0.005$), and *ADIPOQ*, which approached significance (−2.32-fold, $P = 0.059$). In contrast, *FTO* expression was 2.04-fold higher in animals returning from the postbreeding foraging trip ($P = 0.0015$). *RETN* expression was 3.67-fold higher in early lactation compared with early molt ($P = 0.025$). *FTO* expression was 1.55-fold lower in late lactation compared with late molt ($P = 0.036$).

We next evaluated relationships between expression levels of the seven genes that varied with fasting stage and body mass and corticosteroid levels (Fig. 4). Mass was negatively correlated with *LEPR* ($r_S = -0.61$, $P = 0.0093$), *ADIPOQ* ($r_S = -0.60$, $P = 0.010$), *ADIPOR2* ($r_S = -0.50$, $P = 0.039$), and *RBP4* ($r_S = -0.55$, $P = 0.020$). Cortisol was positively correlated with *LEPR* ($r_S = 0.63$, $P = 0.0069$), *ADIPOQ* ($r_S = 0.48$, $P = 0.044$), *RBP4* ($r_S = 0.62$, $P = 0.0079$), and *RETN* ($r_S = 0.61$, $P = 0.0080$), and negatively

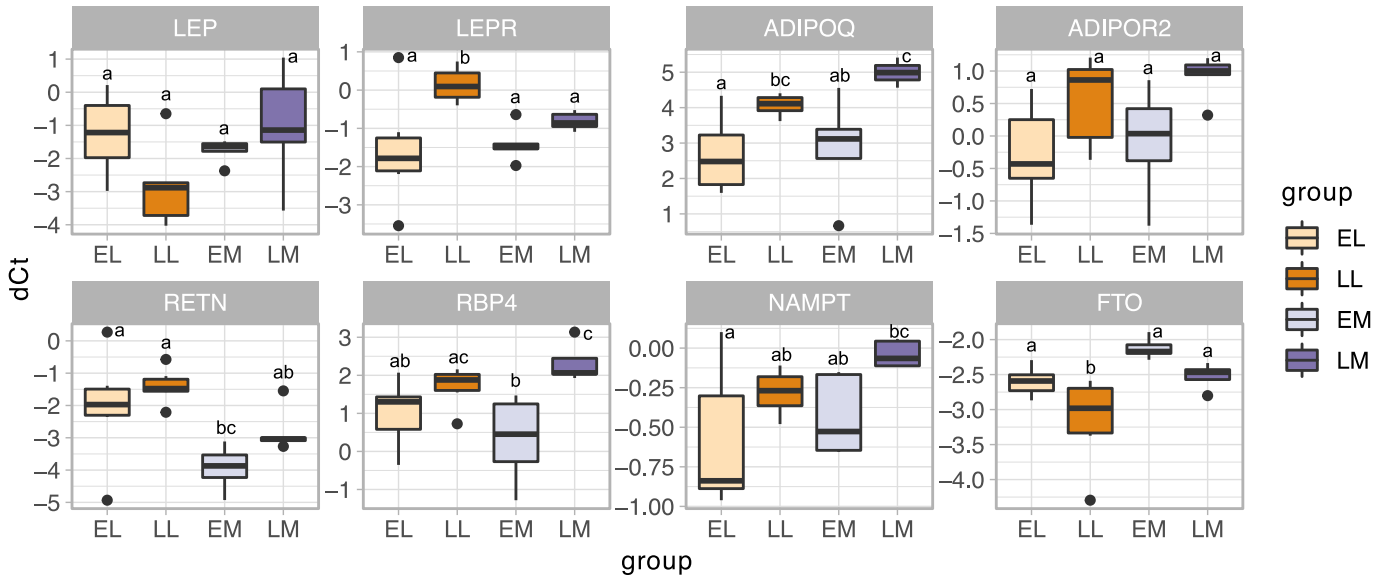

Fig. 3. Mean normalized expression levels (dCt) of adipokine genes measured in four independent cohorts of adult female elephant seals sampled for the study. Different letters denote significant differences between groups (ANOVA followed by Student's *t* test with Benjamini Hochberg correction, $P < 0.05$). *LEP*, leptin; *LEPR*, leptin receptor; *ADIPOQ*, adiponectin; *ADIPOR2*, adiponectin receptor 2; *RETN*, resistin; *RBP4*, retinol-binding protein 4; *NAMPT*, visfatin/nicotinamide phosphoribosyltransferase; *FTO*, fat mass and obesity-associated protein; EL, early lactation ($n = 6$); LL, late lactation ($n = 6$); EM, early lactation ($n = 5$); LM, late lactation ($n = 5$).

correlated with *FTO* ($r_S = -0.67$, $P = 0.0054$). Aldosterone was positively correlated with *LEPR* ($r_S = 0.64$, $P = 0.0069$) and *RETN* ($r_S = 0.65$, $P = 0.0061$) and negatively correlated with *FTO* ($r_S = -0.77$, $P = 0.00028$). *NAMPT* was not associated with mass ($P = 0.21$), cortisol ($P = 0.11$), or aldosterone ($P = 0.83$). Among the adipokines, *ADIPOQ*, *ADIPOR2*, *LEPR*, and *RBP4* were all positively associated ($P < 0.05$). *NAMPT* was positively associated with *ADIPOQ*, *ADIPOR2*, and *RBP4*, while *FTO* was negatively associated with *LEPR* and *RETN* ($P < 0.05$).

Finally, we explored putative associations between gene expression levels and circulating immune markers measured in the same animals in a previous study. *LEP* was negatively associated, while *RETN* was positively associated with hapto-

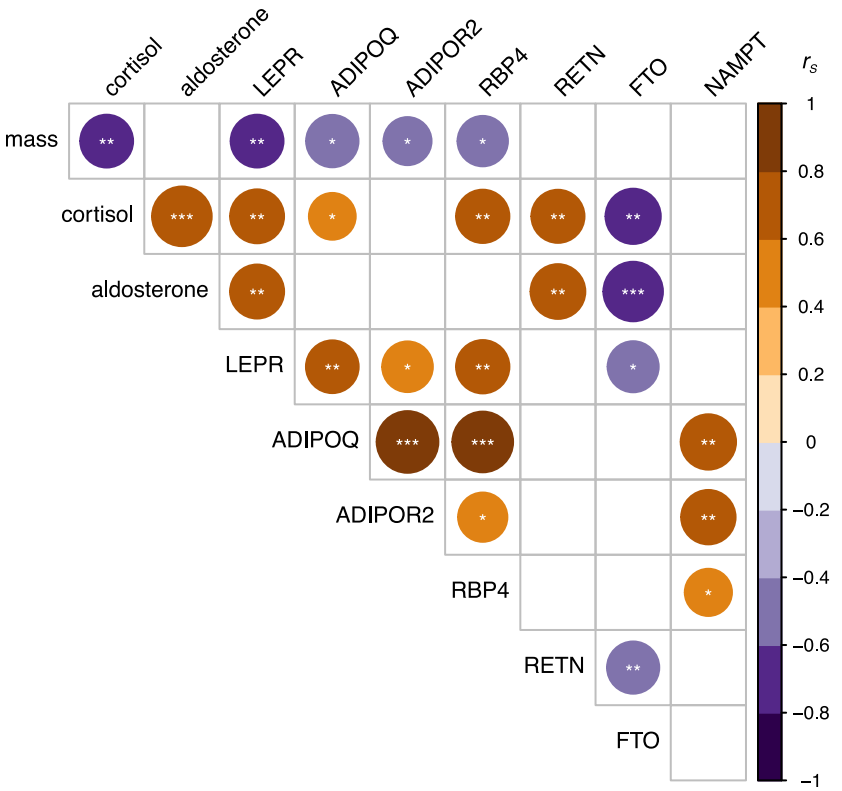

Fig. 4. Correlogram of significant Spearman rank correlations (r_S, $P < 0.05$) between mass, serum corticosteroid hormone levels, and blubber gene expression levels (dCt) of adipokine genes in adult female elephant seals ($n = 22$) of varying fasting stages. Circles denote correlations (orange, positive correlation; purple, negative correlation) that were statistically significant at $^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.0001$. *LEPR*, leptin receptor; *ADIPOQ*, adiponectin; *ADIPOR2*, adiponectin receptor 2; *RBP4*, retinol-binding protein 4; *RETN*, resistin; *FTO*, fat mass and obesity-associated protein; *NAMPT*, visfatin/nicotinamide phosphoribosyltransferase.

globin (*LEP*: $r_S = -0.74$, $P = 0.0011$; *RETN*: $r_S = 0.64$, $P = 0.0071$) and IL-6 (*LEP*: $r_S = -0.65$, $P = 0.0061$; *RETN*: $r_S = 0.73$, $P = 0.0013$, Fig. 5*A*). *ADIPOQ* was negatively associated with IL-6 ($r_S = -0.64$, $P = 0.0071$) and IgE ($r_S = -0.93$, $P < 0.0001$). *RBP4* and *NAMPT* were both negatively associated with IgE (*RBP4*: $r_S = -0.83$, $P < 0.0001$; *NAMPT*: $r_S = -0.81$, $P = 0.00016$). *FTO* was negatively associated with haptoglobin ($r_S = -0.90$, $P < 0.0001$, Fig. 5*B*), IL-6 ($r_S = -0.75$, $P = 0.00078$), and IgM ($r_S = -0.69$, $P = 0.0032$).

DISCUSSION

In this study, we used RT-qPCR to measure expression of 10 adipokine genes in blubber tissue of four independent groups of adult female elephant seals, sampled early and late during prolonged fasting periods associated with lactation and molting. We found that genes encoding four adipokines and two adipokine receptors (*ADIPOQ, RBP4, RETN, NAMPT, LEPR, ADIPOR2*) were expressed more highly in late than early fasting groups, one (*FTO*) was decreased in late compared with early fasting groups, and three (*LEP*, *ANGPTL4*, *RARRES2*) did not vary between groups. Expression of four adipokine genes (*LEPR*, *ADIPOQ*, *ADIPOR2*, *RBP4*) was negatively associated with mass. Expression of *LEPR* and *RETN* was positively associated, while that of *FTO* was negatively associated with both corticosteroids. In addition, expression levels of *ADIPOQ* and *RBP4* were positively associated with cortisol. Lastly, our data suggest that expression of six adipokines (*LEP*, *ADIPOQ*, *RETN*, *RBP4*, *FTO*, *NAMPT*) may be associated with the proinflammatory cytokine IL-6, acute phase protein haptoglobin, and immunoglobulins IgM and IgE.

Weight loss and corticosteroid hormones. Females sampled late in lactation and molting had lower mass and adiposity and elevated circulating corticosteroid levels relative to early fasting groups, consistent with other studies in this species and the known lipolytic and gluconeogenic functions of cortisol that are required to support fasting metabolism (4, 7, 16). The highest corticosteroid concentrations were measured in late lactation, when milk lipid concentration is highest (16), while the lowest were measured during early molt, when females return from their postbreeding foraging trip. Gains in mass and adiposity were higher during the postmolting foraging trip, which is associated with gestation, than during the postbreeding foraging trip, when females mainly replenish protein stores impacted by lactation (1, 9, 37).

Adipokines: RBP4 and resistin. We hypothesized that adipokine genes associated with insulin resistance would be upregulated, while those associated with insulin sensitivity would be downregulated over fasting. Accordingly, expression levels of *RBP4* and *RETN*, two adipokines that contribute to insulin resistance in humans and rodents (34), were elevated in late-fasted compared with early-fasted elephant seals, consistent with the diabetes-like characteristics of these animals (20). *RBP4* expression was also negatively associated with mass in fasting seals. In humans, *RBP4* and *RETN* were positively associated with hepatic expression of phosphoenolpyruvate carboxykinase (*PEPCK*) (34), and high rates of PEPCK flux and glucose recycling have been observed in fasting elephant seals (5).

FTO. Unlike *RBP* and *RETN*, the insulin resistance-associated gene *FTO* was decreased in late-fasted compared with early-fasted elephant seals, similarly to its response to starvation in mice (40). FTO is an mRNA demethylase that regulates gene expression, and mutations that increased its expression in humans and rodents were associated with obesity, decreased energy expenditure, and increased expression of the gene encoding ghrelin hormone (10). Its downregulation during prolonged fasting in elephant seals is consistent with the high rates of energy expenditure that have been observed in fasting animals of this species (7).

Adiponectin. Consistent with data from terrestrial species, *ADIPOQ* expression was increased in late-fasted female elephant seals and was negatively correlated with mass. In contrast, circulating adiponectin levels in weaned elephant seal pups decreased over fasting (44, 45). The discrepancy between our data and those of Viscarra et al. (44, 45) may be attributed to the significant physiological differences between pups and adults, differences in assay sensitivity (qPCR vs. immunoassays), and temporal mismatch between mRNA and protein expression, potentially due to the remarkably low rates of adiponectin peptide production and turnover previously reported in other mammals (19). A mismatch between blubber mRNA and plasma levels of adiponectin was also reported for grey seal pups (3). The stability of plasma adiponectin levels observed during lactation in grey seal females (3), compared with the results observed in the present study, is likely due to similar factors, in addition to differences in fasting duration between the two species (with typically longer fasts in elephant seals).

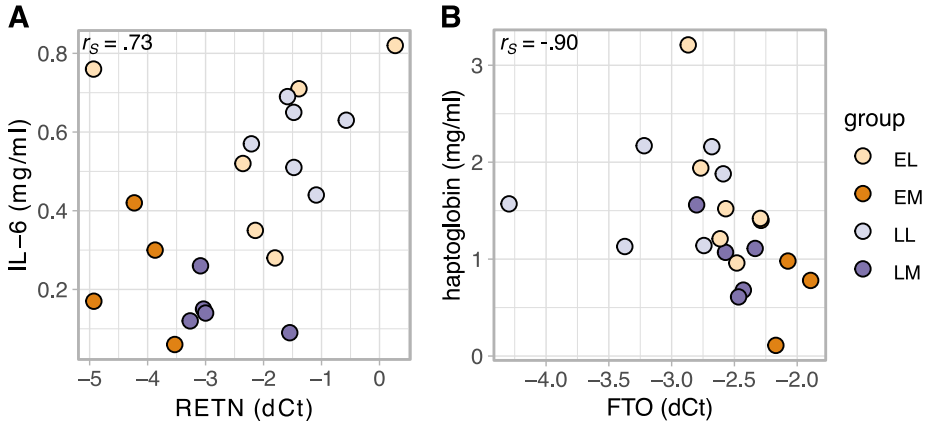

Fig. 5. Significant Spearman rank correlations (r_S, $P < 0.01$) between normalized blubber gene expression (dCt) of *RETN* (resistin) and plasma levels of proinflammatory cytokine IL-6 (*A*) and *FTO* (fat mass and obesity-associated protein) and acute phase protein haptoglobin (*B*) in adult female elephant seals ($n = 22$) of varying fasting stages. IL-6 and haptoglobin were measured in a previous study (Ref. 33). IL-6, interleukin 6; EL, early lactation ($n = 6$); LL, late lactation ($n = 6$); EM, early lactation ($n = 5$); LM, late lactation ($n = 5$).

In humans and mice, *ADIPOQ* increased in response to fasting and caloric restriction, potentially via a mechanism dependent on the sirtuin protein SIRT1 (34). *SIRT1* mRNA expression in muscle increased during the breeding fast in adult male elephant seals (24); a similar increase in its expression and activity in blubber could provide a possible mechanism for *ADIPOQ* upregulation during fasting. Adiponectin signaling is mediated by two receptors, ADIPOR1 and ADIPOR2. While we did not detect expression of the former in this study, *ADIPOR2* was differentially expressed between fasting groups and its expression was highly correlated with *ADIPOQ*. In mice, *ADIPOR2* overexpression increased AMPK activity and fatty acid oxidation and decreased lipogenesis (34), which is congruent with increased AMPK activation and high rates of lipid catabolism observed in fasting elephant seals (44).

NAMPT/visfatin. The insulin-sensitizing adipokine *NAMPT* (visfatin), a rate-limiting enzyme in the NAD^+ biosynthesis pathway, was increased in late-fasted elephant seals, similarly to its upregulation during fasting in mice and humans (32). It has been suggested that NAMPT regulates adiponectin expression via the activity of NAD^+-dependent SIRT1 (47). *NAMPT* and *ADIPOQ* expression were also correlated in this study, and *SIRT1* upregulation has been reported during fasting in elephant seals, as mentioned above (24). However, the insulin-sensitizing effects of NAMPT observed in humans and rodents contrasts with the state of insulin resistance observed in fasting seals, and its biological function in this fasting-adapted species warrants further investigation.

Leptin. Blubber *LEP* expression was not significantly different between fasting groups in this study. Leptin was one of the first adipokines to be discovered and is still considered one of the most important energy-regulating hormones in mammals. It is an anorexigenic hormone that promotes insulin sensitivity and increases energy expenditure and fatty acid oxidation by activating AMPK (26). Due to evidence of positive selection of the leptin gene and a lack of leptin association with body fat in many marine mammals, including male elephant seals, it has been suggested that leptin may not be an important metabolic regulator in these animals (8, 48). However, we found that leptin receptor expression was increased in late-fasted females, with the highest expression levels measured in late lactation, and was negatively correlated with body mass. This suggests that leptin effects may potentially be sex specific and that leptin sensitivity may increase during fasting in female elephant seals, despite low variability of leptin transcript and protein levels. It is possible that leptin may play a role in other functions unrelated to metabolism (e.g., immunomodulation) in blubber of fasting seals, or that changes in local leptin regulation during fasting may be more subtle than those of other adipokines.

Other adipokines. Expression of chemerin (*RARRES2*) and *ANGPTL4* did not differ between fasting groups in this study. *RARRES2* is an anti-inflammatory adipokine that regulates adipogenesis and lipid and glucose homeostasis in human white adipose tissue (34), while *ANGPTL4* is a member of the angiopoietin-like protein family that regulates lipolysis by inhibiting lipoprotein lipase (38). A larger sample size may be required to detect changes in their expression during fasting, if there are any. Alternatively, these adipokines may be constitutively expressed in blubber or may not play a major role in fasting metabolism of elephant seals. Finally, we were unable to detect expression of apelin, apelin receptor, and *ANGPTL2* in elephant seal blubber, but this may be due to the sensitivity of our assays.

Adipokine gene and hormone correlations. We predicted that adipokine expression would be correlated with circulating corticosteroid levels in fasting seals due to similarities in their physiological functions. We also recently demonstrated that corticosteroid elevation induced by adrenocorticotropic hormone (ACTH) administration in juvenile seals resulted in upregulation of *LEP* and *ADIPOQ* in elephant seal blubber (11), similar to what has been reported in humans (25). In this study, we found that *LEPR*, *ADIPOQ*, *RBP4*, and *RETN* were positively associated with cortisol and negatively associated with body mass in fasting female elephant seals, suggesting that the metabolic effects of cortisol may be associated with the expression of this highly coordinated network of adipokine genes in blubber. The lack of cortisol association with *LEP* in this study and its lack of association with *RBP4* and *RETN* in the ACTH study is likely due to differences in cortisol dosage [ACTH elevated cortisol to levels that were 4–5 times those observed in late-fasted seals in this study (11)] and other endocrine and metabolic parameters that vary by life history challenges and sex in this species (acute stress in early fasted juveniles vs. prolonged fasting in adult females) (7). While *NAMPT* expression was positively associated with the other genes in this network (*ADIPOQ*, *ADIPOR2*, *RBP4*), it was not correlated with mass or corticosteroid hormones, either because the small sample size lacked power to detect these relationships or because *NAMPT* may play a different function in blubber. Aldosterone was positively associated with *LEPR* and *RETN*. While a positive association between aldosterone and leptin has been found in humans with metabolic disease (12), aldosterone elevation over fasting in seals may primarily serve to decrease water loss and could be simply coincidental with changes in blubber adipokine expression (29). Finally, we found that *FTO* was negatively associated with both corticosteroids, *LEPR* and *RETN*. A negative relationship between *FTO* and *LEPR* was also shown in mice (43). The function of FTO in physiology of wild animals has not been previously investigated, but it may be more important in immunomodulation than metabolism, as discussed below.

Adipokine gene and immune marker correlations. We hypothesized that adipokine gene expression in blubber would be correlated with circulating immune marker levels due to the well-known immunomodulatory roles of these hormones. Our group previously measured a suite of markers of innate (IL-6, IL-1b, haptoblobin) and adaptive (IgM, IgE, IgG) immune responses in fasting female elephant seals, including the animals used in this study, and found that their circulating levels were higher during breeding than molting and were positively associated with body mass (33). In this study, blubber expression of *RETN* was positively correlated with circulating IL-6 levels in fasting females, similar to what has been reported in other species (32). *RETN* expression and IL-6 levels were highest during breeding, which occurs in a pathogenic, colonial environment. In contrast, *LEP* and *ADIPOQ* expression levels were negatively associated with IL-6, which was consistent with the inhibitory effect of ADIPOQ on IL-6 observed in other species (32). We also found weak associations between haptoglobin, an acute phase protein that increases after parturition in female elephant seals (33) and expression of *LEP* and

RETN. Consistent with the proinflammatory function of RETN in other species (32), *RETN* expression had a positive association with haptoglobin. *FTO* expression was negatively associated with IL-6, haptoglobin, and IgM. A study of obese humans also found a negative correlation between *FTO* and IL-6 (42), and *Fto* knockout mice were shown to downregulate proinflammatory signaling pathways (43). This raises an intriguing hypothesis that FTO may be involved in life history trade-offs between reproduction, immunity, and fasting in seals. Lastly, we found that expression levels of three adipokines (*ADIPOQ*, *RBP4*, *NAMPT*) were negatively associated with IgE, a marker of parasitic infection, which is common in marine mammals (33). However, this relationship is likely indirect: plasma IgE levels were highest in early-fasted animals that had recently returned from foraging, which likely exposed them to parasites, and decreased over fasting, coincident with increased adipokine expression levels we observed in late-fasted groups. Together, our results suggest that differential regulation of several adipokine genes during fasting may be associated with modulation of immune responses to colonial breeding and molting in elephant seals, in addition to regulation of energy homeostasis during fasting.

Perspectives and Significance

This study provides some of the first potential links between corticosteroids, adipokine gene expression, and metabolic and immune responses to prolonged food deprivation in a fasting-adapted mammal, the northern elephant seal. We found that blubber expression of adipokines that are associated with obesity in humans and rodents varied between fed and fasted states in adult female elephant seals. Adipokine gene expression was significantly correlated with body mass and circulating levels of corticosteroid hormones and several immune markers, providing preliminary insights into cellular and endocrine regulation of energy homeostasis and immune responses during prolonged fasting associated with reproduction and molting in a marine mammal. Several of the adipokine genes targeted in this study have not been previously measured in marine or other nonlaboratory mammals, providing putative insights into their adaptive functions and targeted gene expression assays for future studies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.I.K., D.P.C., and D.E.C. conceived and designed research; J.I.K., E.A., A.L.N., G.K.S., A.P.S., D.P.C., and D.E.C. performed experiments; J.I.K., E.A., A.L.N., G.K.S., and A.P.S. analyzed data; J.I.K. and D.E.C. interpreted results of experiments; J.I.K. prepared figures; J.I.K. drafted manuscript; J.I.K., D.P.C., and D.E.C. edited and revised manuscript; J.I.K., D.P.C., and D.E.C. approved final version of manuscript.

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