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Research Article

Fecal Glucocorticoid Metabolites as Biomarkers in Equids: Assay Choice Matters

DANIELLE L. HINCHCLIFFE,[1] *School of Science, Engineering and Environment, University of Salford, United Kingdom*
JESSICA M. D. LEA, *School of Earth and Environmental Sciences, University of Manchester, United Kingdom*
RUPERT PALME, *Department of Biomedical Sciences, University of Veterinary Medicine, Vienna, Austria*
SUSANNE SHULTZ,[2] *School of Earth and Environmental Sciences, University of Manchester, United Kingdom*

ABSTRACT Free ranging animals are exposed to environmental, demographic, and ecological challenges over time, which can affect their health and fitness. Non-invasive biomarkers can provide insight into how animals cope with these challenges and assess the effectiveness of conservation management strategies. We evaluated how free ranging ponies (*Equus ferus caballus*) on the Carneddau Mountain range, North Wales respond to 2 stimuli: an acute stressor of an annual roundup event in November 2014, and spatial and temporal variation in ecological factors in 2018. We evaluated fecal glucocorticoid metabolites using 2 enzyme immunoassays (EIAs): an 11-oxoetiocholanolone EIA (measuring 11,17-dioxoandrostanes [11,17-DOAs]) and a corticosterone EIA. The former assay has been validated in equids, whereas there is limited evidence for the suitability of the latter. We used an additional parent testosterone EIA to measure fecal androgen metabolites in response to the ecological challenges. Following the roundup, the metabolite concentrations measured by the 2 glucocorticoid EIAs were not correlated. The 11,17-DOAs were elevated from the second day following the roundup and then slowly returned to pre-round levels over the next 2 weeks. In contrast, the metabolites measured by the corticosterone assay showed no response to the roundup. For the ecological data, all 3 assays detected a positive correlation between metabolites and social group size in males but not in females. The metabolite concentrations measured by the testosterone and corticosterone assays were highly correlated and were temporally associated with the onset of the breeding season, whereas the 11,17-DOAs were not. The co-variance of metabolites measured by the corticosterone and testosterone assays, and the lack of an acute response in the corticosterone assay to the roundup, suggests that metabolites detected by the corticosterone assay were not primarily associated with increased glucocorticoid production. We recommend using well-validated fecal biomarker assays of hypothalamus-pituitary-adrenal axis activity to evaluate and compare the effect of different management interventions and environmental change. © 2021 The Authors. *The Journal of Wildlife Management* published by Wiley Periodicals LLC on behalf of The Wildlife Society.

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Demographic and ecological factors vary at spatial and temporal scales, which ultimately affects individual health and fitness, within and between populations (Homyack 2010). Environmental conditions impose different challenges for individuals over space and time. Home ranges with limited resources (e.g., food, water, refuges; Pulliam and Danielson 1991) have been associated with reduced fitness (Balestri et al. 2014). Seasonally varying climate imposes challenges through the costs of thermoregulation and variation in resource availability (Lattin et al. 2016). In particular, extreme temperatures can challenge thermoregulation and lead to behavioral changes in foraging and physical activity, as individuals try to maintain a positive energy balance (Iwamoto and Dunbar 1983). Social environments also impose stress through overt aggression, dominance relationships, intraspecific competition for resources, and harassment (Creel et al. 2013).

Physiological measures provide insight into how animals perceive environmental challenges (Wikelski and Cooke 2006). When an organism is exposed to a stressor (i.e., a change or challenge that disrupts physiological homeostasis) the hypothalamic-pituitary-adrenal (HPA) axis is activated to initiate an appropriate centralized stress response (Möstl et al. 2002). The end-products of the HPA axis are glucocorticoids (cortisol and corticosterone), which are steroid hormones that have a key role in mobilizing

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[1]*Current Affiliation: School of Science, Engineering and Environment, Room 302, Peel Building, University of Salford, Manchester M5 4WT UK*
[2]*E-mail: Susanne.shultz@manchester.ac.uk*

glucose to mediate energy balance, and subsequently regulation of whole-organism metabolism, nutrition, reproduction, and immunity (Cox et al. 2010, Wingfield and Romero 2010, Nicolaides et al. 2015). Thus, glucocorticoid concentrations are not equivalent to measuring a stress response directly, rather they represent an end-product from a signaling cascade (MacDougall-Shackleton et al. 2019). Glucocorticoids allow an animal to adaptively respond to predictable (e.g., food availability) and unpredictable (e.g., anthropogenic) challenges (Landys et al. 2006). Long-term elevation or depression of glucocorticoids, however, can have deleterious effects on individual fitness (Wingfield and Romero 2010). Thus, they have been used as a proxy for the effect of different environmental stressors (Madliger et al. 2018) such as habitat suitability, seasonality, extreme events, and management interventions on individuals (Lea et al. 2018).

Androgens are another group of steroid hormones linked to the development of male secondary sexual characteristics (Folstad and Karter 1992) and social breeding behaviors (Lincoln et al. 1972) in males. Chronic elevations of androgen levels are associated with trade-offs such as increased parasite burden (Braude et al. 1999, Malo et al. 2008) and high energetic costs (Wingfield et al. 2001, Miles et al. 2007). Androgens and glucocorticoids are not independent; in addition to a primary role in mobilizing glucose, glucocorticoids also suppress reproductive hormones (Fuzzen et al. 2011). Therefore, there are multiple endocrine biomarkers with the potential to measure stress and provide insights into the physiological pressure or strain on individuals and populations.

Physiological responses to challenges and stressors can reflect animal health, predict long-term population viability (Mason et al. 2009, Balestri et al. 2014), identify factors affecting population performance (Anderson et al. 2017), and help evaluate efficacy of intervention and management (Millspaugh and Washburn 2004, Dantzer et al. 2014). This is particularly important when handling or disturbing captive or free-ranging animal populations results in additional stressors demanding physiological responses. To undertake a broad-scale physiological assessment of a population, especially *in situ*, biologists require reliable, non-invasive biomarkers that cause minimal disturbance. Fecal biomarkers are frequently used to provide a window into an individual's physiological state. Glucocorticoid biomarkers have been developed in an effort to assess individual responses to stressors (Sheriff et al. 2011). Because feces contain glucocorticoid metabolites rather than the parent hormone (Möstl et al. 1999), it is important to carefully consider whether metabolites measured by an assay reflect the biological response of interest (Palme 2019). Moreover, fecal metabolites represent a pooled physiological response across a time window, rather than the snapshot that blood sampling provides. In addition, fecal measurements will have a lag time depending on the animal's gut passage time and activity rhythms (in horses ~24 hours; Palme et al. 2005, Touma and Palme 2005). Despite the importance of validating assays to ensure that their measures are biologically meaningful (Sheriff et al. 2011), many studies continue to apply assays without fully validating them physiologically or biologically in the studied species (Palme 2019).

The Carneddau ponies (*Equus ferus caballus*) are a genetically distinct population (Winton et al. 2013) of feral Welsh mountain ponies inhabiting uplands in Snowdonia National Park in North Wales. The pony social groups maintain relatively stable, overlapping home ranges that vary with elevation and slope (Stanley and Shultz 2012). For most of the year, the population is unmanaged; however, once a year the ponies are driven down from the mountains onto adjacent farmland by people on foot and using all-terrain vehicles and motorbikes. They are identified, undergo health checks, and surplus individuals are rehomed, translocated for conservation grazing schemes, or culled if in poor health. The Carneddau, as with other horse populations, are characterized by social groups that normally have 1 breeding male and ≥1 female (Linklater 2000). Males without a breeding group can form bachelor groups; however, these are not common on the Carneddau because excess young males are removed during the roundup (Stanley et al. 2018). Although the ponies are subject to highly seasonal climate and annual management interventions, the effects of environmental, social, and management factors on their health and fitness are unknown.

We evaluated different endocrine biomarkers to assess how Carneddau ponies respond to 2 different biologically relevant challenge scenarios: an annual roundup; and ecological variation in temperature, habitat, and social group size. We compared 2 enzyme immunoassays (EIAs), where 1 EIA, 11-oxoetiocholanolone EIA (Palme and Möstl 1997), has been designed to measure a group of fecal glucocorticoid metabolites (FGMs) and the other to target a parent glucocorticoid, corticosterone (CJM006; Watson et al. 2013). The 11-oxoetiocholanolone EIA measures 11,17-dioxoandrostanes (11,17-DOAs), a group of cortisol metabolites, and has been validated in equids across a range of studies including radiometabolism, physiological, and biological validations (Möstl et al. 1999, Merl et al. 2000). The corticosterone EIA has been used successfully in several species to measure FGMs (Fanson et al. 2017), but it has not been validated physiologically or biologically in equids, despite being used in several studies (Yarnell et al. 2016, Yarnell and Walker 2017, Lea et al. 2018).

We predicted the physiological response to the acute stress of the annual roundup event measured by the corticosterone assay should be correlated with the 11-oxoetiocholanolone EIA, which has proven to be a reliable measure of HPA activity via FGMs in equids (Möstl et al. 1999, Merl et al. 2000, Vick et al. 2012, Fureix et al. 2013, Gorecka-Bruzda et al 2015). Human handling and transportation increase HPA axis activity in horses (Kirkpatrick et al. 1979, Fazio and Ferlazzo 2003). We predicted the highest FGM concentrations would be in the 24–72 hours following the roundup and handling. In terms of ecological challenges, we

predicted that FGM concentrations would be sensitive to acute stressors such as social competition and acute weather events, whereas testosterone levels would increase prior to the onset of the breeding season.

STUDY AREA

The Carneddau mountain range in Snowdonia National Park, North Wales (53.22°N, 3.95°W), contains an area of approximately 35–40 km^2 of unfenced commons land managed by local farmers. The Carneddau mountains range up to 900 m elevation, but the main study area is between 287 m and 610 m above sea level. The climate in Snowdonia is north temperate, with monthly average temperature ranging between 4°C (Feb) and 15°C (Jul), with an average annual precipitation of 2,280 mm. The vegetation of the Carneddau is characterized by a mixture of dry and wet heath, where both typically occur on acidic, nutrient-poor soils but differ in the composition of dominant vegetation. Sheep and ponies are the only large mammals found on the Carneddau; however, other notable wildlife species include chough (*Pyrrhocorax pyrrhocorax*), peregrine falcons (*Falco peregrinus*), and pine martins (*Martes martes*). The land is used primarily for sheep farming and recreational hiking; as such the ponies are well habituated to human presence, though not to direct physical contact.

METHODS

To identify ponies used in the study, we used demographic data that has been recorded on a regular basis since 2007, including the location and membership of breeding groups. Each individual pony is identified using a photographic and a demographic database that records year of birth, natal group and descriptors including coat color, face and leg markings, and the presence of ear tags and notches. We estimated home ranges based on historical and contemporary sightings for each male and his group (Fig. 1). We produced a shape file of approximate home ranges by merging 250-m-diameter circles surrounding the sampling sites using MapInfo software (Pitney Bowes, Stamford, CT, USA).

We conducted a habitat assessment of the study area following Joint Nature Conservation Committee (1990) to assign the pony home ranges to a dominant land cover type, which can serve as an indicator of vegetation quality. The habitat assessment involved walking around the entire sampling area and noting all dominant vegetation zones (Fig. 1), taking global positioning system (GPS) coordinates of transitional boundaries for demarcation, and then ground

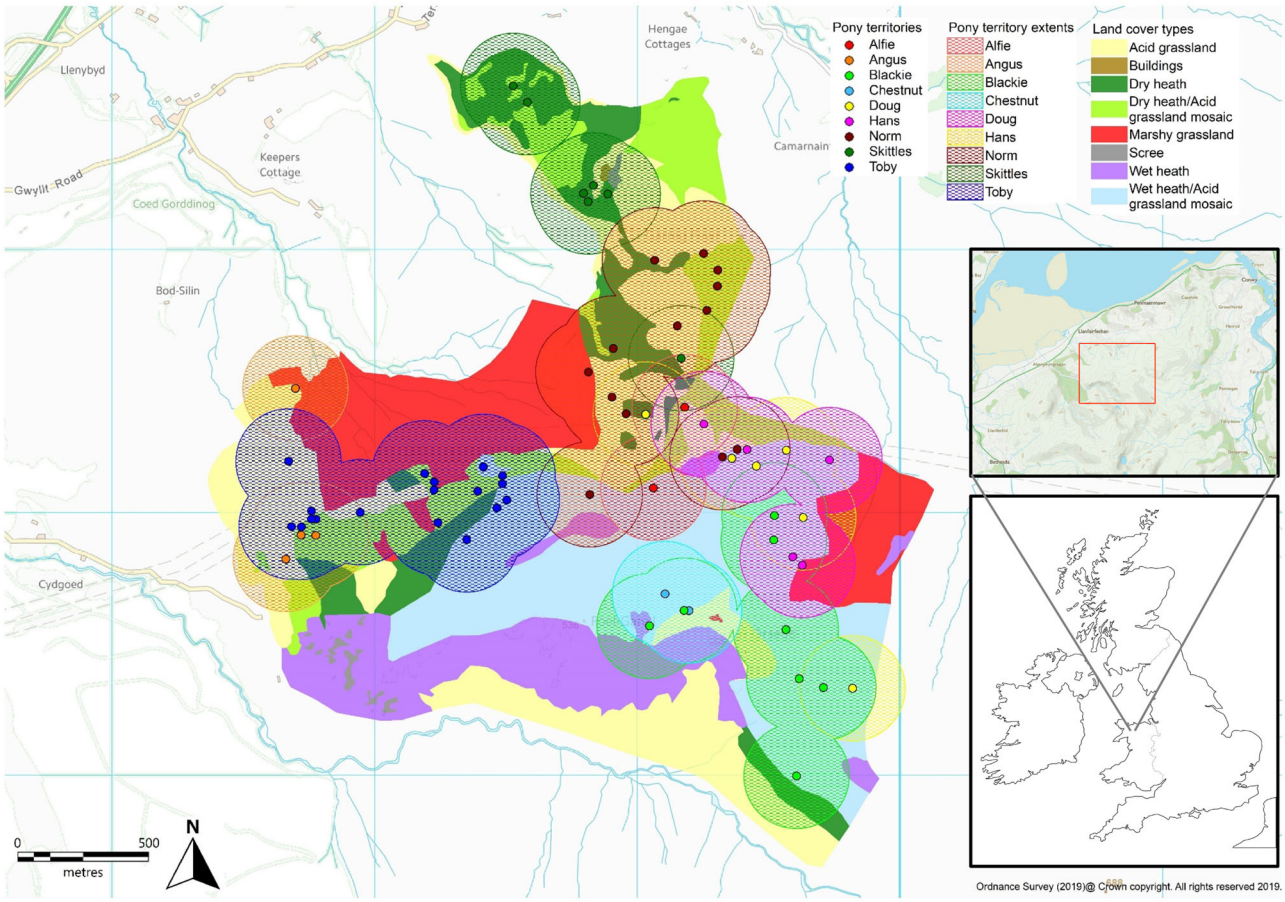

Figure 1. Locations for fecal samples collected during mid-March to May 2018 from the Carneddau pony population in Snowdonia National Park, Wales. There were 10 distinct groups of interest named after the male. We sampled additional individuals from each group, but only males are plotted to simply indicate home range locations.

truthing Google Earth Pro data (Google, Mountain View, CA, USA). We noted all dominant and identifier species (species indicative of a particular land cover type) that could be related to resource availability and overall habitat quality. For example, sheep's fescue (*Festuca ovina*) and mat grass (*Nardus stricta*) are key species for acid grassland. Sheep's fescue is very tolerant to intense grazing and trampling, and commonly grazed by sheep, whereas mat grass is avoided and often grows in marshy areas (Armstrong and Milne 1995). We viewed large areas with binoculars to ensure the same land cover type continued and followed the edge of a predefined printed map.

We then exported and overlaid the home range shape file onto our land cover map in QGIS (QGIS Development Team) and summarized the percent cover of each land cover type in each home range. Finally, we categorized home ranges based on the dominant land cover types (>50% of either dry heath-acid grassland or wet heath).

Annual Roundup Sample Collection

To measure physiological responses during and following an annual roundup event, we collected fecal samples from October to early December 2014. We collected baseline samples, to establish pre-roundup (challenge) metabolite concentrations over 4 weeks before the annual roundup in early November for each of 8 focal individuals (7 females, 1 male). During roundup events, participants on foot, all-terrain vehicles, and motorbikes drive ponies onto farmland and kept them in a fenced paddock overnight. The next day they are put through a livestock race with fixed hurdles and a semi-automatic yoke gate and have their tails trimmed to census which individuals have been rounded up. The following day, the owners moved ponies back onto the commons land of Snowdonia National Park. Therefore, we used this event as a stressor treatment to evaluate how individual ponies responded to an acute stressor. We collected fecal samples daily from 7 adult females and 1 male from 2 focal groups 24 hours after release, which we term day 2 in the analyses. We collected female fecal samples on days 2, 3, 4, 6, 8, 11, 15, and 19; although we did not collect fecal samples from every female on all days, we sampled each female included in the analyses ≥6 times over this period. We collected male samples on days 2, 3, 4, 6, 8, 15, and 19. For all fecal sampling, we observed and identified ponies from 10–20 m distance. We collected fecal samples as soon as they defecated and recorded the date, GPS location, and time of defecation. The maximum elapsed time from defecation to collection, when processing samples from multiple ponies, was <1 hour following defecation. We thoroughly mixed all fecal boluses not touching the ground and collected fecal matter from multiple areas of the mixed dung. The sample was from the upper boluses with minimal exposure to the external environment. We froze all fecal samples at −18°C for 7–10 months, until we were ready to run the assays.

There was 1 day prior to the roundup where the 2 focal bands were close together (inter-band distance <150 m), indicating the possibility of the social interaction leading to a physiological stress response prior to the roundup event. When we did exploratory data analyses to evaluate variation in assays between days, this day was a clear outlier; all sampled individuals had marked elevated hormone concentrations (Fig. S2, available online in Supporting Information). We excluded these samples from the main analysis.

Ecological Sample Collection

We collected fecal samples from February to early May 2018 to capture potential physiological responses to temperature variation, home range location, and social group size. We accessed online climate data for the Carneddau area (53.18°N, 4.00°W; www.meteoblue.com, accessed 14 Jun 2019) to estimate temperature, wind speed, and humidity for each sampling day. This period included the onset of the breeding season (defined as Apr–Jun; Stanley et al. 2018) and the climate changes associated with the transition from winter to spring; the mean minimum and maximum temperatures observed in February were 1°C and 6°C and in May were 7°C and 15°C. The sampling period started with cold weather and a heavy snowfall in late February, a wet and windy March, followed by occasional sunny spells in April and mild weather (~15°C) at the end of that month. We focused on easily recognizable and accessible social groups to collect repeated fecal samples from the same individuals, by walking a same route that covered the homes ranges of 10 focal groups (Fig. 1); on any given day, we collected samples from 1–6 groups. We collected samples from approximately 1000 to 1615. When we encountered a group, we spent approximately 1 hour with the group to collect fecal samples from known individuals. We also collected social data on foraging group size during each sampling period and used the maximum observed group size for each band in our analyses. Most groups consisted of a single adult male, an average of 6 females, and their foals and subadults, although 1 group had 2 males. We recorded weather conditions for the 24 hours prior to sampling collection, to allow for the time lag for gut passage and metabolite excretion. We also took an average of the previous 7 days to evaluate whether there was a signal for a cumulative, or chronic, stress response. The project involved non-invasive sampling and therefore did not require full ethical clearance; related projects have received approval by the University of Manchester Category D panel (non-invasive/non-scheduled procedures).

Steroid Analyses

Corticosterone and testosterone EIAs.—We extracted steroids from fecal samples following thawing and manual homogenization using a shaking method adapted from Walker et al. (2002). We weighed 0.5 g of fecal material from each sample and added 5 ml of 90% methanol. We shook the sample in methanol overnight on an orbital shaker before centrifuging it for 20 minutes at a g force of 598 and then collecting the supernatant. We then evaporated the sample under air and re-suspended it in 1 ml of 100% methanol and stored it at −20°C until analysis.

We analyzed fecal steroid hormone metabolites using a previously described corticosterone and testosterone EIA (Munro and Stabenfeldt 1984, Munro et al. 1991, Young et al. 2004). We obtained antibodies (CJM006 and R156/7, respectively) and horseradish peroxidase conjugated labels from C. J. Munro (University of California, Davis, CA, USA). We purchased standards from Sigma-Aldrich (St Louis, MO, USA). We analytically validated both EIAs in Welsh mountain ponies by measuring corticosterone for pooled fecal extracts through parallelism (corticosterone EIA: $R^2 = 0.93$, $F_{1,7} = 576.40$, $P < 0.001$; testosterone EIA: $R^2 = 0.99$, $F_{1,7} = 617.27$, $P < 0.001$) and matrix interference assessment ($R^2 = 1.00$, $F_{1,7} = 21{,}160.99$, $P < 0.001$ and $R^2 = 0.99$, $F_{1,7} = 810.91$, $P < 0.001$, respectively). To biologically validate the testosterone assay, we compared fecal testosterone metabolites from a castrated bachelor male with 5 intact males and 6 females sampled on the same day. Collectively, there was a significant difference between fecal testosterone metabolite concentrations of adult male and female ponies ($t_{10} = 2.60$, $P < 0.05$). The castrated male had significantly lower measures of fecal testosterone metabolites than the other non-castrated males ($t_4 = 5.29$, $P < 0.01$), but was not significantly different from females ($t_5 = -1.63$, $P = 0.16$; Fig. S3).

11-oxoetiocholanolone EIA.—We extracted steroids from fecal samples following thawing and manual homogenization using a wet-weight shaking extraction (Palme et al. 2013). We combined 0.5 g of fecal material with 80% methanol (5 ml), and vortexed and centrifuged the sample for 15 minutes at a g force of 2,500. We further extracted an aliquot of the supernatant with diethylether and 5% sodium bicarbonate (Merl et al. 2000). We redissolved the dried down ether phase in EIA buffer and stored it at −20°C until analysis. This assay uses an antibody produced against 11-oxoetiocholanolone-3-HS:BSA (1:30000; Ak 7/42/95), 11-oxoetiocholanolone-3-glucosiduronate-DADOO-biotin (1:250000; EL 54) as label and the standard 11-oxoetiocholanolone (5β-androstane-3α–ol-11,17-dione). Details of the EIA including cross-reactions, and its physiological and biological validation for use in horses have been described by Palme and Möstl (1997), Möstl et al. (1999), and Merl et al. (2000). Intra- and inter-assay coefficients of variation of fecal pool samples were <10% and <15%, respectively.

Data Analysis

We conducted all statistical analyses in R (R Core Team 2013). We log10 transformed biomarker concentrations to approximate a normal distribution and tested for collinearity using the corrplot R package (Wei et al. 2017). We used linear mixed effect models to test whether there was variation between metabolite concentrations and environmental factors using the lme4 package (Bates et al. 2012). We initially explored models to determine any suitable interactions. We included individual identification as a random effect to control for repeated sampling effects because we were interested in investigating use of biomarkers at the population level in terms of responses to challenges, rather than variation in individual responses. Because of our relatively small sample size, we used second-order Akaike's Information Criterion (AIC_c) using the MuMIn package (Barton 2009) to compare model fit with minimum daily temperature included as a categorical variable (−5°C to 0°C = freezing, >0°C to 5°C = cold, >5°C to 10°C = mild), in a linear model, and as a quadratic model (Table S1, available online in Supporting Information). Additionally, we also used AIC_c to compare model fit with temperature recorded the previous day, and temperature measured as an average over the previous 7 days. The 2 measures should delineate an acute stress response from a more chronic response, based on length of exposure to the stressor and subsequent physiological response.

RESULTS

We collected 70 female and 10 male fecal samples from 7 females and 1 male over a 1-month period leading up to the roundup and in the 17 days following their release ($\bar{x}$ samples/individual = 8.6). Concentrations of FGMs measured with the 11-oxoetiocholanolone and the corticosterone assay were not correlated ($r = -0.05$, $t = -0.43$, $P = 0.67$). Moreover, fecal 11,17-DOAs increased in the first days following the roundup and started to return to the pre-roundup baseline levels over the next 2 weeks (Table 1; Fig. 2A), whereas FGMs using the corticosterone assay showed no significant response following release relative to the pre-roundup period (Table 1; Fig. 2B). The increase in 11,17-DOAs and the lack of increase in FGMs measured with the corticosterone assay was apparent across individual

Table 1. Post-roundup changes (measured as log ng/g) in Welsh mountain pony female fecal marker concentrations in 2014 relative to the baseline levels for the 11-oxoetiocholanolone (11,17-DOAs) and corticosterone enzyme immunoassay (EIA). Models were linear mixed models with day after release and sex as fixed factor and band (group) and individual identification as random factors.

	11,17-DOAs df = 59				Corticosterone EIA df = 58			
	Estimate	SE	*t*	*P*	Estimate	SE	*t*	*P*
Day 2	0.32	0.06	5.19	<0.001	−0.19	0.11	−1.62	0.11
Day 3	0.25	0.06	4.52	<0.001	−0.07	0.10	−0.69	0.49
Day 4	0.23	0.06	4.31	<0.001	−0.10	0.10	−0.96	0.34
Day 6	0.25	0.06	4.56	<0.001	0.15	0.10	1.49	0.14
Day 8	0.13	0.06	2.26	0.03	0.14	0.10	1.30	0.19
Day 11	0.18	0.06	3.23	0.002	0.14	0.11	1.28	0.21
Day 15	0.14	0.06	2.37	0.02	0.07	0.11	0.63	0.53
Day 19	0.13	0.06	2.22	0.03	0.03	0.11	0.25	0.80
Sex	−0.14	0.04	−3.27	0.02	−0.05	0.09	−0.52	0.63

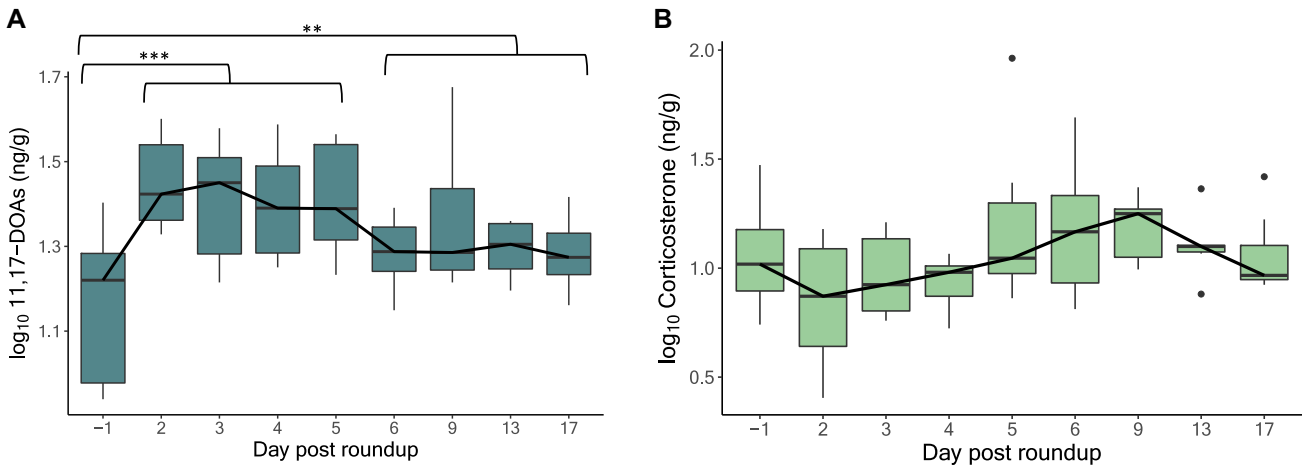

Figure 2. Log concentrations (ng/g) of fecal biomarkers of Carneddau ponies in Wales using A) 11,17-dioxoandrostanes (11,17-DOAs) and B) metabolites measured with the corticosterone enzyme immunoassay (EIA) in the days following the roundup. We averaged samples collected in the month prior to the roundup (Oct 2014) per individual and labeled the average as -1. We held Carneddau ponies on the farms for 24 hours, then released them back onto the mountain. We allowed the ponies 24 hours to return to their home ranges before we resumed sampling opportunistically (day 2 onwards from the roundup) for 7 adult females and 1 male from 2 focal groups. (pairwise comparisons: ** $P < 0.01$, *** $P < 0.001$).

pony profiles (Figs. S1, S2, available online in Supporting Information).

We collected 130 fecal samples from 44 individuals (24 females, 11 males, and 9 juveniles of unknown sex) over 3 months spanning mid-February to mid-May 2018 to evaluate the association between biomarkers and environmental and social conditions from the pre-breeding to the breeding season. Metabolite concentrations measured by the corticosterone and testosterone EIA were very highly correlated across all ponies and in males alone (Fig. 3). The 11,17-DOA and testosterone metabolite concentrations varied across individual ponies, whereas the corticosterone EIA measures did not (11,17-DOAs: $F_{44,\ 77} = 3.44$, $P < 0.001$; testosterone: $F_{42,\ 75} = 3.91$, $P < 0.001$; corticosterone: $F_{42,\ 75} = 1.09$, $P = 0.37$). Our model selection indicated that ambient temperature best predicted fecal metabolites when included as a quadratic term (Table S1). The best fit weather model for the 11,17-DOA biomarker included short-term temperature (i.e., the day

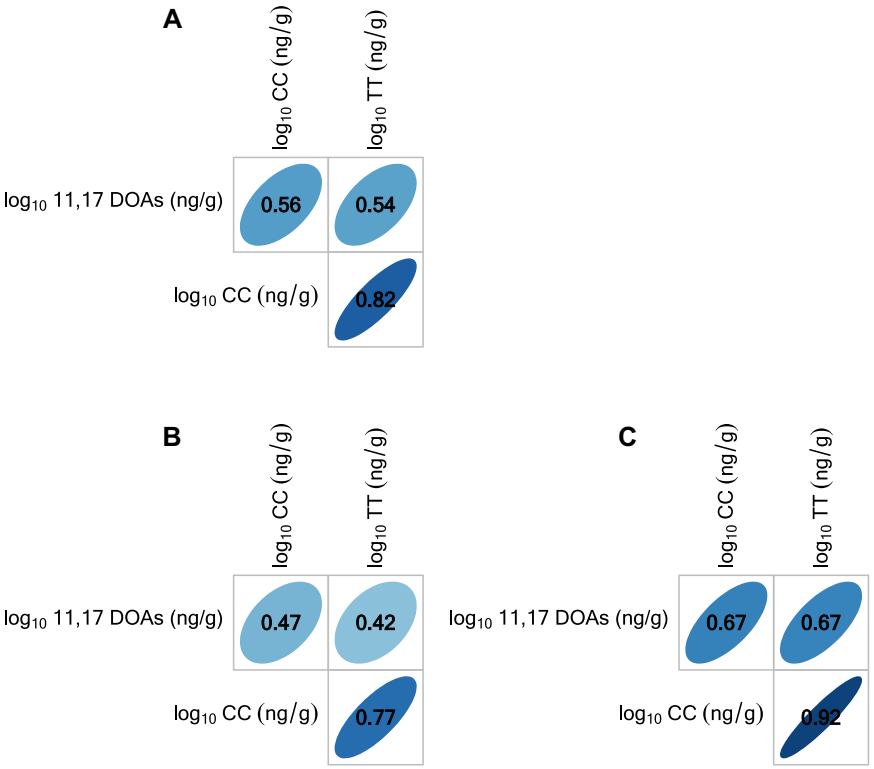

Figure 3. Correlation matrix between biomarkers showing *r* coefficients. We collected all data from the Carneddau pony population in Snowdonia National Park, Wales, mid-March to May 2018. Biomarkers included 11,17-dioxoandrostanes (11,17-DOAs), corticosterone enzyme immunoassays (CC), and testosterone enzyme immunoassays (TT). We present results for all ponies A), males B), and females C). All correlations are significant ($P < 0.001$).

Table 2. Generalized linear mixed effects models to explain fecal metabolite variation in Welsh mountain ponies in 2018 using an 11-oxoetiocholanolone (11,17-DOAs), corticosterone (CC), and testosterone (TT) assay. We measured low temperature the day before feces collection and a mean of daily low temperature for the prior week for CC for all ponies and males. Age classes were defined as adult male (AM), adult female (AF), and young (YU).

		All ponies					Males					Females				
Model	**Factor**	**Estimate**	**SE**	**df**	***F***	***P***	**Estimate**	**SE**	**df**	***F***	***P***	**Estimate**	**SE**	**df**	***F***	***P***
11,17-DOAs	Group size	0.03	0.01	1,80	0.1	0.75	0.03	0.01	1,47	7.4	0.02*	0.0001	0.01	1,22	0.0001	0.99
	Land cover (wet-dry heath)	0.1	0.06	1,41	0	0.85	−0.01	0.09	1,9	0	0.89	−0.04	0.11	1,22	0.11	0.74
	Low temp (linear)	0.02	0.12	2,80	2.6	0.08	0.13	0.1	2,47	3.6	0.04	−0.10	0.14	2,28	1.4	0.26
	(quadratic)	0.23	0.12				0.25	0.1				−0.24	0.01			
	Age-sex (AM-AF)	0.01	0.05	2,41	4.6	0.02*										
	(YU-AF)	0.03	0.06													
	Group size×AM	0.03	0.01	2,80	5	0.01**										
	Group size×YU	−0.01	0.01													
CC	Group size	0.01	0.01	1,80	2.4	0.12	0.02	0.1	146	9.8	0.003**	0.004	0.02	1,22	0.11	0.75
	Land cover (wet-dry heath)	−0.03	0.06	1,39	0.3	0.57	−0.07	0.05	1,8	2.3	0.17	0.05	0.1	1,22	0.2	0.66
	Low temp (linear)	−0.56	0.16	2,80	7.2	<0.001***	−0.49	0.14	2,46	6.1	0.005**	−0.49	0.14	2,28	8.58	0.001***
	(quadratic)	0.23	0.16				−0.04	0.14				0.35	0.15			
	Age-sex (AM-AF)	−0.07	0.04	2,39	1.2	0.32										
	(YU-AF)	−0.001	0.06													
TT	Group size	0.02	0.02	1,38	1.9	0.17	0.02	0.01	1,46	4.5	0.04*	0.002	0.03	1,22	0.001	0.99
	Land cover (wet-dry heath)	−0.13	0.15	1,38	0.5	0.48	−0.08	0.06	1,8	1.6	0.24	−0.22	0.3	1,22	0.54	0.47
	Temp (linear)	−0.73	0.26	2,81	3.2	0.04*	−0.40	0.19	2,46	2.3	0.11	−0.68	0.29	2,28	5.18	0.01**
	(quadratic)	0.06	0.24				0.04	0.19				0.72	0.31			
	Age-sex (AM-AF)	−0.19	0.12	2,38	1.3	0.29										
	(YU-AF)	−0.16	0.15													

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

before; Table S1), whereas the best-fit corticosterone and testosterone models included average temperature of the previous week.

Our final model demonstrated that males in larger groups had significantly higher 11,17-DOA concentrations than those in smaller groups, whereas the relationship was reversed in young ponies (Table 2; Fig. 4). Temperature weakly predicted 11,17-DOA concentrations in males but not across all ponies or in females. Metabolite concentrations measured by the corticosterone and testosterone assays were significantly associated with temperature (Table 2) and with social group size in males. Home range land cover type was not associated with concentrations measured by any of the 3 assays.

We also evaluated whether metabolites co-vary within social groups and whether there were clear seasonal trends over the study days. Fecal metabolites measured by all 3 assays covaried within social groups; there was an interaction between band (group) and date (Table 3). Corticosterone and androgen metabolite concentrations in females and males increased in the pre-breeding season and then declined (Fig. S4).

Table 3. Variation in hormone metabolites of Welsh mountain ponies in 2018 measured by 11-oxoetiocholanolone enzyme immunoassays (11,17-DOAs), corticosterone (CC), and testosterone (TT) assays within social groups. Metabolite concentrations varied between different groups ($^{*}P < 0.05$, $^{**}P < 0.01$) for the 11-oxoaetiocholonolone and corticosterone assay. The group by sampling day interaction suggested that metabolite concentrations across individuals within groups covaried.

Model	Factor	df	*F*	*P*
11,17-DOAs	Group	8,36	2.61	0.02*
	Day	1,75	0.25	0.62
	Temperature	2,75	0.11	0.74
	Group × day	8,75	2.57	0.02*
CC	Group	8,34	2.43	0.03*
	Day	1,73	2.58	0.11
	Temperature	2,73	3.44	0.08
	Group × day	8,73	2.57	0.02*
TT	Group	8,34	1.92	0.09
	Day	1,73	7.67	0.007**
	Temperature	2,73	0.06	0.81
	Group × day	8,73	2.07	0.04*

DISCUSSION

Concentrations of fecal 11,17-DOAs (metabolites measured by the 11-oxoetiocholanolone EIA) were elevated in Carneddau mountain ponies following a roundup before returning to baseline levels; however, metabolites measured with the corticosterone EIA showed no significant change. The ponies showed visible behavioral signs of stress (i.e., avoidance behaviors, body tension, raised head, alert postures, visible sclera) during roundups. Therefore, we considered the event to be an acute stressor, which should be associated with the activation of the HPA axis and release of glucocorticoids. As expected, the concentration of FGMs measured with the 11-oxoetiocholanolone assay increased in the critical window. The lack of increase measured by the corticosterone EIA coupled with the lack of correlation between the results of 2 assays strongly suggests that corticosterone EIA is not appropriate (or sensitive enough) for evaluating ecologically relevant HPA activity in this species.

During the roundup there were several adult males removed from the population, some of which held groups of breeding females and had established breeding home ranges. This resulted in destabilized social groups (Lea 2017), which may explain why cortisol metabolites (11,17-DOAs) remained elevated for 2 weeks after the roundup. This indicates that FGMs could be useful for monitoring the effect of management interventions such as removals,

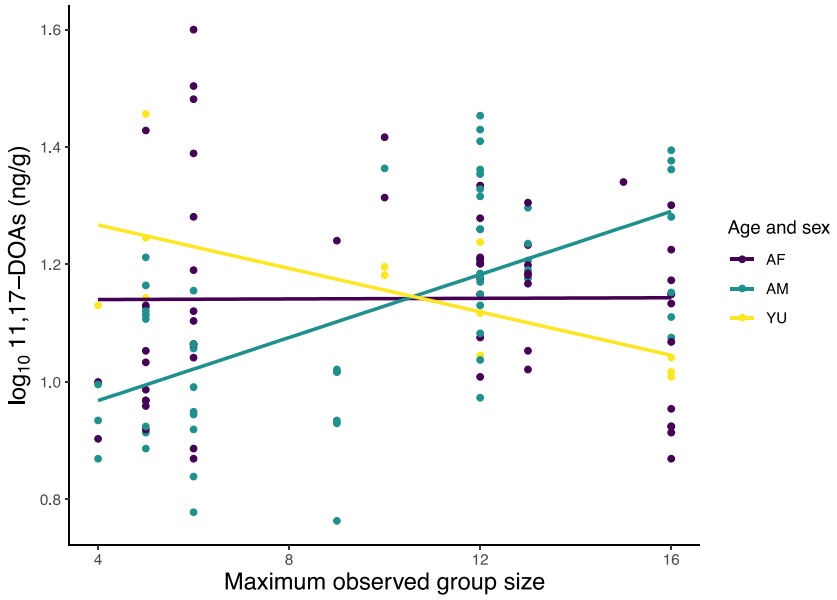

Figure 4. Fecal 11,17-dioxoandrostane (11,17-DOA) concentrations in Carneddau ponies in Wales belonging to foraging groups of different sizes. Ponies are separated by age and sex: adult females (AF) adult males (AM), and yearlings of undetermined sex (YU). We obtained average concentrations for each treatment across the sampling period of February to May 2018.

introductions, and translocations that may have widespread effects on health and physiology.

Home range land cover type was not associated with elevated glucocorticoid metabolites using either assay, suggesting that either this classification did not capture home range quality or that physiological responses to habitat variation did not involve the release of glucocorticoids. Variation in group size (a chronic biotic stressor) did result in changes in concentrations of fecal metabolites. Specifically, metabolites measured by the 11-oxoetiocholanolone, corticosterone, and testosterone EIAs were all elevated in males living in larger groups. The HPA response suggested by the 11,17-DOAs levels may be linked to males having to monitor a greater number of individuals within the group and also experiencing more pressure and incursions by extra-group males. In this population, the breeding groups form dispersed subgroups outside the breeding season, which can help mitigate the costs of competition (Stanley et al. 2018). As groups become larger, males expend more energy to defend and prevent harassment of females by neighboring males (D'Souza-Anjo et al. 2017). Females are less directly affected by group density, the number of sub-groups, and range size (Linklater et al. 1999); however, they have elevated glucocorticoids in response to social disruption, instability, and group turnover (Nuñez et al 2014). Although previous researchers reported associations between individual glucocorticoid levels and social status (Creel et al. 2013, Edwards et al. 2013), few have demonstrated physiological responses at the group level. We showed that metabolites co-varied within social groups, which supports the potential use of multi-level sampling to understand the effect of demography, social organization, and perturbations on individuals, groups, and populations.

The evidence from the roundup strongly indicates that corticosterone EIA is not sensitively measuring HPA activity. The ecological sampling may shed some light on what the assay is detecting. Metabolite concentrations measured with the testosterone and corticosterone assays were very highly correlated and linked to the onset of the breeding season. In addition to both assays being associated with group size in males, they were also associated with ambient temperature. Across ponies, the metabolites measured by both the corticosterone and testosterone EIAs increased prior to the breeding season, and then declined independent of temperature. Running progesterone and estrogen metabolite assays to determine changes as females approach parturition (Graham et al. 2001) would reveal whether there was a comparable correlation between female reproductive hormones and corticosterone, but we did not do this. The concentrations measured by the testosterone EIA were highly correlated with those of the corticosterone EIA, particularly for females, but were not highly correlated with the 11,17-DOAs. This suggests that the corticosterone assay, and potentially also the testosterone assay, is non-specific with cross-reactivity issues with a range of steroid metabolites (Möstl et al. 2005, Ganswindt et al. 2003).

The lack of specificity and lack of sensitivity to a clear biological response following the roundup preclude the corticosterone EIA as appropriate for evaluating adrenocortical activity in this species. We are not the first to document high correlations between fecal androgen and glucocorticoid metabolites using testosterone and corticosterone EIAs (Pribbenow et al. 2017, Lea et al. 2018). Moreover, metabolite concentrations measured with testosterone assays have been shown to increase in response to adrenocorticotropic hormone (ACTH) challenges (Pribbenow et al. 2017), which also suggests non-specificity of parent hormone assays when used on a fecal matrix. Identifying the compounds binding to each of the antibodies is challenging and requires more sophisticated techniques such liquid chromatography coupled with mass-spectrometry (Murtagh et al. 2013). Corticosterone assays are unsuitable for measuring glucocorticoid metabolites in a number of species including giraffe (*Giraffa camelopardalis*; Bashaw et al. 2016), some marsupials (Fanson et al. 2017), and some primates (Heistermann et al. 2006) but are suitable in other species (Edwards et al. 2013, Eleftheriou et al. 2020). A recent review emphasizes the need to thoroughly validate biomarker assays specifically for the species being studied to avoid incorrect inferences about biological responses (Palme 2019), especially when the environmental stressors are complex and interactive. These results reiterate this issue and we strongly recommend ensuring that glucocorticoid assays have been physiologically and biologically validated, ideally by comparing candidate assays across multiple individuals, before applying them to ask biological or ecological questions. This is key because assays that detect a mild to moderate elevation in response to an acute physiological challenge may not be sensitive enough to detect an ecologically relevant response. We suggest that apparent glucocorticoid responses measured with a corticosterone EIA in equids reported by other studies (Yarnell et al. 2016, Lea 2017, Yarnell and Walker 2017) should be revisited and replicated using well-validated assays to confirm findings. A critical message about poor marker selection is that it can lead to inaccurate, or even potentially detrimental, conclusions about the effects of management or the physiological state of a population. In the worst-case scenario, managers may inadvertently miss indicators of stress or challenges facing a population, which in the case of endangered wildlife populations could be catastrophic.

It is difficult to disentangle different factors affecting physiology, but it can be alleviated by using a suite of markers alongside collection of demographic, behavioral, and ecological data (Busch and Hayward 2009). Endocrine biomarkers are particularly useful for establishing the causes and effects of stressors on the physiology of species of conservation interest (Kersey and Dehnhard 2014). Our results suggest that both demographic and ecological factors exert acute and chronic stress for Carneddau mountain ponies. What effect this may have on their fitness is not yet understood. There have been calls for environmental physiologists and physiological ecologists to develop a set of integrated

biomarkers that can be used to address questions about population trends at large spatial and temporal scales. It has only recently been considered possible to carry out large-scale
macrophysiological and macroecological studies for multiple species (Real et al. 2016). By using a toolkit of different biomarkers, it could be possible to identify how multiple shifts in environmental variables have cascading effects on physiology and reproduction that potentially lead to population decline (Todgham and Stillman 2013).

MANAGEMENT IMPLICATIONS

Biomarkers can monitor how populations and individuals respond to both acute and chronic challenges imposed by management interventions or environmental change. Our study provides additional support the 11-oxoetiocholanolone EIA as a valid and sensitive measure of HPA activity in horse fecal samples. In contrast, our results suggest that the corticosterone EIA is not appropriate for measuring fecal glucocorticoid metabolites in equids. Roundups, culling, captures, and translocations are interventions commonly used to manage horse populations, especially where the population size is controlled by removals. The Carneddau pony population faces management issues with rapid population growth and subsequent over-population. This necessitates an active intervention approach to manage numbers. The annual roundup event is also a necessary management tool for the purpose of monitoring the otherwise undisturbed population. This study suggests that there may be a significant physiological effects of these interventions that extends past the event itself via social group distribution. We recommend using biomarkers to identify roundup approaches that are the least disruptive. For example, farmers can minimize time ponies are held in paddocks, avoid separating social groups, and discontinue removing breeding males. The effect of these changes in management could be compared to our results to identify what interventions are perceived as the most stressful and also how to minimize the effect of the interventions. We also advocate the use of contraception to control pony population growth rates to reduce the need to remove and re-home ponies.

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