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RESEARCH ARTICLE

Repeated stimulation of the HPA axis alters white blood cell count without increasing oxidative stress or inflammatory cytokines in fasting elephant seal pups

David C. Ensminger[1,2,*], Daniel E. Crocker[3], Emily K. Lam[1], Kaitlin N. Allen[1] and José Pablo Vázquez-Medina[1]

ABSTRACT

The hypothalamic–pituitary–adrenal (HPA) axis controls the release of glucocorticoids, which regulate immune and inflammatory function by modulating cytokines, white blood cells and oxidative stress via glucocorticoid receptor (GR) signaling. Although the response to HPA activation is well characterized in many species, little is known about the impacts of HPA activation during extreme physiological conditions. Hence, we challenged 18 simultaneously fasting and developing elephant seal pups with daily intramuscular injections of adrenocorticotropin (ACTH), a GR antagonist (RU486), or a combination of the two (ACTH+RU486) for 4 days. We collected blood at baseline, 2 h and 4 days after the beginning of treatment. ACTH and ACTH+RU486 elevated serum aldosterone and cortisol at 2 h, with effects diminishing at 4 days. RU486 alone induced a compensatory increase in aldosterone, but not cortisol, at 4 days. ACTH decreased neutrophils at 2 h, while decreasing lymphocytes and increasing the neutrophil:lymphocyte ratio at 4 days. These effects were abolished by RU486. Despite alterations in white blood cells, there was no effect of ACTH or RU486 on transforming growth factor-β or interleukin-6 levels; however, both cytokines decreased with the 4 day fasting progression. Similarly, ACTH did not impact protein oxidation, lipid peroxidation or antioxidant enzymes, but plasma isoprostanes and catalase activity decreased while glutathione peroxidase increased with fasting progression. These data demonstrate differential acute (2 h) and chronic (4 days) modulatory effects of HPA activation on white blood cells and that the chronic effect is mediated, at least in part, by GR. These results also underscore elephant seals' extraordinary resistance to oxidative stress derived from repeated HPA activation.

KEY WORDS: Adrenocorticotropin, Redox balance, Marine mammals, Glucocorticoids, Antioxidants, Receptor signaling

INTRODUCTION

The hypothalamic–pituitary–adrenal (HPA) axis facilitates organismal responses to metabolic perturbations and environmental stressors (Sapolsky et al., 2000; Tsigos and Chrousos, 2002). Upon activation of the HPA axis, the hypothalamus secretes corticotropin-releasing hormone, which stimulates the anterior pituitary to release adrenocorticotropin (ACTH; Plotsky et al., 1989). ACTH then acts on the adrenal cortex to induce the secretion of corticosteroids including the glucocorticoid cortisol and the mineralocorticoid aldosterone (Haning and Tait, 1970). Cortisol and aldosterone promote energy mobilization and osmotic balance via cellular signaling involving glucocorticoid (GR) and mineralocorticoid receptors (MR; MacDougall-Shackleton et al., 2019). Thus, adrenocorticosteroids impact many physiological processes including lipolysis, immune function and oxidative stress (Wilckens, 1995; Sapolsky et al., 2000; Xu et al., 2009; Costantini et al., 2011).

While glucocorticoids have well-characterized immunosuppressive effects (Claman, 1972; De Bosscher et al., 2000), acute glucocorticoid release also enhances immune function through alterations in white blood cells and cytokine production (Dhabhar and McEwen, 1996; McInnis et al., 2015). As glucocorticoid elevations persist, however, the physiological response shifts to prevent long-term hyperactivity, resulting in decreased white blood cell count and reduced expression of pro-inflammatory cytokines including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α; Claman, 1972; Dhabhar, 2009). This highlights the role of GR-mediated glucocorticoid signaling in inflammation (Baschant and Tuckermann, 2010) and the opposing impacts of acute versus chronic glucocorticoid exposure on immune function (Dhabhar and McEwen, 1997; Dhabhar, 2009). While glucocorticoids, mineralcorticoids and white blood cells play important roles in the production of reactive oxygen species (ROS; Smith and Weidemann, 1993; Yang et al., 2013; Costantini et al., 2011; Spiers et al., 2015; Ferreira et al., 2021), the contrasting actions of GR signaling with stressor duration obscure broad predictions of the role of HPA axis activation on ROS production and redox balance.

ROS are essential for cellular signaling and the immune response (Dröge, 2002; Hamanaka and Chandel, 2010; Yang et al., 2013), but dysregulated ROS generation promotes oxidative stress (Sies, 2019). White blood cells such as neutrophils use superoxide and hydrogen peroxide generated during phagocytosis as part of the respiratory burst, an essential component of the innate immune response (Babior, 1984; Alberts et al., 2008). Moreover, mitochondrial and NADPH oxidase-derived ROS generation increase in response to GR and MR signaling (McIntosh and Sapolsky, 1996; Houstis et al., 2006; You et al., 2009; Spiers et al., 2015). In addition to modulating ROS generation, glucocorticoids have differential impacts on antioxidants (Costantini et al., 2011). Acute glucocorticoid exposure increases antioxidant enzyme expression and activity (Yoshioka et al., 1994; Atanasova et al., 2009) while chronic exposure has the opposite effect (Djordjevic et al., 2010). The three-way interaction between glucocorticoids, immune cells and ROS generation/removal thus complicates extrapolation of the impact of HPA axis activation on redox

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balance in animals undergoing extreme life history events (Stier et al., 2019; Gormally and Romero, 2020; Ensminger et al., 2021).

Northern elephant seals (*Mirounga angustirostris*) molt and develop during prolonged terrestrial fasts and frequently experience sleep apnea, hypoxemia and ischemia/reperfusion (Vázquez-Medina et al., 2012; Allen and Vázquez-Medina, 2019). In many animals, these processes increase oxidative stress and inflammation (Sakamoto et al., 1991; Colominas-Ciuró et al., 2019). Elephant seals, however, can sustain these fasts for months without experiencing oxidative stress or inflammation (Vázquez-Medina et al., 2010, 2013). Fasting-induced increases in antioxidants (Vázquez- Medina et al., 2010, 2011a) allow elephant seals to cope with physiological oxidative stress (Vázquez- Medina et al., 2012, 2013). Whether this adaptation of the redox system extends to the systemic response to HPA axis activation is unknown. To our knowledge, no previous study has experimentally manipulated the HPA axis and looked at redox biology and immune function markers in pinnipeds.

The impacts of glucocorticoids on redox homeostasis in marine mammals are poorly understood. Therefore, we studied whether acute or repeated activation of the HPA axis and GR signaling regulate immune cell function and redox balance in elephant seals. Elephant seals are a unique marine mammal species in which to study the impacts of acute and chronic HPA axis activation as they fast on land for months (Le Boeuf et al., 1973), maintain a functioning HPA axis response (Ensminger et al., 2014; McCormley et al., 2018), and are highly tractable, which allows repeated sampling. Previous work in this species shows that HPA axis activation with exogenous ACTH increases circulating cortisol and aldosterone (Ensminger et al., 2014; McCormley et al., 2018). Additionally, *ex vivo* and *in vivo* transcriptomics studies (Khudyakov et al., 2015, 2017; Deyarmin et al., 2019; Torres-Velarde et al., 2021) highlight the impact of glucocorticoids on expression of genes involved in redox metabolism including polo-like kinase 3, thioredoxin, DNA damage inducible transcript 4 and glutathione peroxidase (GPx) 4. Here, we used exogenous ACTH and a GR blocker to study the effects of acute and chronic HPA axis activation and GR signaling on oxidative stress and immune function in elephant seals. We predicted that ACTH would alter redox balance in elephant seals, with acute ACTH and GR blockade increasing antioxidant enzymes and chronic ACTH increasing oxidative damage. Additionally, we predicted ACTH treatment would alter white blood cell composition, leading to an increase in neutrophils and a decrease in lymphocytes with acute ACTH, and a decrease in neutrophils with chronic ACTH. Finally, we predicted that ACTH treatment would lead to a pro-inflammatory phenotype and that these parameters would be mediated, at least in part, by GR.

MATERIALS AND METHODS

Study site and study animals

All animal procedures were approved by the Sonoma State University and UC Berkeley Institutional Animal Care and Use Committees and were conducted under the National Marine Fisheries Service permit # 19108. Eighteen post-weaned (8 females, 10 males; simultaneously fasting and developing) early fasting (1–2 weeks, pre-molted) elephant seal pups, *Mirounga angustirostris* (Gill 1866), were studied at Año Nuevo State Park, CA, USA.

Field procedures and sample collection

Animals were chemically immobilized with an intramuscular injection of ∼1 mg kg^{-1} tiletamine/zolazepam HCl (Telazol, Fort Dodge Animal Health, Fort Dodge, IA, USA). Immobilization was maintained with intravenous injections of ketamine HCl (Ketaset, Fort Dodge Animal Health). Animals were randomly assigned to one of three groups: (1) daily intramuscular injection of slow-release adrenocorticotropin LA gel (4 females, 2 males; ACTH; Westwood Pharmacy, Richmond, VA, USA) for 4 days, (2) subcutaneous implant of 4 day time-release GR blocker pellets (2 females, 4 males; RU486; Arcos Organics, Fair Lawn, NJ, USA; Innovative Research of America, Sarasota, FL, USA), or (3) ACTH+RU486 treatment (2 females, 4 males). While the sample sizes per sex are small per treatment, there are no major sex effects at this stage in development in this species; hence, we do not suggest any sex effects in our studies (Ortiz et al., 2001; Yochem et al., 2008; Vázquez-Medina et al., 2011a,b).

Animals were given 0.22±0.01 U kg^{-1} (mean±s.e.m.) of ACTH and/or 3.130±0.102 mg kg^{-1} of RU486. ACTH doses were chosen based on previously published work from juvenile elephant seals (McCormley et al., 2018). RU486 implants were positioned laterally, approximately 220 mm superior to the pelvic girdle at the muscle interface, after making a small incision on the skin with a sterile scalpel and removing a blubber core using a 6.0 mm diameter biopsy punch (Vázquez-Medina et al., 2010). ACTH injections were given on the opposite side to where the RU486 implants were positioned. Body mass was collected via the truncated cones method (Crocker et al., 2001), which provides estimates within 5% of that obtained via direct measurement (Crocker et al., 2012). Blood samples were collected from the extradural vein into chilled serum and EDTA vacutainer tubes for analysis at baseline and 2 h post-treatment. ACTH injections on days 2, 3 and 4 were given as remote injections and did not involve animal handling or disturbance. Four days later, animals were again immobilized and sampled 2 h after the last ACTH injection (Fig. 1). This sampling regime does not induce a stress response in elephant seals (Champagne et al., 2012; McCormley et al., 2018). Samples were transported on ice to the laboratory where serum and plasma were prepared and stored at −80°C until analysis.

Whole-blood, serum and plasma analysis

Hematology

Complete white blood cell counts were measured in whole blood using an automated hematology analyzer previously used to analyze pinniped blood (VetScan HM5, Abaxis Inc., Union City, CA, USA; Unal et al., 2018, Thompson and Romano, 2019).

Corticosteroids and metabolites

Cortisol (11-CORHU-E01, Alpco, Salem, NH, USA) and aldosterone (11-AD2HU-E01, Alpco) were measured in serum using EIA kits validated for use in elephant seals (McCormley et al., 2018). Non-esterified fatty acids [NEFA; HR Series NEFA-HR(2), Wako Chemicals, Richmond, VA, USA], and triglycerides (10010303, Cayman Chemical, Ann Arbor, MI, USA) were measured in plasma using enzymatic colorimetric assays as previously described (Ortiz et al., 2003a,b; Viscarra et al., 2012).

Antioxidants

Plasma GPx (703102, Cayman Chemical), glutathione-disulfide reductase (GSR; 703202, Cayman Chemical) and catalase activity (707002, Cayman Chemical), along with total thiols (700340, Cayman Chemical) were measured using colorimetric kits previously used in elephant seals (Vázquez-Medina et al., 2010; Sharick et al., 2015), or validated via parallelism and spiked recovery (total thiols).

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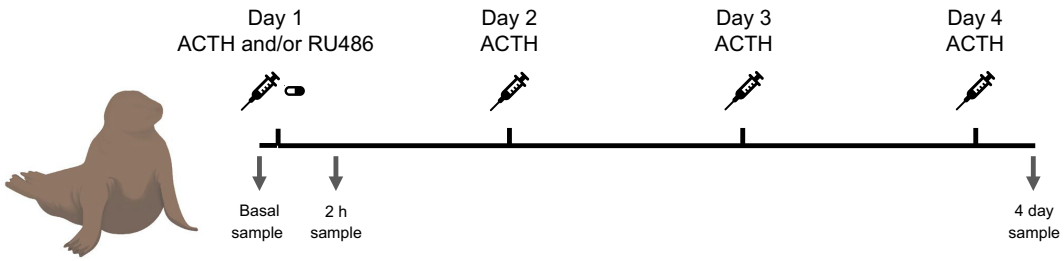

Fig. 1. Field procedures and sample collection. Graphical representation of the experimental design for adrenocorticotropin (ACTH) and glucocorticoid receptor (GR) blocker (RU486) treatment and blood sampling. Seals were given ACTH and/or RU486. Slow-release ACTH gel was administered every 24 h for 4 days, with ACTH administration on days 2, 3 and 4 via remote injection with no animal handling or disturbance. A 4 day time-release tablet of RU486 was implanted on day 1. Blood samples were taken before treatment administration, 2 h post-treatment administration, and 2 h after the fourth day of treatment.

Oxidative damage

Plasma F_2-isoprostanes, a marker for lipid peroxidation, were measured using gas chromatography-mass spectrometry at the Vanderbilt University Eicosanoid Core Laboratory as previously described (Milne et al., 2007; Vázquez-Medina et al., 2010). Protein carbonyls were measured using commercial EIA assays (STA-310, Cell Bio Labs, San Diego, CA, USA) validated for elephant seals via parallelism and spiked recovery.

Cytokines

Plasma transforming growth factor beta (TGF-β; DY240, R&D Systems, Minneapolis, MN, USA) and IL-6 (ELC-IL6-1, RayBioTech, Norcross, GA, USA) were measured using commercial kits previously validated for elephant seals (IL-6; Peck et al., 2016) or validated via parallelism and spiked recovery (TGF-β).

All samples were analyzed in duplicate. The average coefficient of variation for blood, plasma and serum analyses was 6.33% for intra-assay and 3.84% for inter-assay variation.

Data analysis

Data were analyzed using linear mixed models (v.1.1.463, R Development Core Team, Boston, MA, USA; package lme4: https://CRAN.R-project.org/package=lme4) and met the assumptions of the models. Figures were made in RStudio (package ggplot2: https://CRAN.R-project.org/package=ggplot2). For all models, treatment, time point and the interaction of treatment and time point were included as fixed effects and seal ID was included as a random effect. The interaction term of treatment and time point was removed if it did not significantly explain variation in the models (P>0.20). Seal mass, sex and mass-specific treatments were originally included in the models but were removed stepwise as they did not explain variation (P>0.20). Tukey's *post hoc* tests were used to identify specific effects. Line graph data are represented as means±s.e.m., and box and whisker data are presented as median, upper quartile, lower quartile, and 1.5× interquartile range (whiskers). Effect sizes were calculated as partial eta squared (η_p^2). Results were considered significant at P<0.05.

RESULTS

Repeated stimulation of the HPA axis causes differential effects on adrenal steroids

We measured circulating cortisol and aldosterone to corroborate acute and sustained activation of the HPA axis by exogenous ACTH and RU486. Both treatment and time had an effect on serum cortisol and aldosterone (cortisol: $F_{2,52}$=42.549, P<0.001, η_p^2=0.85; $F_{2,52}$=220.069, P<0.001, η_p^2=0.94; aldosterone: $F_{2,52}$=4.880, P=0.023, η_p^2=0.39; $F_{2,52}$=57.087, P<0.001, η_p^2=0.79). There was an interaction between treatment and time which led to differential effects on circulating cortisol and aldosterone ($F_{4,50}$=52.187, P<0.001, η_p^2=0.87; $F_{4,50}$=8.775, P<0.001, η_p^2=0.54). Acute ACTH injection increased both cortisol and aldosterone after 2 h (+366%, +186%; Fig. 2). Similarly, both the cortisol and aldosterone post-ACTH responses were maintained after four daily ACTH injections (+264%, +195%); however, the magnitude of the cortisol response at 4 days was attenuated compared with that at 2 h (−22%; Fig. 2). ACTH+RU486 did not change the response compared with ACTH alone. Moreover, aldosterone increased in response to RU486 alone at 4 days (+92%; Fig. 2A,B). These results show that (1) exogenous ACTH administration increases adrenocorticosteroids in post-weaning elephant seals, (2) repeated ACTH treatment for 4 days decreases the cortisol but not the aldosterone response, and (3) GR blockade with RU486 for 4 days causes a compensatory increase in circulating aldosterone.

Repeated stimulation of the HPA axis does not induce oxidative stress in fasting elephant seal pups

Sustained glucocorticoid release induces oxidative stress in several vertebrates (Costantini et al., 2011). Hence, we measured circulating

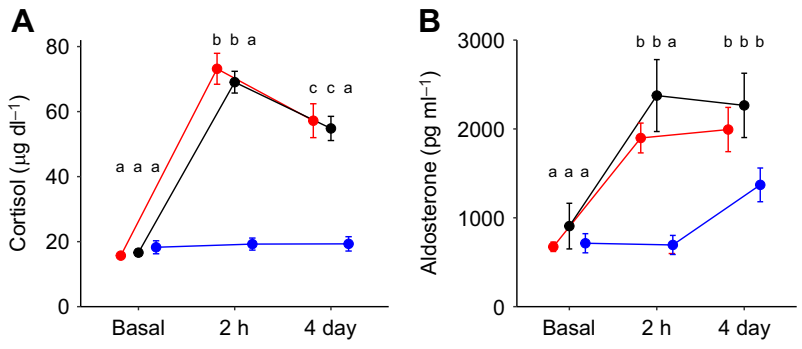

Fig. 2. Repeated stimulation of the hypothalamic–pituitary–adrenal (HPA) axis differentially affects cortisol and aldosterone. Serum adrenocorticosteroid levels during basal (pre-treatment) conditions and after 2 h and 4 days of treatment. (A) Cortisol and (B) aldosterone. ACTH, red (n=6); ACTH+RU486, black (n=6); RU486, blue (n=6). Different letters represent statistical differences of the interaction of time and treatment, respectively (P<0.05) based on Tukey's *post hoc* comparisons of linear mixed models. Data are means ±s.e.m.

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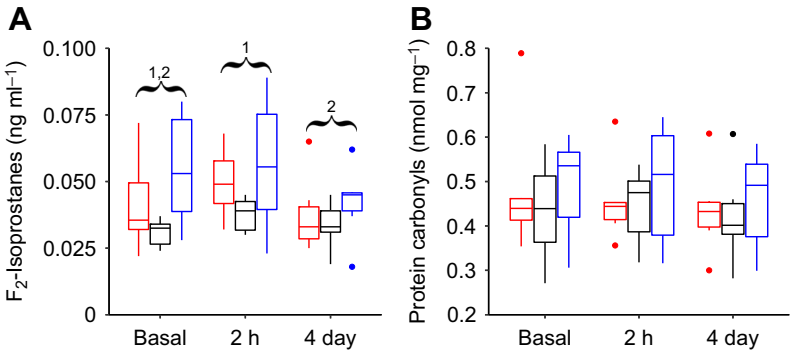

Fig. 3. Neither acute nor repeated stimulation of HPA axis nor GR blockade increases oxidative damage. Plasma oxidative damage markers during basal conditions and after 2 h and 4 days of treatment. (A) F_2-Isoprostanes and (B) protein carbonyls. ACTH, red (n=6); ACTH+RU486, black (n=6); RU486, blue (n=6). Different numbers represent statistical differences between time groups (P<0.05) based on Tukey's *post hoc* comparisons of linear mixed models. Boxplots depict the first quartile and third quartile (box), ±1.5× interquartile range (whiskers) and the median (horizontal line).

antioxidants, lipid peroxidation and protein oxidation to assess the impact of acute and repeated HPA stimulation on oxidative stress in fasting elephant seal pups. While treatment did not alter F_2-isoprostanes, there was an impact of time on F_2-isoprostanes ($F_{2,52}$=2.697, P=0.100, η_p^2=0.26; $F_{2,52}$=4.612, P=0.017, η_p^2=0.22), with concentrations lower at 4 days than at 2 h (−21%; Fig. 3A). There was no impact of treatment or time on protein carbonyls ($F_{2,52}$=0.266, P=0.770, η_p^2=0.03; $F_{2,52}$=2.599, P=0.089, η_p^2=0.13; Fig. 3B). Together, these data show that neither acute nor repeated stimulation of the HPA axis or endogenous GR blockade for 4 days causes systemic oxidative damage despite promoting robust adrenocorticosteroid release in fasting elephant seal pups.

We then measured plasma antioxidants to explore whether manipulation of the HPA axis or endogenous GR blockade alters antioxidant defenses in fasting elephant seal pups. Treatment did not impact catalase or GSR activity ($F_{2,52}$=0.669, P=0.528, η_p^2=0.09; $F_{2,52}$=0.585, P=0.570, η_p^2=0.07). Surprisingly, both catalase and GSR activity decreased at 4 days compared with baseline (−15%, $F_{2,52}$=9.705, P<0.001, η_p^2=0.38; −5%, $F_{2,52}$=5.113, P=0.011, η_p^2=0.21; Fig. 4A,B). Treatment did not impact GPx, but, in contrast to catalase and GSR, GPx activity increased at 4 days compared with baseline and 2 h ($F_{2,52}$=0.602, P=0.561, η_p^2=0.07; +12%, +11%, $F_{2,52}$=6.921, P=0.003, η_p^2=0.29; Fig. 4C). There were trends for the impact of treatment and time on total thiols ($F_{2,52}$=3.225, P=0.069, η_p^2=0.30; $F_{2,52}$=2.913, P=0.070, η_p^2=0.16). RU486 tended to increase total thiols compared with ACTH+RU486 (+8%), while concentrations tended to be higher on day 4 compared with 2 h (+7%; Fig. 4D). There was no interaction between treatment and time for total thiols ($F_{4,50}$=1.946, P=0.129, η_p^2=0.21). These data show that increased antioxidant defenses do not account for the absence of systemic oxidative damage during acute or repeated stimulation of the HPA axis or endogenous GR blockade in elephant seals.

Stimulation of the HPA axis does not induce lipolysis in postweaning elephant seals

In most animals, glucocorticoid release increases fuel availability through the liberation of stored energy. Hence, we measured circulating NEFA and triglyceride levels to assess the impacts of acute and repeated stimulation of the HPA axis on circulating lipids. NEFA was not altered by treatment, time or the interaction of treatment and time ($F_{2,52}$=0.663, P=0.530, η_p^2=0.08; $F_{2,52}$=0.771,

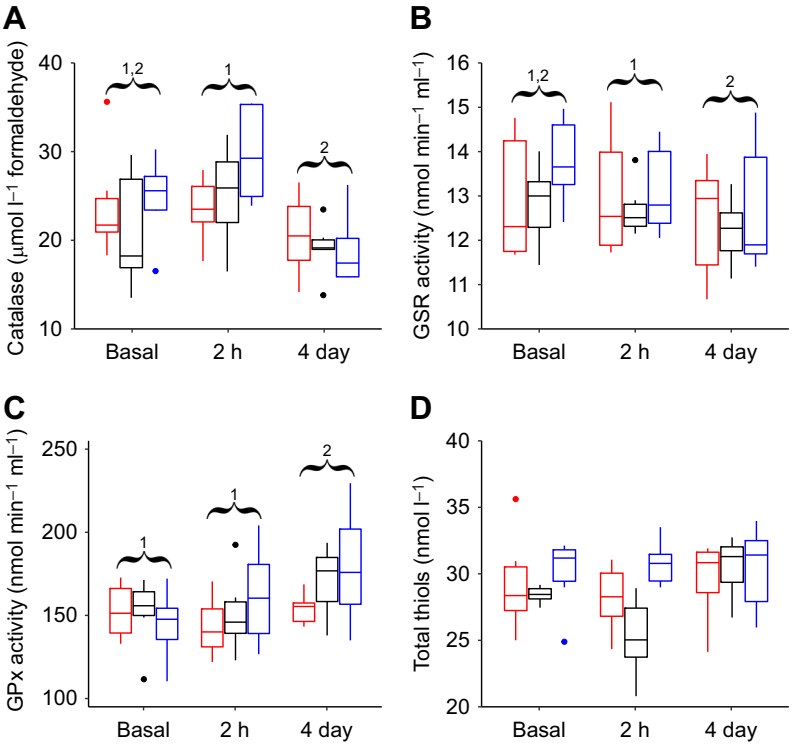

Fig. 4. Neither acute nor repeated stimulation of the HPA axis nor GR blockade alters circulating antioxidants. Plasma antioxidants during basal conditions and after 2 h and 4 days of treatment. (A) Catalase activity, (B) glutathione disulfide reductase (GSR) activity, (C) glutathione peroxidase (GPx) activity and (D) total thiols. ACTH, red (n=6); ACTH+RU486, black (n=6); RU486, blue (n=6). Different numbers represent statistical differences between time groups (P<0.05) based on Tukey's *post hoc* comparisons of linear mixed models. Boxplots depict the first quartile and third quartile (box), ±1.5× interquartile range (whiskers) and the median (horizontal line).

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Table 1. Plasma lipids and cytokines in elephant seal pups during basal conditions and after adrenocorticotropin (ACTH) and glucocorticoid receptor (GR) blocker (RU486) administration

	ACTH			ACTH+RU486			RU486		
	Basal	2 h	4 days	Basal	2 h	4 days	Basal	2 h	4 days
NEFA (mmol l^{-1})	1.56±0.21	1.55±0.25	1.66±0.22	1.24±0.09	1.94±0.44	1.2±0.14	1.19±0.13	1.14±0.12	1.61±0.38
Triglycerides (mg ml^{-1})	21.46±4.45	21.37±4.13	11.6±1.44*	15.45±0.96	18.19±2.81	10.39±1.3*	16.75±1.92	15.45±1.29	17.46±4.7*
TGF-β (pg ml^{-1})	146±31.5	163.6±16.2	121.5±22.3	85.2±9.3	150.5±32.6	121.1±23.5	185.5±35.8	227.6±54.1	136.3±56.6
IL-6 (pg ml^{-1})	113±41	141±20	116±27	41±11	135±29	88±32	149±20	147±27	72±33

NEFA, non-esterified fatty acids; TGF-β, transforming growth factor beta; IL-6, interleukin-6. *Significant difference from baseline (P<0.05) based on Tukey's *post hoc* comparisons of linear mixed models.

P=0.471, η_p^2=0.05; $F_{4,50}$=2.144, P=0.100, η_p^2=0.22; Table 1). Similarly, triglycerides were not affected by treatment or the interaction of treatment and time ($F_{2,52}$=0.630, P=0.546, η_p^2=0.08; $F_{4,50}$=2.135, P=0.101, η_p^2=0.08). However, triglycerides were impacted by time ($F_{2,52}$=4.391, P=0.021, η_p^2=0.24), with triglyceride concentrations being lower at 4 days compared with baseline or 2 h (−26%, −28%; Table 1). These data show that neither ACTH nor GR signaling alter rates of lipolysis in post-weaned (simultaneously fasting and developing) elephant seals.

Acute and repeated stimulation of the HPA axis alters white blood cell count without affecting cytokine levels

We measured white blood cell count and cytokine levels to examine the role of acute and repeated stimulation of the HPA axis and GR signaling on the immune system in elephant seals. Neither treatment nor time altered total white blood cell count ($F_{2,52}$=0.238, P=0.791, η_p^2=0.03; $F_{2,52}$=0.432, P=0.653, η_p^2=0.03). There was a trend for an interaction; however, *post hoc* analysis revealed no differences ($F_{4,50}$=2.607, P=0.057, η_p^2=0.27; Fig. 5A). Treatment did not affect the neutrophil percentage, but this increased at 4 days compared with baseline and 2 h ($F_{2,52}$=2.625, P=0.108, η_p^2=0.27; +4%, +8%, $F_{2,52}$=7.984, P=0.001, η_p^2=0.33; Fig. 5B). In contrast, both treatment and time impacted lymphocyte percentage ($F_{2,52}$=4.433, P=0.032, η_p^2=0.39; $F_{2,52}$=11.070, P<0.001, η_p^2=0.41). ACTH lowered lymphocyte percentage compared with ACTH+RU486 or RU486 (−22%, −24%) and this was also lower at 4 days than either baseline or at 2 h (−23%, −29%, Fig. 5C). Similar to lymphocyte percentage, both treatment and time altered the neutrophil:lymphocyte ratio ($F_{2,52}$=5.927, P=0.014, η_p^2=0.46; $F_{2,52}$=13.132, P<0.001, η_p^2=0.45). ACTH increased the neutrophil:lymphocyte ratio compared with ACTH+RU486 or RU486 (+28%, +40%; Fig. 5D). The neutrophil:lymphocyte ratio was higher at 4 days compared with baseline and 2 h (+29%, +47%; Fig. 5D). These results show that acute and repeated stimulation of the HPA axis have differential effects on white blood cell count in elephant seals. While acute stimulation of the HPA axis does not have an effect on the neutrophil:lymphocyte ratio, repeated activation increases the neutrophil:lymphocyte ratio, suggesting a shift in the immune response, which is mediated by GR.

We then measured plasma cytokine levels to explore whether HPA axis activation or GR signaling alter circulating cytokine levels. Neither treatment nor time altered TGF-β or IL-6 levels (TGF-β: $F_{2,52}$=2.112, P=0.155, η_p^2=0.22; $F_{2,52}$=2.640, P=0.086, η_p^2=0.14; IL-6: $F_{2,52}$=1.404, P=0.276, η_p^2=0.16; $F_{2,52}$=2.632, P=0.087, η_p^2=0.13; Table 1). These data show that despite altering white blood cell proportions, repeated stimulation of the HPA axis does not increase cytokine expression in elephant seals,

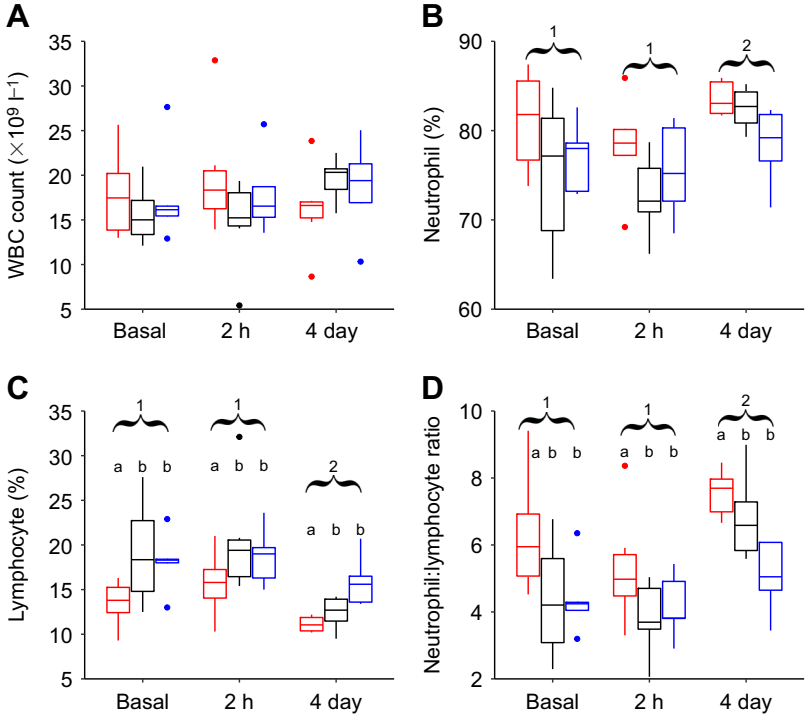

Fig. 5. Acute and repeated stimulation of the HPA axis alters white blood cell count. White blood cell count and composition during basal conditions and after 2 h and 4 days of treatment. (A) Complete white blood cell (WBC) count, (B) neutrophil percentage, (C) lymphocyte percentage and (D) neutrophil to lymphocyte ratio. ACTH, red (n=6); ACTH+RU486, black (n=6); RU486, blue (n=6). Different numbers represent statistical differences between time groups (P<0.05) and different letters represent statistical differences between treatment groups based on Tukey's *post hoc* comparisons of linear mixed models. Boxplots depict the first quartile and third quartile (box), ±1.5× interquartile range (whiskers) and the median (horizontal line).

underscoring these animals' extraordinary resistance to inflammation while simultaneously fasting and developing.

DISCUSSION

A variety of stressors activate the HPA axis, altering corticosteroid concentrations, redox balance and inflammation across taxa. While the stress response has been heavily explored in terrestrial vertebrates, considerably less is known about the downstream impacts of this response in marine mammals. Here, we show that acute and chronic activation of the HPA axis elicit differential responses on adrenocorticosteroids and that GR blockade causes a compensatory increase in aldosterone but not cortisol in elephant seal pups. Additionally, we found that neither manipulation of the HPA axis nor blockade of endogenous GR signaling induces oxidative stress or lipolysis in post-weaned elephant seal pups. Our data also show that stimulation with exogenous ACTH alters the proportion of white blood cells without altering cytokine levels, and that this effect is driven by GR signaling.

In our experiments, ACTH stimulation increased circulating cortisol and aldosterone; however, the impact of repeated ACTH exposure and the role of GR feedback differed between the two adrenocorticosteroids. The magnitude of the cortisol response to ACTH stimulation decreased after 4 days of repeated ACTH exposure, as reported previously in juvenile elephant seals (McCormley et al., 2018). This response may result from desensitization of the HPA axis, stronger negative feedback or adrenal exhaustion (Rich and Romero, 2005; Welberg et al., 2006; Gądek-Michalska et al., 2013). HPA axis desensitization was previously observed in adult male elephant seals (Ensminger et al., 2014), suggesting that repeated stimulation with ACTH desensitizes the HPA axis independent of age.

Our results also show low individual variability in cortisol levels in response to ACTH, suggesting tight regulation of cortisol during early postnatal development. In contrast, the cortisol response to ACTH is largely variable in juvenile and adult elephant seals (Ensminger et al., 2014; McCormley et al., 2018), suggesting that this variability is impacted by postnatal maturation. Our results about the effect of ACTH stimulation on aldosterone are consistent with those of juvenile elephant seals (McCormley et al., 2018), but contrary to those for adult males, suggesting that life history stage impacts negative feedback on aldosterone secretion (Ensminger et al., 2014). Contrary to cortisol, there was a compensatory impact of GR blockade on aldosterone levels, which increased after 4 days of repeated ACTH stimulation, further supporting the hypothesis that aldosterone is an important component of the stress response in marine mammals (Thomson and Geraci, 1986; Houser et al., 2011). This change in aldosterone levels could result from a potential shift in aldosterone signaling between GR and MR (Gaeggeler et al., 2005; Gauer et al., 2007); however, this idea needs further exploration. Though chronically elevated aldosterone may have less impact on land than at sea because of low urinary output (Adams and Costa, 1993), it could negatively impact osmotic balance while at sea as a result of aldosterone's influence on sodium concentrations (Morris, 1981; Ortiz et al., 2000). Moreover, aldostoerone signaling via MR increases oxidative stress and alters white blood cell function, increasing inflammatory cytokines (Christ and Wehling, 1999; Ferreira et al., 2021), suggesting further potential downstream impacts of HPA axis activation. Furthermore, these differential impacts on aldosterone and cortisol also show differential sensitivity of the zona glomerulosa and zona fasciculata to acute and repeated ACTH stimulation and endogenous GR blockade.

Despite robust HPA axis activation, which elevated both cortisol and aldosterone, we found no effects of ACTH stimulation or GR blockade on oxidative stress in fasting elephant seal pups. While redox metabolism varies with tissue type, circulating levels of lipid and protein oxidation are a reliable metric for systemic oxidative stress across vertebrates (Costantini et al., 2011). In elephant seals, prolonged fasting has strong impacts on circulating markers of redox metabolism (Vázquez-Medina, et al., 2010, 2011a, 2015). Cortisol increases oxidative stress in several vertebrates (Costantini et al., 2011; Spiers et al., 2015). Similarly, aldosterone induces oxidative stress via activation of NADPH oxidases (Sun et al., 2002; Miyata et al., 2005). Our results, however, show that neither cortisol/aldosterone nor GR signaling regulates circulating antioxidants, lipid peroxidation or protein oxidation in elephant seal pups. These results support previous observations of the extraordinary capacity elephant seals possess to cope with oxidative stress derived from prolonged food and water deprivation (Vázquez-Medina et al., 2010, 2011a, 2013), sleep apnea, hypoxemia and ischemia/reperfusion (Vázquez-Medina et al., 2012; Allen and Vázquez-Medina, 2019). Both acute and repeated ACTH injection alter blubber and muscle expression of genes involved in redox balance in juveniles (polo-like kinase 3 and thioredoxin; Khudyakov et al., 2015; Khudyakov et al., 2017; Deyarmin et al., 2019). Similarly, sustained exposure to glucocorticoids upregulates the expression of the phospholipid hydroperoxidase GPx4 while downregulating peroxiredoxin 6 expression in elephant seal muscle cells in primary culture (Torres-Velarde et al., 2021). Therefore, the lack of response here suggests that either pups have an altered redox response to cortisol compared with other life history stages, likely due to the combination of fasting and development, or that the effect of cortisol on oxidative stress is tissue specific. In support of the latter hypothesis, a previous meta-analysis shows tissue-specific differences in cortisol-induced oxidative stress across taxa (Costantini et al., 2011). Future research should focus on identifying the interplay between tissue-specific and systemic oxidative responses to further elucidate these relationships.

While our treatments did not alter circulating antioxidant enzymes or oxidative damage, we found opposing patterns in lipid peroxidation and antioxidants involved in reducing lipid hydroperoxides (GPX and total thiols) within the short (4 day) fasting progression. Lipid peroxidation decreased from day 1 to day 4 and was not associated with changes in NEFA or triglyceride levels (data not shown) (Pérez-Rodríguez et al., 2015). Moreover, GPx and total thiols increased with time, supporting the hypothesis that fasting promotes a positive redox balance in part by stimulating the glutathione system, as previously shown in fasting elephant seal pups (Vázquez-Medina et al., 2010, 2011a; Ensminger et al., 2021). Interestingly, both catalase and GSR decrease from 2 h to 4 days. Combined with the increase in GPx and total thiols, these data suggest a potential shift in resources away from recycling glutathione through GSR and toward increasing lipid hydroperoxide removal and stimulating glutathione synthesis (Vázquez-Medina et al., 2011a). Of note, the previously observed decrease in catalase and GSR opposed patterns in muscle and red blood cells further suggests tissue-specific effects in redox metabolism or differential effects of short and long fasting duration (4 days versus 2 months; Vázquez-Medina et al., 2010, 2011a).

Similar to oxidative stress markers, we found no impact of ACTH or GR blockade on NEFA or triglycerides; however, triglycerides decreased with fasting progression. While changes in triglycerides may represent a fasting-derived decrease in stored fat supplies

(Williams et al., 1999), the constant NEFA concentrations suggest that these seals were not fat limited (Jenni-Eiermann and Jenni, 1992). As elephant seals have a high fat-based metabolism, tight regulation of cortisol-induced lipolysis may support fat metabolism during prolonged fasting (Crocker et al., 2014). However, while breeding females show correlations between cortisol and NEFA, males only exhibit this relationship during the molt (Ensminger et al., 2014; Fowler et al., 2016). As such, our results differ from results found in other life history stages, suggesting that life history plays a strong role in the downstream effects of HPA axis activation on lipolysis in elephant seals and that animals in certain life history stages (weaned elephant seal pups and breeding males) might limit the impact of adrenal stimulation on fat metabolism during prolonged fasts. More work is needed to understand the mechanisms underlying this phenomenon.

While there was no impact on oxidative balance or lipolysis, repeated ACTH stimulation shifted the composition of white blood cells, lowering the lymphocyte proportion and subsequently increasing the neutrophil:lymphocyte ratio. While white blood cells take longer to respond to stressors (Gross, 1990; DuRant et al., 2015), they stay elevated over the duration of chronic stress exposure (Goessling et al., 2015). When combined with findings showing that changes in white blood cells are more sensitive than cortisol to a wider range of stressors (Müller et al., 2011), these results support the hypothesis that neutrophil:lymphocyte ratios may be a better marker for assessing chronic stress exposure in wildlife, and that the observed changes in neutrophil:lymphocyte ratio are likely driven by both neutrophils and lymphocytes (Keogh and Atkinson, 2015; Davis and Maney, 2018). Despite shifts in white blood cell proportions, cortisol and aldosterone, we found no impact of either ACTH stimulation or GR blockade on pro-inflammatory cytokines. Similar cytokines remain stable across the fast despite increases in cortisol (i.e. IL-1β; Ortiz et al., 2003a,b; Vázquez-Medina et al., 2013; Peck et al., 2016), suggesting that elephant seals possess physiological mechanisms to limit inflammation. This is further supported by the intrinsic anti-inflammatory properties of elephant seal serum (Bagchi et al., 2018), though more research is needed to understand the uncoupling of both cortisol and HPA axis activation from the inflammatory response, as activation of the renin–angiotensin–aldosterone system increases with fasting progression in elephant seal pups, along with muscle TNF-α muscle expression and protein abundance (Suzuki et al., 2013; Vázquez-Medina et al., 2010, 2013).

Overall, this study shows that neither repeated stimulation of the HPA axis nor blockade of endogenous GR signaling causes systemic oxidative stress or inflammation, or alters lipolysis in simultaneously fasting and developing weaned elephant seals. Hence, these results underscore elephant seals' robust tolerance of repeated and sustained cortisol elevations. Furthermore, our results support the hypothesis that animals in metabolically demanding life history stages or living in areas with repeated stressors may rely on physiological processes that help mitigate the deleterious impacts of a prolonged stress response (Huber et al., 2017; Stier et al., 2019; Ensminger et al., 2021). These data also support the hypothesis that aldosterone is an important component of the stress response in marine mammals and highlight a potential role of GR signaling in osmotic regulation (Gaeggeler et al., 2005). Though the sample size precludes an explicit examination of the interaction of treatment and fasting on neutrophil:lymphocyte ratio, these data highlight the need for future research to examine the use of this metric as a more consistent indicator of chronic stress in marine mammals (Davis and Maney et al., 2018).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: D.C.E., J.V.; Methodology: D.C.E., D.E.C., J.V.; Software: D.C.E.; Validation: D.C.E., D.E.C., J.V.; Formal analysis: D.C.E.; Investigation: D.C.E., D.E.C., E.K.L., K.A., J.V.; Resources: D.C.E., D.E.C., E.K.L., K.A., J.V.; Data curation: D.C.E.; Writing - original draft: D.C.E.; Writing - review & editing: D.C.E., D.E.C., E.K.L., K.A., J.V.; Visualization: D.C.E., J.V.; Supervision: D.C.E., D.E.C., E.K.L., K.A., J.V.; Project administration: D.C.E., D.E.C., E.K.L., K.A., J.V.; Funding acquisition: D.C.E., J.V.

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Data availability
Data are available from figshare: https://doi.org/10.6084/m9.figshare.16629148

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