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RESEARCH ARTICLE

Synergetic effects of immune challenge and stress depress cortisol, inflammatory response and antioxidant activity in fish-eating *Myotis*

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ABSTRACT

One of the most common tools in conservation physiology is the assessment of environmental stress via glucocorticoid measurement. However, little is known of its relationship with other stress-related biomarkers, and how the incidence of an immune challenge during long-term stress could affect an individual's overall stress response. We investigated here the relationship between basal and post-acute stress fecal cortisol metabolite (FC) with different antioxidant enzymes, oxidative damage and immune parameters in the fish-eating bat, *Myotis vivesi*. We found that in both basal and post-stress conditions, FC was highly related with a number of antioxidant enzymes and immune parameters, but not to oxidative damage. We also assessed changes of FC through the seasons. Basal FC samples and stress reactivity after short-duration stress displayed similar levels during summer, autumn and early winter, but lower concentrations in late winter. Stress reactivity after long-duration stress was greater in summer and early winter. Finally, we tested the effect of a simultaneous exposure to a long, strong stress stimulus with an immune response stimulation by administrating adrenocorticotropic hormone (ACTH) and phytohemagglutinin (PHA) after 42 h. Results showed that when both stimuli were administrated, FC concentrations, inflammation and some antioxidant activity were lowered in comparison with the control and individual administration of the challenges. Our findings support the idea that animals maintain constant basal glucocorticoid levels when living in challenging environments, but response to acute stress differs seasonally and immune defense mechanisms and stress responses might be compromised when confronted with multiple challenges.

KEY WORDS: ACTH, Antioxidants, Chiroptera, Cortisol, Immune response, Stress

INTRODUCTION

One of the most common tools in conservation physiology is the rapid assessment of environmental stress via the measurement of glucocorticoid 'stress' hormones. These steroid hormones are ubiquitous in vertebrates, and occur at low (baseline) levels in all individuals (Wingfield et al., 1997). Glucocorticoids (GC) are usually measured in plasma (Romero and Reed, 2005) and in other fluids (saliva, hair, urine and feces) (Sheriff et al., 2011a). In many cases when animals experience inclement weather (Kitaysky et al., 2007) or risk of predation (Clinchy et al., 2013; Sheriff et al., 2011b), GCs increase in the circulation and, subsequently, in feces. The degree of stress response activation often correlates with the overall health of an individual, hence conservation physiologists often experimentally induce short-term stress (capture and handling) to make inferences of an individual's general ability to cope with other sources of stress (Cabezas et al., 2007; Cote et al., 2006; Rodas-Martínez et al., 2013; Romano et al., 2010; Romero, 2004; Romero and Wikelski, 2001; Wikelski et al., 2002).

GCs also play important roles in non-stressful situations, mediating energy balance through the stimulation of glycogenesis, proteolysis and lipolysis (Rose and Herzig, 2013). GCs are known to vary with predictable changes in the environment on a daily (day/night periods, high tide/low tide periods) or seasonal basis (Dickmeis, 2009; Michael Romero, 2002; Woodley et al., 2003), and they also change with life history traits such as reproduction (Kenagy and Place, 2000; Klose et al., 2006), migration (Carruth et al., 2000; Landys-Cannelli et al., 2002; Piersma et al., 2000) or social interactions (Goymann et al., 2001; Muller and Wrangham, 2004; Sands and Creel, 2004; Sapolsky, 1982).

The assessment of GCs in plasma of wild animals can prove difficult in many cases. Circulating hormone levels increase within 3–5 min after capture in birds and small mammals (Romero and Reed, 2005), thus obtaining plasma samples within this time frame is sometimes difficult in many field settings. Furthermore, plasma provides hormone concentration at a single point in time and this might be not representative of long-term hormone exposure levels for GC that exhibit both regular and erratic changes with time (Harper and Austad, 2000). In contrast, non-invasive techniques, such as fecal glucocorticoids (FC) assays, may provide an accurate assessment of stress without the immediate bias of the capture-induced increases in GCs (Harper and Austad, 2000; Millspaugh et al., 2001; Touma et al., 2003). Furthermore, FCs reflect an average level of circulating GCs over a time period, rather than a point sample, and therefore may provide a more accurate assessment of long-term GC levels (Harper and Austad, 2000). Therefore, the use of non-invasive fecal samples has become a preferable measure of GC (Harper and Austad, 2000; Palme et al., 2005; Sheriff et al., 2011a) in the field.

GC excretion and immune systems are often negatively correlated as high levels of GC often down-regulate immune reactivity (Cain and Cidlowski, 2017; Martin, 2009; Sapolsky et al., 2000). Thus, there appears to be a trade-off between immunity and GC stress

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responses where a high activity of one system constrains the activity of the other (Homberger et al., 2013). It has been experimentally shown that acute stress (i.e. short-term stress from minutes to hours) boosts the immune response by re-locating the cellular components (leucocytes) on the skin (Dhabhar, 2013; Dhabhar and Viswanathan, 2005). However, chronic stress (i.e. long-term stress from days to months) causes immunosuppression by shifting the cytokine balance from a TH1 profile (pro-inflammatory) to a TH2 profile (anti-inflammatory), hence decreasing the number of circulating leucocytes (Dhabhar, 2013). However, whether stress stimulates or inhibits an immune response also depends on the immune parameter assessed. For example, chronic exposure to corticosterone reduced the production of antibodies of barn owl nestlings (*Tyto alba*) but did not significantly affect the constitutive innate immunity (Stier et al., 2009).

An associated cost of the immune system and GC stress response is the production of reactive oxygen species (ROS) (Costantini and Moller, 2009; Knight, 2000). Cortisol mobilizes energy reserves to deal with stressors (Krischbaum et al., 1993; Sapolsky et al., 1986), thus increasing mitochondrial respiration and the production of ROS as a by-product (Gutierrez et al., 2006). ROS are highly reactive molecules that are ubiquitous in all aerobic organisms (Dowling and Simmons, 2009). The role of adrenal steroids in the regulation of systemic antioxidants is not known, but the synthesis of adrenal steroids themselves has been linked to ROS production due to oxi-reductive reactions resulting from the interaction of cytochrome P-450 with steroids during the biosynthesis of these hormones from cholesterol (Hornsby, 1986; Hornsby and Crivello, 1983). A negative relationship between plasma GC and mitochondrial superoxide production was found for grey partridges (*Perdix perdix*), which might highlight the regulatory role of GC in the context of resistance to oxidative stress (Homberger et al., 2013). Nevertheless, there have been reports of GC inducing oxidative stress. For example, dexamethasone (a synthetic GC) significantly enhanced ROS levels in hippocampal slice cultures of Sprague-Dawley rats (You et al., 2009). It has also been reported that chronic stress exposure promotes oxidative damage through frequent and sustained activation of the hypothalamic–pituitary–adrenal (HPA) axis (Aschbacher et al., 2013; Lin et al., 2004). In the common lizard (*Lacerta vivipara*), carotenoid (an antioxidant) was positively correlated with corticosterone under basal conditions, but under stress conditions corticosterone was negatively related to carotenoids and caused oxidative stress (Cote et al., 2010). Similarly, nestlings of barn owl (*T. alba)* showed a significant reduction in resistance to oxidative stress after 12 days of implantation of corticosterone-releasing pellets (Stier et al., 2009).

Here we tested the relationship between fecal cortisol metabolites and different antioxidant enzyme activity [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)], oxidative damage (protein carbonylation) and immune parameters (humoral response and inflammatory response) in the fish-eating bat (*Myotis vivesi*, Menegaux 1901), which is endemic to the islands of the Gulf of California, Mexico. Islands where these bats roost are exposed to seasonal changes in ambient temperature (T_a), ranging from 45°C in summer to 5°C during winter (Carpenter, 1968). Bats roost under rocks during the day, which provides partial isolation from T_a (Salinas-ramos et al., 2014). Lactation in this bat occurs during summer and it enters torpor (Salinas-Ramos et al., 2014) in the winter, two factors that are known to influence GC levels (Gustafson and Belt, 1979; Hiebert et al., 2000; Reeder et al., 2004; Willis and Wilcox, 2014). We used this bat as a model to describe the cortisol profile and its response to acute stress, and the changes induced in antioxidant and immune response by acute stress (endogenous GC inducement by 24 h movement restriction) and by chronic stress (exogenous GC inducement by ACTH injection). We hypothesized that bats under acute stress would have an enhanced immune response, and that GC concentration would be positively related to antioxidant activity. In contrast, we predicted that when bats were exposed to chronic stress, GC would have a suppressor effect on the immune response.

MATERIALS AND METHODS

Study site

This study was conducted in Partida Norte Island (28°52′30″ N, 113°21′7″ W), a 1.4 km^2 island located in the midriff region of the California Gulf, Mexico (Carreño and Helenes, 2002). This island supports the largest known colony of fish-eating bats (~8000 adults) (Flores-Martínez et al., 2005). Fieldwork was conducted for 5 days each during summer (July 2014), autumn (October 2014), early winter (December 2014) and late winter (February 2015). These periods include the months when lactation occurs (July), when bats enter torpor (December and February), and a transition period (October). Sample collection during spring was not conducted because the population was composed almost exclusively of pregnant females (Maya, 1968) and we considered that our manipulations might have been too invasive for these animals.

Animal handling and sample collection

All bats captured were sampled for blood and fecal samples, and their weight and forearm length were measured. We captured only adult females directly from their roost sites between 06.00 and 07.00 h. As the population female–male ratio was higher, and males have been found to abandon the island during spring and summer (Gerardo Herrera et al., 2019), the number of male captures was not enough for a solid statistical comparison. Bats were placed in individual small cotton bags, where the acute stress stimuli consisted of their immobilization. Two blood samples were taken from each bat for antioxidant activity, carbonyl determination and bactericidal activity (BA). A first blood sample (within 30 min of capture) for antioxidant analysis was obtained by bleeding bats from the right forearm vein (150 µl). This first sample was considered as basal, as enzymatic antioxidants are primarily transcriptionally regulated, and BA activity in plasma has been shown not to change after 1.5 h of capture (Strobel et al., 2015). After the first blood sample, bats were injected subcutaneously in the footpad with phytohemagglutinin (PHA) to promote an inflammatory response (described further below) (Vinkler et al., 2014). Bats were maintained in the bag for 6 h after capture and a second blood sample was drawn from the left forearm vein (150 µl). Antioxidant parameters measured at this point were denoted with a '6'. Blood was drawn by hypodermic needle (27Gx 13 mm BD), collected with heparinized capillary tubes, and placed in 1.5 ml Eppendorf tubes. All samples were kept on ice during collection and handling in the field, and plasma and erythrocytes were separated after centrifugation at 3450 ***g*** (Digital ZipSpin Centrifuge, LW Scientific, Lawrenceville, GA, USA) and stored in liquid nitrogen in 0.2 ml tubes. Samples were transferred from liquid nitrogen to dry ice and shipped to the laboratory where they were stored at −80°C until analysis.

Fecal samples were collected directly from the cotton bag of each bat captured. Bags were examined every 30 min for defecations and the collection time was recorded. Samples were stored in liquid nitrogen until transportation to the laboratory, where they were kept

at −20°C until their metabolite extraction (≤6 months after collection).

Bats were fed with shrimps twice while in captivity following previous studies with captive individuals (Carpenter, 1968). The first meal was provided approximately 5–6 h after capture, and a second meal was provided 12 h after capture. Shrimp was offered in small pieces to bats with the aid of tweezers until they no longer accepted the food. Although *M. vivesi* has been reported to obtain water mainly from its food (Carpenter, 1968), water was also offered with a needleless syringe. Minimal food transit time (mFTT) was measured for each bat, adding blue vegetable pastry dye to one of the meals. We then calculated mFTT per season (mean±s.d.): 9.6±2.5 h (N=7) during autumn, 9.8±2.1 h (N=8) during early winter, 10.6±4.63 h (N=10) during late winter, and 8±3.5 h during summer (N=19). As the shortest mFTT found was 6 h, we considered all feces deposited before that time as basal samples. Time of collection of basal samples after capture was 0.64±0.7 h (N=7) during autumn, 1.8±0.9 h (N=8) during early winter, 1.7±1.4 (N=10) during late winter, and 1.6±1.1 h (N=19) during summer. According to this, in order to compare stress reactivity between seasons samples were grouped as follow: stress reactivity 1 (SR1) were the samples collected between 6 and 14 h after capture, and stress reactivity 2 (SR2) were the samples collected between 15 and 24 h. After 24 h, bats were returned to their sites of capture.

The study was conducted in strict accordance with the recommendations and permits approved by Mexican authorities (permits 13/13 from Secretaría de Gobernación and 01947/13 from Dirección General de Vida Silvestre to L.G.H.M.). All sampling procedures and experimental manipulations were performed according to the Principles of the Mexican Official Ethics Standard 062-ZOO-1999. No other approval was required to conduct the study, as there is no Institutional Animal Care and Use Committee or animal ethics board at our institution.

Fecal cortisol assay

The protocols used to extract corticosteroid metabolites from feces have been previously used and validated to detect the activation of the HPA axis in response to stress in different wild mammals (Mateo and Cavigelli, 2005; Ordóñez-Gómez et al., 2016). However, the methods were modified in order to adjust to the low mass of samples (~0.05 g). Accordingly, each sample was dried out at 65°C in a scientific oven (Precision Scientific 25EM), pulverized and weighed. Samples were placed in 3 ml glass tubes, 600 µl of 100% ethanol was added, and then they were vortexed for 1 min every 30 min for 3 h and incubated at 4°C for 12 h. Samples were vortexed again for 3 min and centrifuged at 4°C for 15 min at 4481 ***g***. The supernatant was decanted into 1.5 ml tubes and samples were centrifuged again at 4°C for 10 min at 14,900 ***g***. The process was repeated until all solid matter was removed and the samples were dried on a hot plate with nitrogen flow. Samples were then resuspended by adding 160 µl of 100% ethanol, and kept at −24°C until radioimmunoassay (RIA). Fecal extraction efficiency was measured by the recovery of ^{125}I-cortisol (16.00%, 74.6±11.9% coefficient of variation, mean±s.e.m., N=8).

Cortisol concentrations were quantified in the samples with a solid phase ^{125}I RIA method using cortisol CORT-CT2 CIS kits (Bio International, Gif-sur-Yvette, France), following the protocol for saliva samples as indicated by the manufacturer. The calibration range for the assay was 0–2000 nmol l^{-1}. Samples were incubated for 30 min at 37°C and radioactivity was measured using a Packard Cobra II scintillation counter (Canberra Industries, Meriden, CT, USA) for gamma radiation. This kit presents a low cross-reactivity with corticosterone (2.5%) and cortisone (2.2%). As cortisol levels in this species were unknown, no dilutions were made in order for samples to enter within the standard curve of the kit. All extracts were assessed with one replica, in a total of two assays. Intra- and inter-assay coefficients of variation were 0.73 and 4.92%, respectively. Differences of slopes derived from each linear regression were tested using the method proposed by Zar (1984) which is equivalent to an analysis of covariance (ANCOVA) that is used to compare more than two lines. Parallelism was assessed, and no significant differences ($F_{2,9}$=0.0733, P=0.9298, N=2) were found between the slopes of a serial dilution curve of pooled bat fecal extracts and the slope of the standard curve. The slope of standards spiked with diluted fecal extract exhibiting high accuracy (B=−27.28, R^2=0.837, P=0.0038), indicating that the assay reliably measures FC across its range of concentrations. The minimum detection limit for this assay was 14 ng of GC per 50 µg of bat feces.

Body condition determination

Body condition has been measured as a non-destructive method to estimate nutritional state and provide a proxy of an animal's physiological state (Jakob et al., 1996). Two methods were used. First, we estimated the scaled mass index (SMI) (Peig et al., 2009), which relies on body mass and linear measures of body size to determine a condition index with the following formula: $pM_i = M_i\,(L_0/L_i)^{bSMA}$, where M_i is the mass (g), L_i is the forearm length (mm), L_0 is the forearm arithmetic mean, bSMA is the scaled exponent estimated using online software (http://www.kimvdlinde.com/professional/rma.html), and pM_i is the predicted body mass for an individual when the linear body measurement is standardized to L_0. The second method used was the hematocrit percentage (%H). When blood samples were collected and centrifuged (as described above), total volume of plasma and erythrocytes were measured with the aid of a micropipette. Hematocrit was calculated as the percentage of erythrocytes corresponding to the total volume of blood collected. The %H is directly related to plasma volume, rate of erythrocyte production and destruction, dehydration, toxins and direct blood loss, and it may thus be used as an index of the 'health' of the oxygen transport system (Bolliger and Everds, 2012). The %H was calculated for both basal and post-stress blood samples (6 and 12 h).

Phytohemagglutinin challenge

The delayed cutaneous hypersensitivity response was quantified as an indicator of non-specific inflammatory response (Huyghe et al., 2010; Kamhawi et al., 2000; Vinkler et al., 2014) in all bats captured. This response was assessed by injecting 50 µl of a PHA solution [3 mg PHA ml^{-1} of phosphate-buffered saline (PBS)] in the right foot and 50 µl PBS in the left foot. PHA activates a variety of cell types and therefore the response to its injection is complex, but it can serve as an index for heightened immune cell activity (Kennedy and Nager, 2006; Martin et al., 2006a). Thickness of the foot was measured before injection and at 6, 12 and 24 h afterwards using digital calipers (Mitutuyo Cd-6″ CSX, ±0.01 mm). Cellular immune response was estimated as the change in thickness of the PHA-injected foot minus the change in the control foot. Larger localized swelling was indicative of increased immune activity. Measurements were made in triplicate and the mean was used for the analyses.

Bactericidal activity

The anti-microbial capacity of plasma was assessed with the Liebl and Martin Ii protocol (Liebl and Martin Ii, 2009) as a measurement of serological components (Merchant et al., 2003) [non-specific antibodies (Ochshenbein et al., 1999), complement cascade (Esser,

1994; Nordahl et al., 2004) and lysozyme activity (Selsted and Martinez, 1978)]. Before assay, the bacteria *Escherichia coli* (ATCC no. 8739) was reconstructed according to manufacturer instructions. The stock solution of the bacteria was diluted to 1×10^5 microbes ml^{-1}. Plasma was diluted (1:23) in sterile PBS, 25 µl of working solution was added, and samples were incubated for 30 min at 37°C. After the first incubation, 500 µl of Soy Broth (TSB) were added to samples, which were incubated at 37°C for an additional 12 h. After the second incubation the samples were analysed spectrophotometrically (Beckman DU-650) at 340 nm. The proportion of killed bacteria was calculated as 1 − (sample A_{340}/control A_{340}), where A is absorbance at 340 nm. All samples were analysed in duplicate.

Combined ACTH and PHA stimulation

The effects of a strong stress and immune response stimulation (IRS) on antioxidant activity and GC in four experimental groups were determined. Each group contained five females captured with mist nets at 21.00 h in summer. The first group (ACTH–PHA), was injected intraperitoneally with 20 µg of ACTH (1.0±0.1 µg ACTH g^{-1} body weight) within 5 min of capture, and immediately challenged with a subcutaneous injection of 50 µl of a PHA solution (3 mg PHA ml^{-1} saline–PBS) in the right foot and 50 µl of PBS in the left foot. The second group (ACTH–PBS) was injected intraperitoneally with 20 µg of ACTH, followed by a subcutaneous injection of 50 µl of PBS in both feet. The third group (saline–PHA) was injected intraperitoneally with 20 µg of saline solution (0.9% NaCl) followed by a subcutaneous injection of 50 µl of PHA in the right foot and 50 µl of PBS in the left foot. The fourth group (saline–PBS) was injected intraperitoneally with 20 µg of saline solution (0.9% NaCl) followed by a subcutaneous injection of 50 µl of PBS. After treatments, bats were kept individually in small cotton bags for 42 h, and checked every 2 h for fecal samples. Feces were collected and maintained in liquid nitrogen for cortisol analyses. A blood sample (300 µl) was collected 24 h after the treatment and maintained in liquid nitrogen, until its shipment to the laboratory in dry ice, where it was stored at −80°C before analysis of antioxidant activity and carbonyl concentration. Bats were fed twice a day during their captive period, and the food was dyed with blue vegetable pastry dye. Bats were allowed to eat freely, and the amount they ate was recorded. However, as not all bats ate the same amount, and the number of defecations of each bat was not equal, paired analysis of ANOVA was not possible. mFTT was 10.7±3.6 h (mean±s.d., N=17) so FC samples were grouped as basal samples (mFTT, 3.1±2.8 h) collected within 5 h of capture; second samples were collected within 6–12 h, third samples within 12–24 h, and fourth samples after 24 h.

Protein extraction from erythrocytes

Erythrocytes are unique, highly specialized and the most abundant cells in humans. Although their primary function is transportation of O_2 and CO_2 between the lungs and tissues (Pandey and Rizvi, 2011), they also have an innate antioxidant mechanism due to their high content of reduced (ferrous) iron that they need to be functional as an oxygen carrier, and which ultimately leads to the production of ferric iron ions (Franco et al., 2019). Therefore, erythrocytes are thought to be active scavengers of ROS by virtue of their large antioxidant reservoirs (Agar et al., 1986; Toth et al., 1984; Van Asbeck et al., 1985), providing antioxidant protection for themselves and also to other tissues and organs in the body. Furthermore, erythrocytes are highly sensitive to protein oxidation by ROS, which affects hemoglobin and several other proteins from the cytoskeleton (Pandey and Rizvi, 2011). Hence, due to the above mentioned and the small volumes of our blood samples, we used. Hence, due to the above mentioned and because of the small volumes of our blood samples. We decided to use the plasma fraction of the blood samples for FC and BA measurements, and erythrocytes fraction for antioxidant and protein damage measurements.

The above mentioned is the justification for using only erythrocytes for antioxidant activity, which the reviewer asked us to put plasma samples for FC and BA measurements, and erythrocytes for antioxidant and protein damage measurements.

Erythrocytes samples were first washed twice with 0.9% NaCl by centrifugation at 4500 ***g*** for 10 min. Protein was then extracted by adding lysis buffer [100 µl of 1 mol l^{-1} dithiothreitol (DTT), 100 µl of 0.1 mol l^{-1} phenylmethylsulfonyl flouride (PMSF), 1 complete mini protease inhibitor tablet, 10 ml T-PER] and centrifuged at 20,200 ***g*** at 4°C for 15 min. Protein samples were separated in four aliquots to prevent freezing and thawing. Before each assay, total protein concentration was determined spectrophotometrically at 595 nm from each aliquot, using a commercial Bradford reagent (Bio-Rad, Hercules, CA, USA) (Bradford, 1976).

Antioxidant enzyme activity

Protein from erythrocyte samples was used to assess antioxidant enzyme activity spectrophotometrically (GENESYS 10S UV-Vis; Thermo Fisher Scientific, Madison, WI, USA) as described elsewhere (Conde-Pérezprina et al., 2012; Hernández-Arciga et al., 2018). Samples were diluted 1:10. SOD activity was determined through the xanthine–xanthine oxidase system, based on protocols by Paoletti et al. (Paoletti et al., 1986). The superoxide anion formed through this system reacts with Nitro Blue Tetrazolium (NBT) and generates a formazan salt, which was measured every 30 s for 5 min at 560 nm. One unit of enzymatic activity in this assay is considered to be the amount of SOD needed to inhibit 50% of the superoxide reaction with NBT. CAT activity was measured using the protocol established by Abei (Aebi, 1984), which evaluates H_2O_2 decrease at 240 nm every 15 s for 3 min. One unit of catalase activity (UCAT) was considered to be the amount of enzyme necessary to catalyse 1 µmol of H_2O_2 per minute. GPx activity was evaluated at 340 nm with the protocol described by Ahmad et al. (Ahmad et al., 1989). One unit of GPx activity indicates how much enzyme is required to neutralize H_2O_2 using NADPH (Flohé and Günzler, 1984).

Protein oxidative damage

In order to assess oxidative damage to proteins, we evaluated carbonyl content in proteins as biomarkers of cellular stress (Davies et al., 1999). Carbonyls are introduced into proteins either by direct oxidation of amino acids or indirectly by oxidation products of carbohydrates or polyunsaturated fatty acids, and such damage to proteins leads to a loss of functional and structural efficiency (Mateos and Bravo, 2007; Mirzaei and Regnier, 2008). Carbonyls were determined by using the 2,4-dinitrophenylhydrazine (DNPH) alkaline method (Mesquita et al., 2014) and adjusting the optimal volumes for use in 96-well plates. Twenty microliters of DNPH (10 mmol l^{-1} in 0.5 mol l^{-1} H_3PO_4) were added to 20 µl of sample protein. Samples were incubated for 10 min in the dark with constant agitation. Then 10 µl of NaOH (6 mol l^{-1}) were added and further incubated in the dark for 10 min at room temperature. Absorbance was determined at 450 nm against a blank where the protein solution was substituted by an equal volume of buffer solution. Carbonyl content was calculated as (A_{450}/E)/total protein content of sample, where E=extinction factor of 46.1.

Journal of Experimental Biology (2020) 223, jeb234914. doi:10.1242/jeb.234914

Statistics

To test the effect of movement restriction on FC concentrations, we performed a paired *t*-test in all comparison groups, except for the comparison between the SR1 and SR2 groups in early winter, where we performed a Wilcoxon paired test.

To test differences between seasons, three different multivariate discriminant analyses with Wilks' lambda test were performed. Multivariate normality (Mardia's test) and covariance (Box's *M* test) for each dataset were assessed. The variables used for the first discriminant analysis were the three fecal cortisol time samples: basal, SR1 and SR2. The dataset had multivariate normality (*s*kewness: b=2.43, z=12.95, d.f.=10, P=0.23; kurtosis: b=15.5, z=0.26, d.f.=10, P=0.80), and had equal covariance (M=38.62, $F_{18,446.26}$=1.33, P=0.29). The variables used for the second discriminant analysis were the basal data of cortisol, SOD activity, CAT activity, GPx activity, BA activity, PHA and carbonyl content; data had multivariate normality (skewness: b=11.51, z=69.05, d.f.=56, P=0.90; kurtosis: b=43.78, z=–1.29, d.f.=56, P=0.19), but no equal covariance (M=430.98, $F_{63,585.5}$=2.62, P=2.1×10^{-9}). The variables used in the third discriminant analysis were the post-stress data of cortisol, SOD activity, CAT activity, GPx activity, BA activity and carbonyl content; data had multivariate normality (skewness: b=21.08, z=73.77, P=0.7799; kurtosis: b=57.1, z=−1.21, P=0.228) and equal covariance (M=1185.8, $F_{84,461.2}$=0.656, P=0.99). Therefore, we used the regularized method for the second discriminant analysis and the linear method for the first and third discriminant analyses.

To evaluate if any other stress parameter (antioxidant activity and immune response) was related to basal and post-stress cortisol, a multiple regression model was performed. Multivariate normality was tested with Mardia's test. Both basal data (skewness: b=17.36, z=57.9, d.f.=56, P=0.41; kurtosis: b=38.63, z=–2.92, d.f.=56, P=0.06; SOD and BA were log transformed) and SR data (skewness: b=11.73, z=35.18, d.f.=35, P=0.46; kurtosis: b=28.72, z=–1.59, d.f.=35, P=0.1111) were normally distributed. Only variables which significantly contributed to the model after a stepwise regression control analysis were included (P<0.05). For the basal multiple regression, carbonyl content (P=0.22), PHA (P=0.82), SMI (P=0.41) and hematocrit percentage (P=0.09) were not included in the final analysis. For the post-stress analysis, carbonyl content (P=0.21), SMI (0.26), CAT (P=0.43) and hematocrit percentage (P=0.65) were not included in the final analysis.

A two-way ANOVA test was performed to compare the four treatments challenged with ACTH and IRS, with the four sample set points (basal, second, third and fourth). Tukey's *post hoc* tests were performed to compare differences between treatments within each time set point, changes in FC with each treatment over time, and differences in total cortisol excreted over the 48 h experiment between each treatment. Finally, we performed a one-way ANOVA with Tukey's *post hoc* tests to determine the effect of each ACTH–immunization treatment in antioxidant activity and inflammation response.

All univariate analyses and graphs edition were performed in GraphPad Prism 7 (www.graphpad.com), and multivariate analyses were performed in JMP 9 (www.jmp.com).

RESULTS

Validation of movement restriction as a stressor

In order to verify that physical immobilization inside the cotton bags was being perceived by bats as a stressor, FC samples were obtained during SR1 and SR2. As shown in Fig. 1, FC levels in stressed samples were significantly higher compared with their paired basal samples (P<0.05).

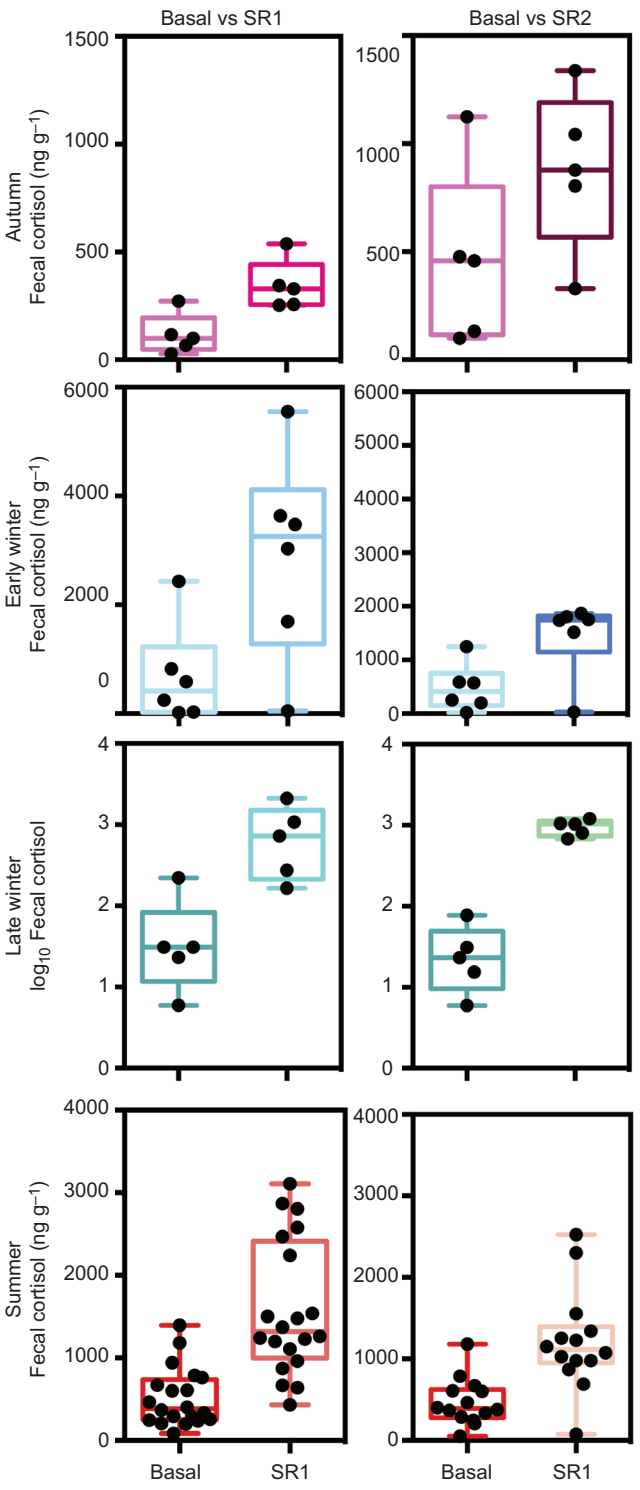

Fig. 1. Fecal cortisol metabolite change after acute stress by movement restriction. Box and whisker graph with mean, minimum and maximum values. Left column: comparison between FC basal samples (collected 0–5 h after capture) and stress reactivity 1 (SR1) samples (collected 6–14 h after capture). Right column: comparison between FC basal samples and stress reactivity 2 (SR2) (collected 15–24 h after capture). A Student's paired *t*-test was performed in each case (N=5). All comparisons were significant (P<0.05).

Relationship with other physiological parameters

A significant relationship of FC basal levels with basal antioxidant activity and bactericidal activity was found ($F_{4,11}$=9.25, r^2=0.77, P=0.0016) (Fig. 2A). Under basal conditions, SOD (β=28.40,

Journal of Experimental Biology (2020) 223, jeb234914. doi:10.1242/jeb.234914

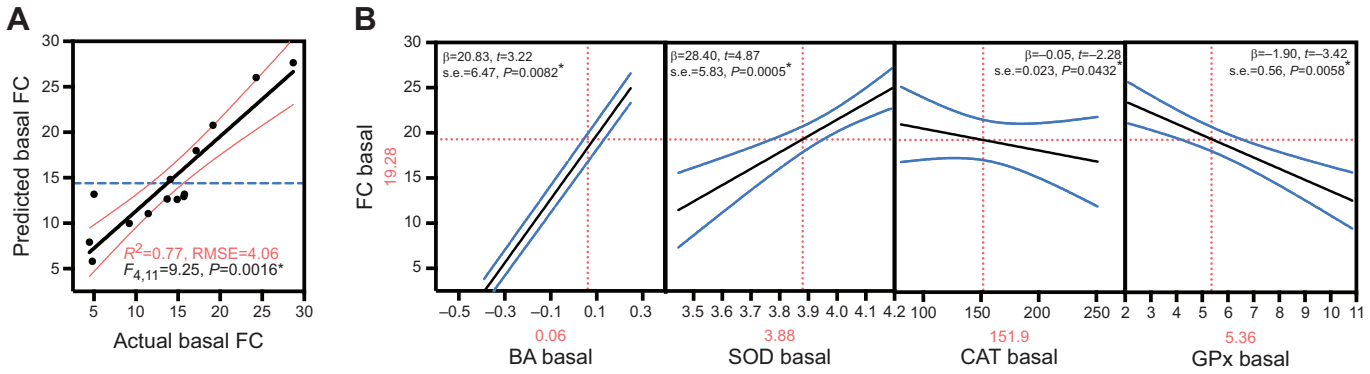

Fig. 2. Multiple regression model for basal samples. (A) Actual versus predicted FC model plot; blue dashed line represents mean response. (B) Prediction profiler between FC and BA, SOD, CAT and GPx. Blue solid lines represent confidence intervals, red dotted lines represent *x* and *y* predicted values at a given point; *N*=18. RMSE, root mean square error.

P=0.0005), GPx (β=−1.9, *P*=0.0058), CAT (β=−0.05, *P*=0.0432) and BA activity (β=20.83, *P*=0.0082) were good predictors of bat FC (Fig. 2B). Together, these variables explained 77% of the total variance, significantly more than what they can explain on their own (BA r^2=0.28, *P*<0.0416; SOD r^2=0.26, *P*=0.0497; PHA r^2=0.07, *P*=0.94; CAT r^2=0.15, *P*=0.1550; GPx r^2=0.006, *P*=0.76; SMI r^2=0.13, *P*=0.1792). The regression model predicted that when bats have high BA and SOD activity, medium levels of CAT activity and low GPx activity, animals would have high levels of basal FC (Fig. 2B).

For SR1 FC samples, the multiple regression model was also significant ($F_{4,13}$=42.42, r^2=0.92, *P*<0.0001) and accounted for 92% of the variation (Fig. 3A). SOD6 (β=0.003, *P*=0.0002), GPx6 (β=3.83, *P*=0.0515), PHA (β=–11.21, *P*=0.0403) and BA 6 (β=23.17, *P*=0.0039) activity were significant in the model (Fig. 3B), and together they explained more than the univariate models (SOD6 r^2=0.005, *P*=0.8084; GPx6 r^2=0.32, *P*=0.0236; PHA r^2=0.38, *P*=0.4266; BA6 r^2=0.36, *P*=0.0142). The multivariate regression model predicts that after being exposed to a stress stimuli (6–12 h), bats would have high levels of SOD, medium levels of GPx and BA activity, and low levels of PHA response (Fig. 3B).

Seasonal changes

The discriminant analysis with basal, SR1 and SR2 FC as covariates was significant (Wilks' lambda=0.46, $F_{9,58.5}$=2.44, *P*=0.0198), and both canonical dimensions explained 96.2% of the total variance. Autumn, summer and early winter presented equally high levels of basal FC compared with late winter. SR1 levels were higher in summer, followed by early winter, and SR2 FC levels were higher in summer and early winter compared with autumn and late winter (Fig. 4A).

The discriminant analysis of basal physiological measurements was significant (Wilks' lambda=0.19, $F_{18,76.8}$=3.36, *P*<0.0001), indicating a significant seasonal separation. Both canonical dimensions explained 90.95% of the total variance. Summer, early winter and autumn were characterized by high basal cortisol values, compared with late winter. Summer had higher carbonyl content and BA plasma activity, while early winter and autumn had higher SOD and CAT activity. Late winter had a higher GPx activity compared with the other seasons (Fig. 4B).

The discriminant analysis of post-stress physiological measurements was significant (Wilks' lambda=0.08, $F_{21,32}$=2.22, *P*=0.02), with both canonical dimensions explaining 92.41% of the total variance. Results revealed that bats in late winter had the highest levels of SR1 FC and GPx activity, whereas in summer bats had the highest levels of SR2 FC, SOD, BA activity and carbonyl content (Fig. 4C). Furthermore, the discriminant analysis also revealed that there were some individual bat samples from early winter that, based on their physiological parameters, were predicted to be similar to bats in summer or autumn. Therefore, it is highly probable that early winter represented a buffering season.

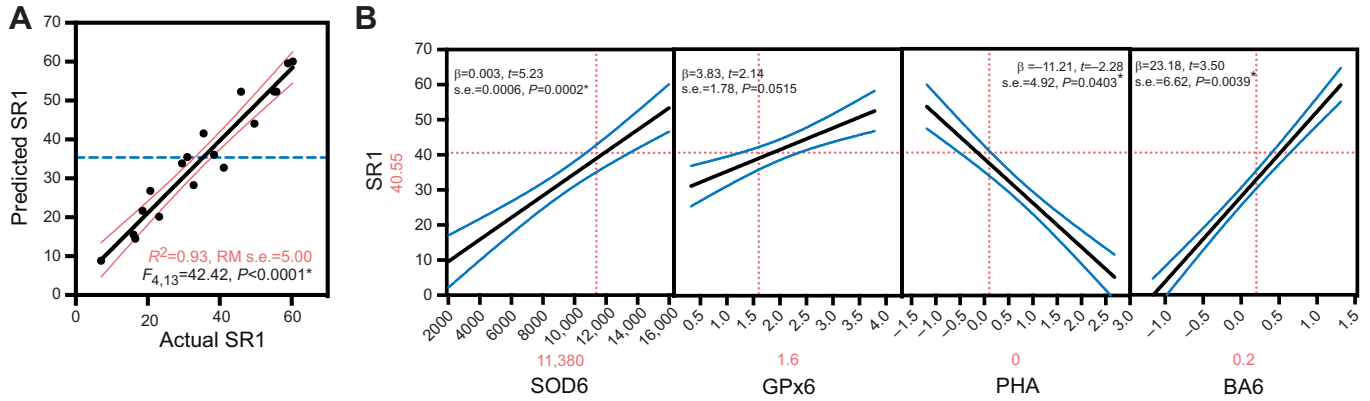

Fig. 3. Multiple regression model stress reactivity 1 samples. (A) Actual versus predicted FC model plot; blue dashed line represents mean response. (B) Prediction profiler between FC and SOD, GPx, PHA and BA. Blue lines represent confidence intervals, red dotted lines represent *x* and *y* predicted values at a given point; *N*=16. RMSE, root mean square error.

Journal of Experimental Biology (2020) 223, jeb234914. doi:10.1242/jeb.234914

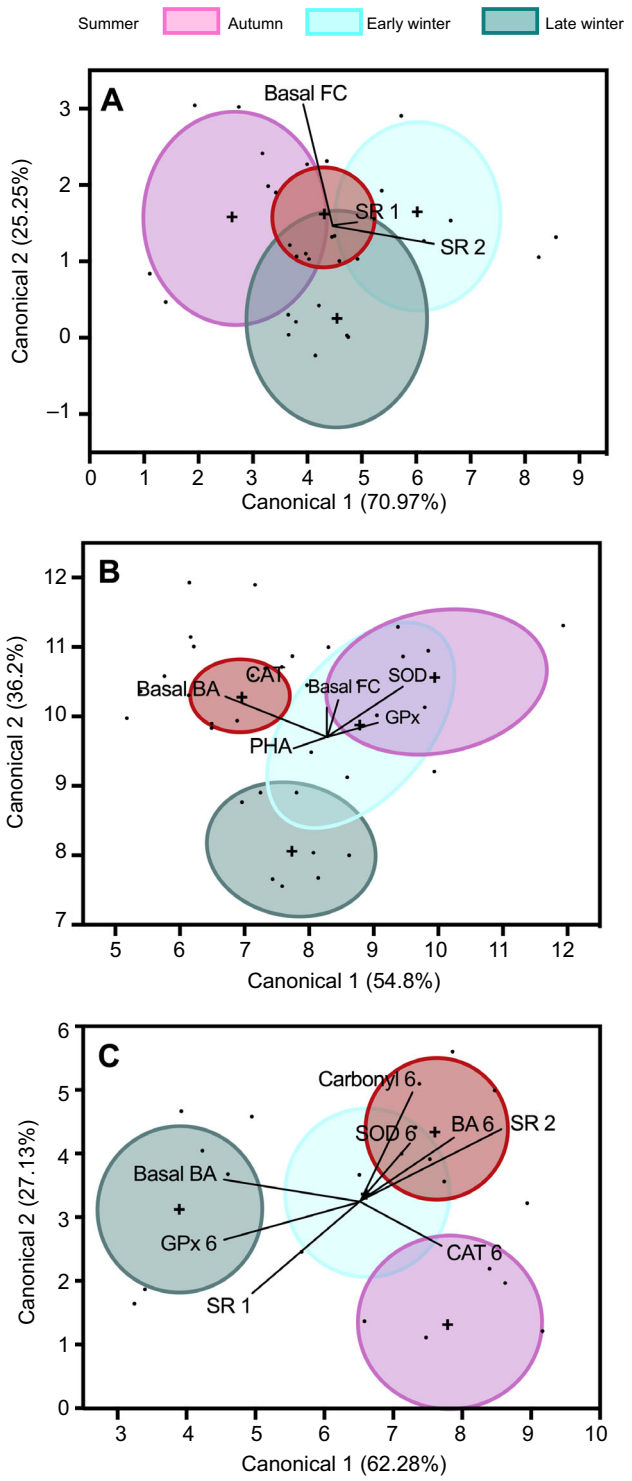

Fig. 4. Discriminant analysis by season. The canonical plot shows the two dimensions that best separate the groups. The percentage of variation explained by each dimension is indicated. Each group mean is denoted by a plus (+) marker. A 95% confidence level ellipse is plotted for each mean. If two groups differ significantly, the confidence ellipses tend not to intersect. The length and direction of each ray in the biplot indicate the degree of association of the corresponding covariate to the classified groups. Autumn, *N*=7; early winter, *N*=9; late winter, *N*=11; summer, *N*=25. (A) Linear discriminant analysis of fecal cortisol (FC) as physiological parameters characterizing seasonal change. (B) Regularized discriminant analysis of basal physiological parameters as indicators of change in seasons: FC antioxidant activity (SOD, CAT and GPx) and immunocompetence (BA activity and PHA response). Baseline samples were collected 0–5 h after capture. (C) Linear discriminant analysis of post-stress physiological parameters as indicators of change in seasons: FC of SR1 data (collected 6–14 h after capture) and SR2 (collected 15–24 h after capture), as well as antioxidant activity (SOD, CAT and GPx) and immunocompetence (BA activity and PHA response) collected after 6 h of capture. Wilks' lambda test was significant for the three models (P<0.05).

ACTH and IRS

ACTH–PBS and saline–PBS treatments ($F_{3,55}$=3.29, P=0.0271) had higher FC concentrations at the second and fourth sampling points than in basal samples, but not at the third sampling point (P<0.05) (Fig. 5A). There were significant differences when comparing FC concentrations between treatments among sample-time groups ($F_{3,55}$=7.63, P=0.0002); values from the second and fourth time periods were higher in the ACTH–PBS treatment than in the saline–PBS (P<0.0211), saline–PHA (P<0.007) and ACTH–PHA (P<0.0152) treatments (Fig. 5B).

The total GC excreted in the 42 h of the experiment was significantly different among groups ($F_{3,55}$=2.97, P=0.0059); the ACTH–PBS treatment had the highest total FC concentration compared with saline–PBS (P<0.0001), saline–PHA (P=0.0003) and ACTH–PHA (P=0.0036) treatments (Fig. 5C).

Inflammation after IRS varied with treatment ($F_{3.16}$=33.26, P<0.0001). Inflammation in the ACTH–PHA and saline–PHA treatments was higher than in treatments without PHA (P<0.05) (Fig. 6). Inflammation after 12 and 24 h post-IRS also varied among treatments ($F_{3.16}$=32.27, P<0.0001). It was higher in the ACTH–PHA and saline–PHA treatments than in the ACTH–PBS (P<0.003) and saline–PBS (P<0.0001) treatments, but, specifically after 24 h, inflammation was lower in the ACTH–PHA treatment than the saline–PHA treatment (P=0.0199) (Fig. 6). CAT activity varied among treatments ($F_{3,8.2}$=33.87, P<0.0001); it was lower in the saline–PBS treatment than in ACTH–PBS (P=0.0026) and saline–PHA treatments (P<0.0001). No other treatment comparisons (Fig. 6) were significantly different after 24 h. GPx activity varied significantly among treatments ($F_{3,16}$=7.68, P=0.0021); activity was higher for the saline–PHA treatment than the saline–PBS (P=0.0047), ACTH–PBS (P=0.0061) and ACTH–PHA treatments (P=0.0087; Fig. 6). SOD activity varied among treatments ($F_{3.16}$=6.48, P=0.0044); the activity in the ACTH–PHA treatment was lower than in the saline–PBS (P=0.0035) and ACTH–PBS treatments (P=0.0262), but it was not significantly different from the saline–PHA treatment (P=0.2152; Fig. 6).

DISCUSSION

Relationship with other physiological parameters

We tested the relationship between cortisol (as the main GC) and redox state parameters (enzymatic antioxidant activity and protein oxidative damage) in both basal and post-stress conditions in *Myotis vivesi*, and determined if they could be complementary fitness indicators of the stress response for wild animals. We found that individual redox state parameters were poor predictors of cortisol levels in both basal and post-stress situations. Even when there were significant relationships, most correlations of single parameters with FC had low determination coefficients (r^2<0.50). In turn, models that considered the pooled effect of several parameters explained a high proportion of the variation. Basal BA, CAT, SOD and GPx activity explained 77% of the variation of basal FC, whereas post-stress BA, SOD and GPx activity and PHA explained 93% of the variation in post-stress FC.

It is well known that chronic exposure to GCs impairs antioxidant defenses (Fontella et al., 2005; Lin et al., 2004; McCoy et al., 2010;

Journal of Experimental Biology (2020) 223, jeb234914. doi:10.1242/jeb.234914

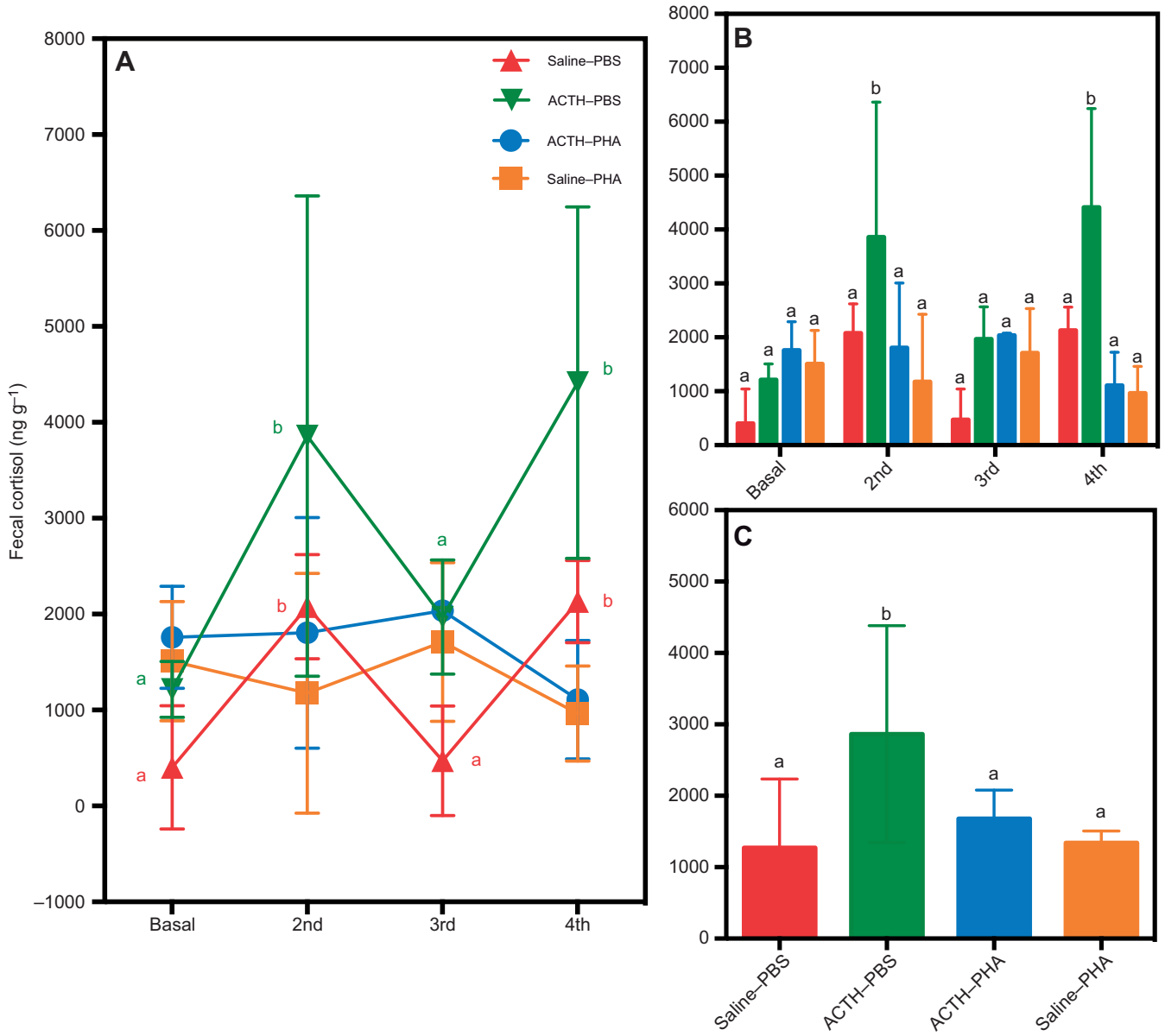

Fig. 5. Fecal cortisol metabolite change after combined ACTH and PHA stimulation. Two-way ANOVA analysis (mean±s.d., *N*=5). (A) FC change over time after treatment injection; multiple comparison between sample times for each treatment group (identified by colors); lowercase letters denote significant differences ($P<0.05$). (B) FC multiple comparison of treatments by sampling time groups; lowercase letters denote significant differences ($P<0.05$) within each time group. (C) Comparison of total FC excreted during experiments between treatment groups; lowercase letters denote significant differences ($P<0.05$).

McIntosh et al., 1998; You et al., 2009). However, to our knowledge, this study is the first attempt to determine how oxidative stress parameters relate to fecal cortisol levels during an acute stress stimulus in a natural setting. Oxidative stress implies an imbalance between oxidant and antioxidant agents; hence enzymatic antioxidants are central for redox state balance (Fregoso-Aguilar et al., 2016) and are expected to reflect animal fitness. However, the relationship between GCs and the immune response seems to be more complex. PHA-induced swelling was considered an index of T cell-mediated immunocompetence (Martin et al., 2006b; Tella et al., 2008). However, it has been proved that T cell mediation increases after re-exposure to PHA and after 24 h of injection, and that other leucocytes (basophils, neutrophils and eosinophils) are responsible for localized vasodilatation, infiltration and edema (Ilkov and Vinkler, 2015; Kennedy and Nager, 2006; Martin et al., 2006b; Turmelle et al., 2010; Vinkler et al., 2012). Additionally, the number of lymphocytes present previous to PHA injection positively affects the number of lymphocytes infiltrating the tissue (Dhabhar and McEwen, 1996). Higher initial basophil and lymphocyte frequencies in peripheral blood were associated with increased swelling, and high pre-treatment blood lymphocyte levels led to intense lymphocyte migration into inflamed tissue, while high initial basophil levels resulted in lower cellular infiltration (Ilkov and Vinkler, 2015), suggesting that the levels of basal leucocyte populations determines the intensity of the response when it is presented with a novel antigen. Twelve hours after PHA injection, neutrophils, thrombocytes and macrophages peaked and remain high at 24 h, and lymphocytes are most abundant after 24 h (Aapanius, 1998; Dhabhar, 2013; Dhabhar and Viswanathan, 2005; Martin et al., 2006b; Wingfield, 2003). Our findings revealed that basal FC levels had no direct relationship with inflammatory response. However, this relationship changed when bats were exposed to short-term novel stress

Journal of Experimental Biology (2020) 223, jeb234914. doi:10.1242/jeb.234914

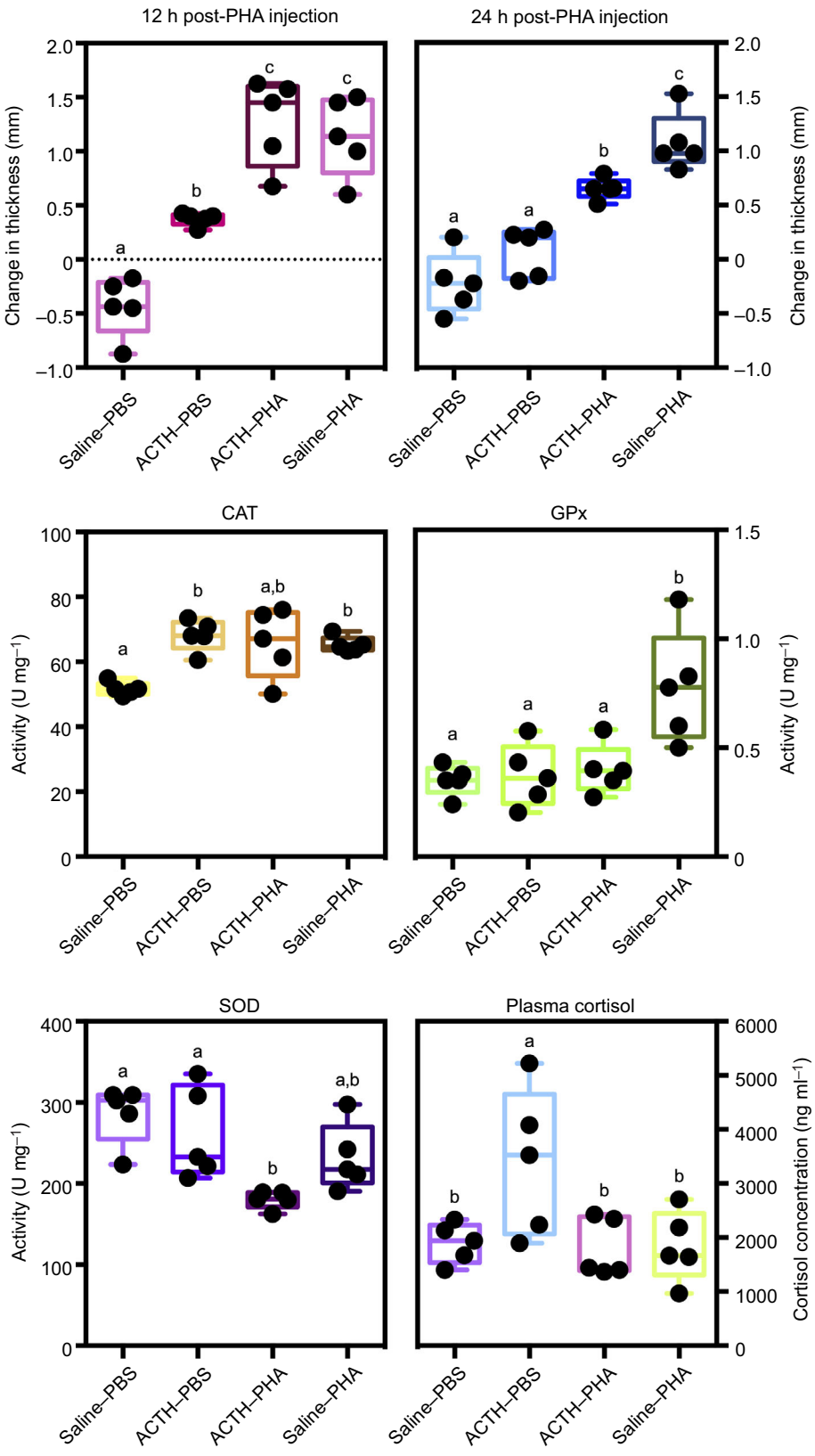

Fig. 6. Effect of combined ACTH and PHA stimulation on inflammatory response and antioxidant activity. One-way ANOVA (mean ±s.d., *N*=5); lowercase letters denote significant differences between treatments ($P<0.05$).

conditions, and inflammation and FC levels became negatively related, supporting the anti-inflammatory role of GC. Furthermore, FC levels were always positively correlated with BA, suggesting that GCs enhanced humoral activity in the short term. In our study, BA primarily measured the complement cascade and lysozyme activity (Liebl et al., 2013), and it has being previously reported that GCs increase the expression of complement 3, an element of the cascade (C3) in peripheral monocytes (Galon et al., 2002); GCs exert similar effects in lung epithelial cells and hepatocytes (Sacks et al., 2003). GC may therefore have a counter-repressive action on local

complement functions (Sacks et al., 2003). Body condition was not found to have a direct relation to basal or post-stress FC levels.

Seasonal changes

Significant effects of season or weather conditions such as temperature, humidity and availability of food and water on fecal GC concentrations have been shown for several species of mammals and birds, with most studies reporting higher levels during harsher conditions in winter or during the dry season (reviewed in Touma and Palme, 2005). Other seasonal activities, such as breeding, lactation and hibernation are also known to influence basal GC levels in a wide range of species. However, in contrast to our expectations, we found little variation in FC levels of *M. vivesi* through the year. Basal FC samples displayed similar levels during summer, autumn and early winter, although lower concentrations in late winter (Fig. 1; Fig. 4A).

High levels of baseline GCs are routinely assumed to indicate poor conditions for individuals or populations, as elevated GC levels trigger re-allocation of resources away from normal activities of reproduction and survival to cope with the environmental challenge and reducing fitness (Bonier et al., 2009). Therefore, the lack of variability of basal FC through the year indicates that fish-eating bats are well adapted to seasonal ambient conditions. The low FC during late winter could be due to daily torpor and hibernation during the winter period (Salinas-Ramos et al., 2014) as GCs are known to play an important role in torpor (Hiebert et al., 2000; Willis and Wilcox, 2014). Similar to our findings, high GC levels during the beginning of the hibernating season have been reported for the little brown bat (*Myotis lucifugus*) with a progressive decrease by the end of the season (Gustafson and Belt, 1979).

In contrast, FC concentration differed among seasons after an acute stress stimulus. FC concentration at SR1 was higher in autumn and late winter, but when the stress continued (SR2), FC concentrations were higher in summer and early winter. This is consistent with the antioxidant activity and bactericidal capacity of *M. vivesi* after 12 h of stress in summer and early winter (Hernández-Arciga et al., 2018). This finding indicates that bats responded more strongly to stress stimuli during autumn and late winter, but quickly resumed their negative feedback to normal levels. In contrast, bats were more susceptible to stress during summer and early winter, probably due to the weather (compared with the other seasons), plus having low body condition (reported elsewhere: Hernández-Arciga et al., 2018) during late winter (which could suggest reduced foraging) as an additional factor. All antioxidant activity under basal conditions was related to FC and was higher during autumn, while SOD and BA activities were more related to SR2, and were higher in summer and late winter, highlighting the importance of SOD protection against superoxide radical $O_2^{\bullet}$ during prolonged stressful conditions. GPx and CAT activities were more related to SR1 during late winter and autumn, and appeared unable to maintain their function for long periods of time.

ACTH and immunization challenge

IRS had a dampening effect on the stress response of animals. All bat groups had similar basal FC, but those bats treated with ACTH–PHA had significantly lower FC excretions than stressed bats that did not receive PHA (ACTH–PBS). Both bat groups without the IRS (saline–PBS and ACTH–PBS treatments) had the same change pattern over time, with FC peaks at the second and at fourth sample times, only differing in the intensity of response. However, IRS bat groups (saline–PHA and ACTH–PHA treatments) showed no change in FC concentration over time, and the group injected with exogenous ACTH had a lower stress response. A possible explanation for this pattern is that in the face of an immune challenge, organisms react by lowering their GC response in order to cope with the immune threat. For instance, a tropical population of house sparrows (*Passer domesticus*) under low stress conditions had a normal PHA inflammation response in comparison with sparrows under stress conditions, which presented a suppressed inflammation response to PHA (Martin et al., 2005). Also, it has been demonstrated in air-breathing fish (*Anabas testudineus*) demonstrated that an immune challenge suppresses the cortisol-driven stress response (Simi et al., 2017). Therefore, these results suggests that animals which encounter an immune challenge may prioritize immune response over stress response, or that animals living in areas where disease threats are high might maintain low baseline and stress-induced levels of GC (Martin et al., 2005). However, our results reveal a complex interaction between ACTH injection and PHA injection. The PHA injection compromised cortisol secretion as the ACTH–PHA group had lower cortisol than the ACTH–PBS group, and was similar to the control group (saline–PBS), which had only the endogenous GC concentration. At the same time, the inflammation response was also compromised as it was lower in the ACTH–PHA group. This could be explained by reports that have shown that stimulation with PHA increases GC receptor activity threefold in lymphocytes (Neifeld et al., 1977), and consequently GCs exert an inhibitory effect on lymphocytes early in the cell cycle shortly after PHA stimulation (Niefeld and Tormey, 1979). Accordingly, our results show that the inflammation produced by PHA injection was the same in bats treated with ACTH–PHA and saline–PHA after 12 h, but after 24 h the inflammation in the saline–PHA group was greater than in the ACTH–PHA group, when lymphocyte infiltration had been demonstrated to be at its highest point (Martin et al., 2006b). Hence, we propose that under a mild stress situation, acute stress can enhance cell-mediated immunity, but this is inhibited after a certain threshold of stress intensity, even if the stress is for a short time. Furthermore, the experiment was performed during summer, when *M. vivesi* have been shown to be more susceptible to stress (Hernández-Arciga et al., 2018), which could also explain why they lowered their stress and immune responses when challenged with both stimuli at the same time.

The activity of each antioxidant enzyme was affected in different ways by the treatments. CAT activity was equally enhanced by the strong stress treatment (ACTH–PBS) and by the immunization treatment (saline–PHA), but it did not change when bats were exposed to stress and immunization simultaneously (ACTH–PHA treatment). However, GPx activity was not enhanced by stress, but was modified by immunization (saline–PHA), suggesting that GPx scavenger activity is strongly related to the immune oxygen burst, rather than to the stress response, as reported in Beck (2001) and Hernández-Arciga et al. (2018). In the case of SOD activity, it remained the same in almost all groups, but its activity dropped when bats were simultaneously exposed to stress and an immune challenge (ACTH–PHA). Thereafter, our results demonstrate that each enzymatic antioxidant reacts differently to stress and immune challenge, and their activity is compromised when animals confront both a stressful situation and an immune challenge at the same time.

Our findings support the idea that animals maintain constant basal GC levels when living in challenging environments, but their response to acute stress differs seasonally and their immune defense mechanisms might be compromised when confronted with multiple challenges. Bats exhibited a positive relationship between antioxidant activity and GCs, which could suggest that during short periods of time, high GC reactivity would be beneficial and might positively influence the prospects of survival. Therefore, we

strongly encourage more studies on the short-term effects of GC on different physiological parameters in order to further understand its implication in fitness, survival and evolution and make them available as effective markers of animal welfare.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: U.H.-A., L.G.H.M., M.C.R.; Methodology: U.H.-A., R.A.V., M.C.R.; Validation: U.H.-A., R.A.V.; Formal analysis: U.H.-A.; Investigation: U.H.-A., R.A.V.; Resources: L.G.H.M., M.K., M.C.R.; Writing - original draft: U.H.-A.; Writing - review & editing: L.G.H.M., M.K., R.A.V., M.C.R.; Visualization: U.H.-A., L.G.H.M., M.K., M.C.R.; Supervision: L.G.H.M., M.K., J.J.F.-M., M.C.R.; Project administration: L.G.H.M., J.J.F.-M.; Funding acquisition: L.G.H.M., M.K., M.C.R.

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