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The paradox of spring: Thyroid and glucocorticoid responses to cold temperatures and food availability in free living Carneddau ponies

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ABSTRACT

In seasonal environments, maintaining a constant body temperature poses challenges for endotherms. Cold winters at high latitudes, with limited food availability, create opposing demands on metabolism: upregulation preserves body temperature but depletes energy reserves. Examining endocrine profiles, such as thyroid hormone triiodothyronine (T3) and glucocorticoids (GCs), proxies for changes in metabolic rate and acute stressors, offer insights into physiological trade-offs. We evaluated how environmental conditions and gestation impact on faecal hormone metabolites (fT3Ms and fGCMs) from late winter to spring in a free-living population of Carneddau ponies. Faecal T3Ms were highest in late February and March, when temperatures were lowest. Then, fT3Ms concentrations decreased throughout April and were at the lowest in May before increasing towards the end of the study. The decline in fT3M levels in April and May was associated with warmer weather but poor food availability, diet diversity and diet composition. On the other hand, fGCM levels did not display a clear temporal pattern but were associated with reproductive status, where pregnant and lactating females had higher fGCM levels as compared to adult males and non-reproductive females. The temporal profile of fT3Ms levels highlights metabolic trade-offs in a changing environment. In contrast, the ephemeral but synchronous increase in fGCM concentrations across the population suggest a shared experience of acute stressors (i.e., weather, disturbance or social). This multi-biomarker approach can evaluate the role of acute stressors versus energy budgets in the context of interventions, reproduction, seasonality and environmental change, or across multiple scales from individuals to populations.

1. Introduction

Animal populations residing at high latitudes endure extreme seasonality. Cold winters are challenging for endotherms as increasing energy is needed to generate heat for maintaining body temperature at the same time as resource availability declines (Morrison et al., 2008). This leads to an energy conflict between metabolic needs and low food availability and quality, as individuals cannot afford to continuously burn through energy reserves throughout winter without being able to replenish them (Cruz-Neto and Bozinovic, 2004). Endotherms must keep their core body temperature within specific range and can do so within their thermoneutral zone at low energetic costs. When ambient temperatures go below the thermoneutral zone, referred as the lower critical temperature, the body must increase metabolic rate to maintain core

body temperature and allow normal body function (Mejdell et al., 2020; Morrison et al., 2008). For herbivores at high latitudes, the availability and quality of food decreases throughout winter, with early spring (i.e., before vegetation growth begins) being the most challenging period for animals to maintain a positive energy balance (Kuntz et al., 2006; Owen-Smith, 2008). Low food availability stops herbivores from being able to upregulate their metabolic rate, as required for thermoregulation in winter and early spring. A negative energy budget through the winter and into early spring can lead to a decline in fat stores and body condition (Albon et al., 2017).

Endotherms have developed several strategies to cope with the seasonal energy challenge associated with high latitude winters. Some species migrate either to lower elevation or lower latitudes to avoid the harsh conditions (Hsiung et al., 2018). Other species enter a

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hypometabolic state of torpor or hibernation/estivation to lower basal metabolism and reduce energy use (Heldmaier et al., 2004). However, non-migratory, non-hibernating species who cannot escape this energy challenge employ energy conservation strategies. These include reducing expensive activity such as locomotion, lowering heart rate and basal metabolic rate (Arnold, 2020). Changes in activity budget and heart rate have been documented across a range of non-migratory, non-hibernating large herbivores, providing support for hypometabolism as an adaptive metabolic response to winter conditions (Arnold, 2020). These studies include: red deer *Cervus elaphus* (Turbill et al., 2011) Svalbard reindeer *Rangifer tarandus platyrhynchus* (Trondrud et al., 2021) alpine ibex *Capra ibex ibex* (Signer et al., 2011); moose *Alces alces* (Græsli et al., 2020); muskoxen *Ovibos moschatus* (Schmidt et al., 2020) llamas *Lama glama* (Riek et al., 2017) Przewalski horses *Equus ferus przewalskii* and Shetland ponies *Equus ferus caballus* (Brinkmann et al., 2014, 2012). In some of these studies, heart rate, body temperature and activity levels were on average 50 % less in winter than in summer. In addition to heart rate and activity, endocrine profiles can provide more direct measures of seasonal changes in metabolic rate in response to temperature and energy availability.

Two endocrine markers that can evaluate metabolic rate (e.g., nutritional or thermal stress) and environmental stressors (e.g., social stress, predation risk or nutritional stress) are triiodothyronine (T3) and glucocorticoids (GCs), respectively (Shultz et al., 2021; Tarlow and Blumstein, 2007; Wasser et al., 2010). In addition to measuring circulating levels of the active compounds in blood, they can also be measured non-invasively in faeces or urine. Some hormones are primarily excreted in their native form (e.g., T3) and other hormones are heavily metabolised (e.g., steroid hormones including GCs); here we refer collectively to faecally eliminated compounds as faecal T3 metabolites (fT3Ms) and faecal GC metabolites (fGCMs).

Serum GCs and fGCMs have been employed as biomarkers of general physiological 'stressors'. Elevated GC and fGCM concentrations have been linked to increased predation (Boonstra et al., 1998; Clinchy et al., 2004), social instability (Edwards et al., 2013; Nuñez et al., 2014; Sapolsky, 1992; Van Meter et al., 2009), human disturbance (Dantzer et al., 2014) or low food availability (Foerster and Monfort, 2010; Pride, 2005). However, a suggested link between elevated GCs, fGCMs and food limitation is physiologically not straightforward. GCs are involved with the mobilization of energy reserves in three phases: first by stimulating gluconeogenesis and inhibiting glucose uptake by peripheral tissue (for a review see (Kuo et al., 2015)), second by metabolising fatty acids when glucose reserves are depleted, and finally by breaking down proteins as the last resort (Wingfield and Romero, 2015). Elevated GCs increase energy availability and are an adaptive response, which can become maladaptive when energy reserves are already low or when GC leads to the breakdown of proteins (de Bruijn and Romero, 2018). Moreover, acute GC and fGCM signals during a period of food restriction may indicate an acute stressor such as social conflict or predation risk rather than a stress response to caloric restriction, or simply be an adaptive response. Thus, as GCs and fGCMs are associated with acute mobilisation of energy, they have been used as markers for nutritional stress (Foerster and Monfort, 2010; Pride, 2005), despite no clear physiological pathways and no information on what physiological response is used by an individual to cope with nutritional stress (i.e., down or upregulating metabolic rate).

Triiodothyronine (T3) is a more direct biomarker for energy use and oxygen consumption as it regulates metabolic rate (Behringer et al., 2018; Cristóbal-Azkarate et al., 2016; Silva, 2006). Specifically, T3 is a key regulator of obligatory thermogenesis and energy saving strategies, with high levels linked to increasing heat production (MURAMATSU et al., 1986; O'Malley et al., 1984), and low levels associated with low food availability (Bahnak et al., 1981a; Jeanniard du Dot et al., 2009; Bahnak et al., 1981a; Jeanniard du Dot et al., 2009; Bahnak et al., 1981b; Jeanniard du Dot et al., 2009). Therefore, decreases in serum T3 and/or fT3M levels can indicate periods of metabolic or nutritional

stress and offer a mechanistic link between resource availability and physiological state (e.g., hypometabolism). Thyroid hormones and metabolites have been used to identify thermal stress (Chen et al., 2021; Hunnink et al., 2020), nutritional stress (Dias et al., 2017; Jeanniard du Dot et al., 2009) and a combination of the two (Cristóbal-Azkarate et al., 2016). Therefore, fT3Ms may enable us to understand what triggers metabolic responses such as hypometabolism, which may increase survival of non-hibernating species by allowing individuals to conserve energy, but is linked to serious costs such as lower body condition (Arnold et al., 2006) and potential death (Schmidt et al., 2020).

Although hypometabolism may be an adaptive response to reduce energy consumption over winter months, the energetic requirements of pregnant females can provide an opposing pressure during this time. Pregnancy and lactation impose high energetic costs, especially in ungulates which carry large expensive young (Owen-Smith and Ogutu, 2013). In deer and Przewalski's horses, pregnant females graze more often, have elevated metabolic and heart rates (Boyd, 1988; Pekins et al., 1998; Pohlin et al., 2017), and gestating howler monkeys have elevated fT3M concentrations (Dias et al., 2017). These studies suggest that pregnant females have to upregulate their metabolic rate to cope with the high energetic demands of gestation, which conflicts with being in a hypometabolic state to conserve energy reserves throughout winter. For example, in female muskoxen hypometabolism was associated with lower reproductive output and growth, and increased abortion rates (Desforges et al., 2021). This conflict between hypometabolism for winter survival and hypermetabolism imposed by gestation could lead to increased allostatic load in pregnant and lactating females, corresponding with higher GCMs levels seen in many gestating mammal species (Edwards and Boonstra, 2018).

Thus, combining the quantification of GC and T3, or their metabolites (GCMs, T3Ms), can offer an insight into how the body regulates energy consumption and energy availability in response to environmental challenges, and the role of physiological pathways in adaptive response to challenges (Costa-e-Sousa and Hollenberg, 2012; Sapolsky, 2002; Shultz et al., 2021). A few studies support T3 and GC in playing an important role regulating metabolic trade-offs in large ungulate species that undergo hypometabolism in winter (Hoffman and Robinson, 1966) found considerable loss of body weight, accompanied by histological evidence of reduced thyroidal and adrenal activity during late winter in white tailed deer. The authors associated this to reduced fat stores as natural food supplies become less available or are of poorer quality, and energetic requirements for food increase during this period. This pattern in body weight and thyroidal and adrenal activity is reverted in March-April. In line with these results, (Bahnak et al., 1981a; Bahnak et al., 1981a; Bahnak et al., 1981b; Hamr and Bubenik, 1990) found that serum T3 levels decreased in fall, stayed low during winter and increased in spring, and that this response was mediated by nutritional status. Specifically, the artificially feed white deer showed higher T3 values, while extremely low T3 levels were found in malnourished individuals at the end of winter (Bahnak et al., 1981b). Brinkmann et al. (2016) studied Shetland ponies kept in semi-extensive conditions and found that compared with summer, field metabolic rate and heart rate decreased substantially in winter, and that this was associated to a decrease in serum T3 levels. Finally, (Huber et al., 2003) report fGCM levels in red deer to peak during December and January to decrease sharply in February and March and increase again in April. Related to this, the authors found a significant negative relationship between fGCM levels and minimum ambient temperature, the relation being best fitted with a non-linear regression model. Overall, these studies suggest that by reducing metabolic activity via lower levels of thyroid hormones herbivores effectively reduce the need to mobilize energy reserves in winter, which should be reflected in low GC and fGCM levels.

Equids are found at higher latitudes (Cao et al., 2023), and readily adapt to differences in resource availability (Gersick and Rubenstein, 2017), making them an ideal study group to investigate physiological trade-offs in response to low food availability combined to cold climate.

The Carneddau pony is a unique breed of horse (*Equus caballus*) that is found in the Carneddau mountain range in Snowdonia, Wales (Winton et al., 2013). They are free-living, unprovisioned and predator-free population, meaning that the only variability in their environment is natural seasonality in food availability and temperatures. Often confronted to temperatures outside their thermoneutral zones (5–25 °C – (Morgan, 1998)) these ponies exhibit visible signs of winter adaptation, including a thick winter fur coat that is shed at the end of spring, sheltering behaviour (i.e., against a rock or each other), sunbathing, and most births occurring throughout spring. Additionally, the vegetation across Snowdonia National Park is mostly shrubs and grasslands, appearing as a uniform food resources. The next research step is to quantify the physiological adaptations and diet shifts of this pony population to survive the winter.

Here we evaluated changes in fT3Ms and fGCMs levels measured in six groups of free-living Carneddau ponies in the Snowdonia National Park, Wales, to identify their metabolic responses to the dual challenges of cold temperature and resource limitation during late winter and early spring. We analysed how the levels of these hormones change in response to: a) diet composition determined via faecal DNA metabarcoding, b) above ground food availability assessed via NDVI (Normalised Difference Vegetation Index), c) ambient temperature and d) reproductive status (reproductive or non-reproductive active females or stallions). We predicted that the ponies will increase thyroid secretion (increase metabolism) in response to low ambient temperature, allowing the ponies to maintain core body temperature as long as body condition and energy reserves allow. Optimal foraging theory predicts that ponies should expand their diets to include lower quality fallback foods during periods of food scarcity (Schoener, 1971) as has been shown in moose (Jesmer et al., 2020). Thus, at the end of winter and in early spring, when food availability is lowest, we predicted that individuals will diversify their diet with a reduction in the relative abundance of grass consumed. Low food availability and diet diversification should be associated with decreases in fT3M levels as individuals down-regulate metabolic rate (hypometabolism) to minimise energy use. In turn, increased food availability in spring should lead to fT3M levels being positively associated with NDVI as a proxy for vegetation biomass (Borowik et al., 2013). On the other hand, we predicted that fGCM levels will be higher during particularly challenging periods, such as cold spells or during low food availability. Lastly, we also predicted higher metabolic rates and allostatic load, and thus greater fT3M and fGCM concentrations, during late gestation and lactation (Boyd, 1988; Pekins et al., 1998; Pohlin et al., 2017).

2. Methods

2.1. Study population

The free-living Carneddau pony population resides in a 51.8 km² enclosed area in the Carneddau mountains, Snowdonia National Park, Wales. The vegetation in the area is characterised by acid grasslands, heath and bog, including arctic-alpine plant species (Ratcliffe, 1959). The population is free ranging but managed by local farmer communities and are accustomed to the proximity of humans. The management of the ponies does not include food provisioning, medical care or population control (e.g., culling, castration or translocation), but they are rounded up yearly to assess population size and health, with the latest estimate of about 300 individuals (Stanley et al., 2018). Pony groups form social units, generally composed of one dominant stallion and associated mares, subadults and foals (Linklater et al., 1999). The six focal groups varied in group size (9 ± 3.94 s.d.) individuals (min:5-max:18), and home range properties (i.e., elevation, home range size, topography heterogeneity - see supplementary table 1 for information regarding composition and home range characteristics of the study groups).

The reproductive status of female ponies was evaluated based on observation and foaling dates, with females placed into one of three

reproductive categories: pregnant (defined as starting from the day of estimated conception to the day of parturition), lactating, or non-reproductive (neither pregnant nor lactating). Visual inspection of the fT3M and log fGCM concentrations profiles showed little difference between pregnant and lactating females, so we group them into one category: reproductive females.

Data was collected non-invasively from the Carneddau pony population, and observations were conducted by minimising approach and distress to individuals. This study complies the U.K. Animals (Scientific Procedures) Act, 1986 and was ethically approved by the University of Manchester (permit D060).

2.2. Faecal sample collection

Covid travel restrictions limited the sampling period to between 18th of March and 1st of June 2021. All faecal samples were collected during this time period. All groups were followed as randomly as possible to ensure equal and random sampling. Each group was mainly visited weekly or at least once every two weeks, with at least four days in between sampling dates of the same group. This resulted in six visits per group, with exception of one group (Skittles) which was visited for a total of eight visits. Individuals were observed on foot between 10 am and 5 pm, at 5–30 m distance, and faecal samples from adult individuals were collected immediately after defecation to ensure individual identification and avoid deterioration of samples. After collection of each faecal sample, the date, time, group and individual name. To ensure homogenisation of the sample, 4–5 faecal boluses were collected across the faecal piles and then mixed in the collection bag. The sample bag was then stored in a cooler with ice packs for the remaining of the field day and then transferred to a -20 °C freezer until analysis.

2.3. Faecal T3Ms validation and analysis

Before this study, the use of T3 metabolites measured in faeces had not been validated for the assessment of changes in metabolic physiology of horses. Validations are necessary to ensure that the hormone of interest (T3Ms) is reliably measured in faeces (Touma and Palme, 2005; Wasser et al., 2010). We conducted both an analytical and biological validation using faecal samples collected from two leisure horses and one leisure pony that had been placed on a calorie restricted diet to manage weight and pre-laminitis symptoms. A parallelism test was run to analytically validate the protocol, confirm that the antibody in the assay binds effectively to T3Ms in faeces and that there was minimal matrix interferences. A pool sample was created from six Carneddau pony samples (randomly selected across the study period and containing three adults females and three stallions) and then serially diluted from x2 concentration to a 1:16. The slope of the concentration vs logit10 binding of the serially diluted pooled samples was compared to the slope of the standard curve (Gesquiere et al., 2018). Once parallelism of both lines was visually confirmed, the optimal binding concentration (50 % binding is the optimal binding point for assay accuracy (Weiss et al., 2009)) was determined as 2× concentration and selected for all future analyses.

The biological validation was based on the well-established negative effect of dietary restriction on T3 secretion (Keech et al., 2010; Wasser et al., 2010). The two leisure horses were placed on restricted grazing paddocks and the leisure pony was placed on a temporary diet of soaked hay with limited access to grass. The feeding restriction experienced by these two leisure horses and one leisure pony were implemented by their owners, and faecal sampling was performed opportunistically. The samples were collected over a month (May–June 2021), starting at one week previous, to 3 weeks post diet/enclosure change (see supplementary results and Fig. 1). The change between pre/post diet restriction fT3M levels of samples collected from the three leisure animals was analysed using mixed effect model with individual as a random effect. Faecal T3M levels significantly decreased after diet restriction (linear

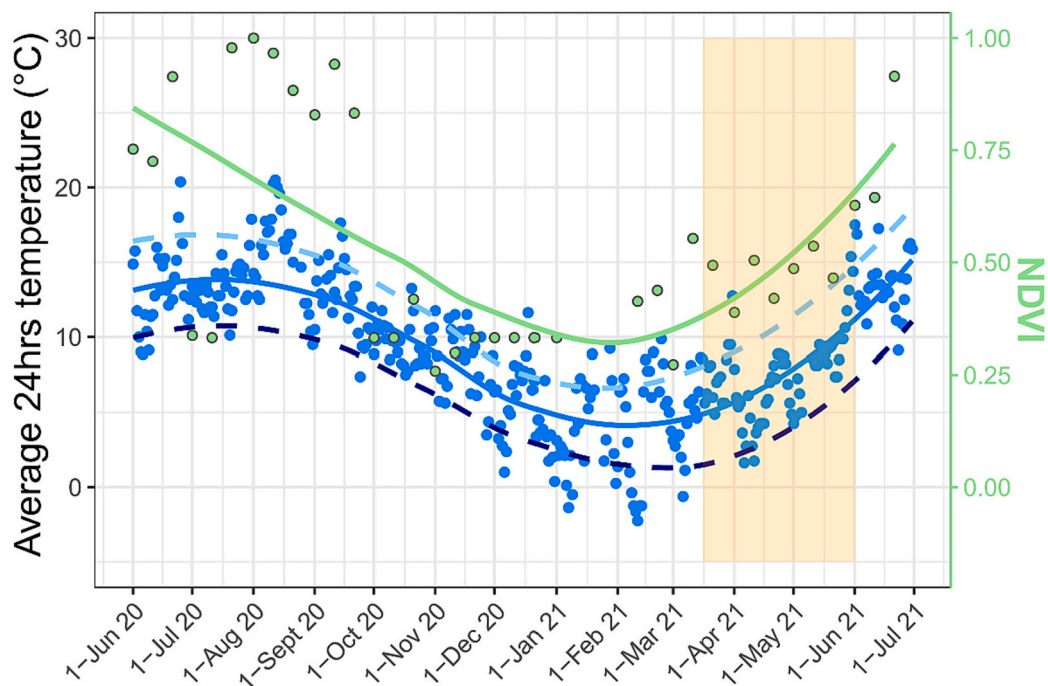


Fig. 1. Scatterplot of NDVI and average, minimum and maximum 24 hrs air temperature (°C) at the study location for June 2020 to July 2021. The blue data points and solid line represent the average 24 hrs temperature (°C), the light and dark blue dotted line are the maximum and minimum (respectively) air temperature (°C) over a 24 hrs period, and the light-green data points and line represent the NDVI values.

model (see supplementary); estimate = -0.017 ± 0.005 , $t_{16,308} = -3.330$, $p = 0.004$) validating that our protocol to quantify fT3Ms can be used to reliably assess thyroidal function in horses.

Faecal samples were processed following a modified method described in (Wasser et al., 2010). Briefly, first 0.5 g faecal sample was mixed with 5 mL of 70 % ethanol, hand-vortexed (2 min) and centrifuged (2500 g for 15 min). Second, 1 mL of the supernatant was taken, dried down in a Genevac™ miVac Centrifugal Concentrator and finally, reconstituted in 500 μ L of assay buffer. fT3M assays were conducted at the University of Manchester's laboratory using a commercial DetectX® Triiodothyronine (T3) Enzyme Immunoassay kit, and procedure was followed according to the manufacturer's instructions. The plate shaken for five seconds prior to reading and absorbance read at 450 nm using a Thermo Scientific™ Multiskan™ FC Microplate Photometer. The optical density measurements were then uploaded to MyAssays (My Assays Ltd., 2022), which produced the standard curve, the samples concentrations, and the coefficient of variability (CV) between duplicate wells.

The intra-assay CV for the fT3M ELISA was 8.4 % and the inter-assay CV was 8.6 % ($n = 2$ control samples).

2.4. Faecal GCMS analysis

To quantify GCM levels in faeces of ponies a previously physiologically and biologically validated method was used (Hinchcliffe et al., 2021; Merl et al., 2000; Möstl et al., 1999; Palme, 2019). The extraction process was based on (Merl et al., 2000)'s study and is resumed in the following steps: first 5 mL of 80 % methanol was added to 0.5 g of faecal matter, hand-vortexed (2 min) and centrifuged (2500g for 15 min); second, 1 mL of the supernatant was transferred and combined with 5 mL of diethylether and 0.25 mL of 5 % NaCO_3 , hand vortexed and centrifuged (15 min); third, the supernatant was dried down in a Genevac™ miVac Centrifugal Concentrator; and finally redissolved in 500 μ L of assay buffer. The second step of the extraction above is necessary due to low concentration of fGCMS being naturally found in equids faecal matter (Merl et al., 2000).

The fGCM analysis was performed with an 11-oxoetiocholanolone

enzyme immunoassay (EIA) previously described in detail (Palme and Mostl, 1997), validated and applied in horses (Hinchcliffe et al., 2021; Möstl et al., 1999).

The intra-assay CV for fGCM EIAs was 2.1 % and the inter-assay CV was 3.2 % ($n = 8$ control samples).

2.5. Diet metabarcoding

Diet composition was assessed from faecal samples through faecal DNA metabarcoding. This technique produces an accurate and detailed snapshot of what the animal recently consumed, and if coupled with thyroid hormone concentrations, can inform on the nutritional quality of certain food types or link sub-optimal foraging with nutritional stress (Jeanniard du Dot et al., 2009; Shultz et al., 2021).

First, plant DNA was extracted from the faecal samples using QIA-GEN QIAamp DNA mini-stool kit (Qiagen, German) - the manufacturer's instructions were followed using 0.2 g of each faecal sample. Secondly, quantification of the amount of DNA extracted per sample was performed using the Invitrogen Qubit kit and Invitrogen™ Qubit™ 4 Fluorometer. Third, plant DNA was amplified for the P6 loop of the trn-L (UAA) chloroplast gene region (Taberlet et al., 2007). Lastly, Sequencing was carried out by Illumina MiSeq. Sequencing used paired-end reads (2x150bp) and a MiSeq V2 Reagent kit. The final library loading concentration was 10pM and a 10 % spike-in of 4pM PhiX control V3 was included. Further details on the cycle times and PCR process are found in the supplementary material.

In order to analyse the DNA readings from the sequencing process, the forward and reverse adapters present in the amplicon sequences were removed using Cutadapt 2.1 pipeline (Martin, 2011). The sequencing generated 3,258,818 raw reads, which were filtered and merge using the DADA2 (v1.18.0) (Callahan et al., 2016) package in R Studio (RStudio, 2020). Any samples of <5000 reads ($n = 10$) were removed (Avramenko et al., 2015). The remaining reads were compared to a sequence reference library, combining libraries from Gill et al. (2019); and Kowalczyk et al. (2019). Taxonomy was assigned to family level for 44 % of amplicon sequence variants' (ASV) and to genus level

for 42 %. A final phylogenetic tree of the reads was generated by the DECIPHER package (Wright, 2016) in R Studio and constructing a maximum likelihood tree in the phangorn (Schliep, 2011) and phytools (Revell, 2012) packages in R Studio.

The Phyloseq package (McMurdie and Holmes, 2013) was used to compute alpha diet diversity and relative read abundance (RRA) of the main vegetation families found in the faecal samples. Alpha diet diversity estimates diet richness using the Shannon-Weaver index. A low dietary alpha diversity means that the faecal sample contain a low number of different vegetation families. Measures of RRA correlate with biomass per vegetation family consumed, and therefore is used as an indicator of proportion of each taxa consumed (Willerslev et al., 2014). Grass being the major food source of equids, only the RRA of Poaceae was considered in the analyses of the best predictors of fT3M and fGCM levels.

2.6. Food availability and climate variables

To assess changes in food availability, NDVI values were extracted from NDVI maps generated by Global Land Service of Copernicus (300 m resolution every 10 days). NDVI is an estimate of vegetation greenness, a proxy for vegetation biomass and food availability for herbivores, where high NDVI values represent 'greener' vegetation (Borowik et al., 2013). For this, the raster calculator tool in QGIS (QGIS Development Team, 2016) was used to subtract values of the red band from the near-infrared band and divided by the sum of the red and near-infrared bands.

To investigate the influence of ambient temperature on fT3M and fGCM concentrations, data for daily minimum, maximum and average temperature was calculated based on eight daily raw temperature measures from the nearby Capel Curig weather station extracted from www.timeanddate.com. Retrospective-integrated ambient temperature data have been shown to correlate better with T3Ms level changes in urine than with the temperature on the day of urine collection (Hassi et al., 2001). To account for this, in addition to daily minimum, maximum and average temperature values on the day of faecal collection, the average minimum, average and maximum temperature across 48 hours and seven days before the day the faecal sample were also calculated. In total six temperature variables were generated: the minimum, maximum and average temperature at 48 hours and 7 day timescale.

Climate variables initially included NDVI, temperature (minimum, average and maximum at a 48 hours and 7 day window) and rainfall (minimum, average and maximum at a 48 hours and 7 day window). After a PCA analysis, only NDVI and temperature variables were kept (further detailed in supplementary material; Supplementary Figs. 2 and 3).

NDVI and average temperature values were calculated for the whole 2021 year (Fig. 1) to put the ambient temperature and food availability values during the study period in a broader context for better interpretation of the results.

2.7. Statistical analyses

We investigated the effect of intrinsic (reproductive state) and extrinsic (ambient temperature, food availability and diet composition) on the faecal T3M concentrations in free-living Carneddau ponies (Table 1). The global models for both fT3M and fGCM concentrations included reproductive state [reproductive females (pregnant or lactating), non-reproductive female and stallion], NDVI (quadratic term), alpha diet diversity, RRA of Poaceae (logged for normality) and a temperature variable (°C) (quadratic term) as fixed effects, and individual ID nested with group as random effect (Table 1). Due to high correlation between the temperature variables [average correlation coefficient $r = 0.638 \pm 0.226$ (r min: 0.146, r max: 0.960)], only one could be kept for further modelling (Dormann et al., 2013). Moreover, PCA analysis indicated that all temperature variables loaded in a similar

Table 1

Variables used in the general linear mixed effect models.

Variable	Description/Levels
<i>Dependent variables</i>	
Levels of fT3Ms* (ng/g)	Continuous Variable (CV) (range = 0.279–1.703; mean \pm SD = 0.941 \pm 0.347)
Levels of fGCMs† (ng/g)	CV (range = 0.950–8.847; mean \pm SD = 3.373 \pm 1.432)
<i>Independent variables (fixed effects)</i>	
Ambient temperature	
7 day average temperature (°C)	Average temperature for 7 days prior to the faecal sample collection. CV (range = 3.321–14.250; mean \pm SD = 7.177 \pm 2.106)
Average 48 hours temperature (°C)	Averaged temperature 48 hours prior to the faecal sample collection. CV (range = 2.688–15.938; mean \pm SD = 7.520 \pm 2.388)
NDVI	Normalised Difference Vegetation Index. CV (range = 0.395–0.631; mean \pm SD = 0.488 \pm 0.074)
Diet	
Alpha diet diversity	Diet richness estimated using Shannon-Weaver index. CV (range = 1.465–3.344; mean \pm SD = 2.730 \pm 0.327)
RRA of Poaceae	Relative Read Abundance (RRA) of the Poaceae (grass) family. CV (range = 0.042–0.855; mean \pm SD = 0.393 \pm 0.274)
Elevation (m a.s.l.‡)	CV (range = 254 - 661; mean \pm SD = 415.700 \pm 108.195)
Sex / Reproductive category	Factor variable: 3 levels (stallions, reproductive females (pregnant or lactating), non-reproductive female)
<i>Independent variables (random effects)</i>	
Individual	Factor variable: 61 levels (61 individuals: 55 adult females and 6 stallions)

* Faecal T3 metabolites.

† Faecal glucocorticoid metabolites.

‡ Meters above sea level.

direction (supplementary Fig. 3), therefore individual models was favoured over inclusion of a PC1 variable to detect differences in time window sensitivity between fT3M and fGCM concentrations. We therefore ran seven global models for fT3M and seven global models for fGCMs, each varying in the temperature variable (null model and six temperature variables). AiCc based model selection was performed to select the best global model out of the seven temperature global models for both the fT3M and fGCM analyses. The average temperature across the past 7 days was selected as the temperature variable best explaining fT3M variation and average temperature 48 hours prior to faecal sample collection better explained the variation in fGCM concentrations. We only included the best global model in our results, the other global models outputs and model selection can be found in the supplementary material (supplementary table 6-33).

To test our predictions mixed-effect linear models were conducted using the lme4 package (Bates et al., 2015). Model selection and averaging was conducted with the dredge function of the package MuMin, which generates all possible model combinations and evaluates model fit based on AICc information criteria (Barton, 2020), using a difference between AICc (Δ AICc) of less than two as a threshold for top model selection and model averaging (Symonds and Moussalli, 2011). Elevation was kept in preliminary models but soon removed due to the lack of patterns in the data (see Supplementary section I and supplementary table 2 for more detail). After inspection of the spread of the raw data, both NDVI and the temperature variables were included as a quadratic term in the following models. Both fT3M and fGCM data was checked for normal distribution, but only fGCM levels were skewed and logged to restore normality. The global model for fT3M concentrations included reproductive state [reproductive females (pregnant or lactating), non-reproductive female and stallion], NDVI (quadratic term), alpha diet diversity, RRA of Poaceae (logged for normality) and 7 day average

temperature (°C) (quadratic term) as fixed effects, and individual ID nested with group as random effect (Table 1). The global model exploring variation in log fGCM concentrations (logged for normality) contained the same variables as the global model for fT3Ms, to the exception that the temperature variable was replaced for average 48 h temperature (°C) (Table 1). Moreover, date of sample collection was not included in the global model because the temporal pattern of sampling was unbalanced across categories and individuals (i.e., some dates did not include samples for all individuals or categories). Instead, a singular model including date was performed to investigate the presence of acute temporal spikes in the fGCM data. In all models, continuous variables were centred around the mean, and scaled residuals inspected for outliers and model fit (Hartig, 2020). Multicollinearity of the variables in the global models was lower than 1.821. Post-hoc pairwise comparisons were conducted with the emmeans package (Lenth et al., 2018). Summary statistics report the mean ± standard error of the mean unless noted. The reported importance (Tables 2 and 3) is the sum of Akaike weights over all models including the explanatory variable. The effect size was calculated by dividing the absolute value of a predictor's coefficient by the standard deviation of the predictor. A Bonferroni correction was applied to the p-value of the temperature variables in our global model, to account for the false positives and amplified p-values from multiple comparison testing (Cabin and Mitchell, 2000).

All statistical analyses were performed on R version 4.1.0 (R Core Team, 2020).

3. Results

3.1. Climate and vegetation patterns in 2021

NDVI and average 24 hours temperature (°C) were calculated for a 12 month period prior to the end of data collection (June 2020-July 2021). Both NDVI and average 24 hours temperatures were highest in summer 2020, decreasing throughout the fall of 2020 to reach the lowest values in winter 2020 (December – March). NDVI and average 24 hours temperature then increased again from March 2021 until summer 2021. Our study period, highlighted below in orange, includes both the end of the low NDVI and average 24 hours temperature and the increase in spring (Fig. 1).

Table 2

Full model-averaged estimates for four environmental variables predicting faecal T3M concentrations (ng/g) in the Carneddau free-living pony population. Alpha diet diversity, relative read abundance (RRA) of Poaceae, 7 day average temperature (°C) and NDVI were centred and standardised individually, prior to computation of the model and model averaging. 7 day average temperature (°C) and NDVI were all included as a polynomial factor (degree 2). RRA of Poaceae in faecal samples was logged for normality. Only the variables kept after model selection ($\Delta AICc < 2$) and averaging were presented in this table. #Post Bonferroni correction the p-value = 0.021.

Parameter	Estimate	Unconditional SE	Confidence interval	Effect size	Z value	p-value	Relative importance
Dependent variable: fT3Ms							
Independent variables: Reproductive state, NDVI ² , 7 day average temperature ² , Dietary alpha diversity and log RRA of Poacea.							
Random effect: Individual ID nested in group name.							
(Intercept)	1.132	0.052	1.029, 1.235		21.560		
Dietary alpha diversity	-0.107	0.028	-0.162, -0.051	0.382	3.779	<0.001	1.00
Log (RRA Poaceae (grass))	0.154	0.036	0.082, 0.226	0.428	4.183	<0.001	1.00
7 day average temperature (°C)							
Linear term	0.234	0.309	-0.381, 0.848	0.076	0.745	0.456	
Quadratic term	-0.848	0.285	-1.413, -0.282	0.298	2.937	0.003#	1.00
NDVI							
Linear term	0.136	0.307	-0.475, 0.746	0.044	0.435	0.663	
Quadratic term	1.395	0.294	0.812, 1.979	0.475	4.688	<0.001	1.00

3.2. Diet composition

The most abundant families consumed by the study pony population were identified as Poaceae (38 %, grasses), Ericaceae (11 %, heather and bilberry), Juncaceae (10 %, rushes), Rosaceae (5 %, e.g., hawthorn, rowan from the rose family) and other families compromised ≤3 % each (Fig. 2). Throughout the study period, the ponies' diet was initially dominated by Poaceae in March to mid-April, to then diversified include other families such as Ericaceae and Juncaceae in mid-April to early May and Rosaceae in mid to late May (Fig. 2).

3.3. Faecal T3M concentrations

Between March to June 2021, a total of 100 faecal samples were collected (Adult females $n = 64$, Stallions $n = 36$) across 61 individuals (Adult females $n = 55$, Stallions $n = 6$) (Supplementary Table 4 for details). Faecal T3M concentrations showed a sharp 45 % decline between March to mid-May (1.450 vs 0.646 ng/g), remained low during most of May and then increased at the end of the study period (Fig. 3). This pattern matched NDVI and average weekly temperatures trends, with a positive quadratic relationship between fT3M levels and NDVI (Table 1, Fig. 3A) and warmer average ambient temperatures (Table 1, Fig. 3B). Moreover, increased fT3M concentrations were significantly linked to a decrease in alpha diet diversity and increase in RRA of Poaceae (Table 1, Fig. 3C and D).

3.4. Faecal GCM concentrations

Faecal concentrations of T3 and GC metabolites were not correlated ($r_{99} = 0.076$, p-value = 0.448, $N = 100$). Log fGCM levels showed a decreasing trend across the study period (Fig. 4A), with a negative quadratic relationship between average temperature over the past 48 hours and log fGCM concentrations (Table 2). Log fGCM levels were higher at both extreme of the previous 48 hours temperature range (Fig. 4B). Additionally, pregnant, or lactating females had higher log fGCM levels than non-pregnant or non-lactating females and stallions (Fig. 5).

Date of sample collection was not included in the global model because the temporal pattern of sampling was unbalanced across categories and individuals (i.e., some dates did not include samples for all individuals or categories). Instead, a singular model including date was performed to investigate the presence of acute temporal spikes in the

Table 3

Full model-averaged estimates for three environmental variables predicting log faecal GCM concentrations (ng/g) in the Carneddau free-living pony population. Average 48 hours temperature and log (RRA Poaceae) were centred and standardised individually. A post-hoc emmeans analysis was run to evaluate the pairwise comparison between reproductive states. *Z-value was reported for the variables with the global model, whereas t-value was reported for the post-hoc test of significance between the levels of reproductive state (Reproductive females = Pregnant/Lactating females; Non-reproductive = Non-pregnant/Non-lactating females; Stallions). Only the variables kept after model selection ($\Delta AICc < 2$) and averaging were presented in this table. #Post Bonferroni correction the p-values are 0.028 and 0.042 respectively.

Parameter	Estimate	Unconditional SE	Confidence interval	Z value*	Effect size	p-value	Importance
Dependent variable: log fGCMs							
Independent variables: Reproductive state, NDVI ² , average 48 hrs temperature ² , Dietary alpha diversity and log RRA of Poacea.							
Random effect: Individual ID nested in group name.							
Intercept	1.362	0.068	1.228, 1.496	19.901			
Log (RRA Poaceae (grass))	0.033	0.045	-0.017, 0.150	0.738	0.073	0.461	0.5
Reproductive State				18.738		<0.001	1.00
Pregnant/lactating ♀ VS Non-reproductive ♀	0.316	0.095	0.085, 0.547	3.329	0.333	0.005	
Pregnant/lactating ♀ VS Stallions	0.286	0.085	0.066, 0.506	3.367	0.336	0.011	
Non-reproductive ♀ VS Stallions	-0.030	0.094	-0.281, 0.221	-0.316	0.032	0.947	
Average 48 hours temperature (°C)							
Linear	-0.964	0.329	-1.617, -0.312	2.898	0.293	0.004#	
Polynomial	0.921	0.330	0.266, 1.576	2.756	0.279	0.006#	1.00

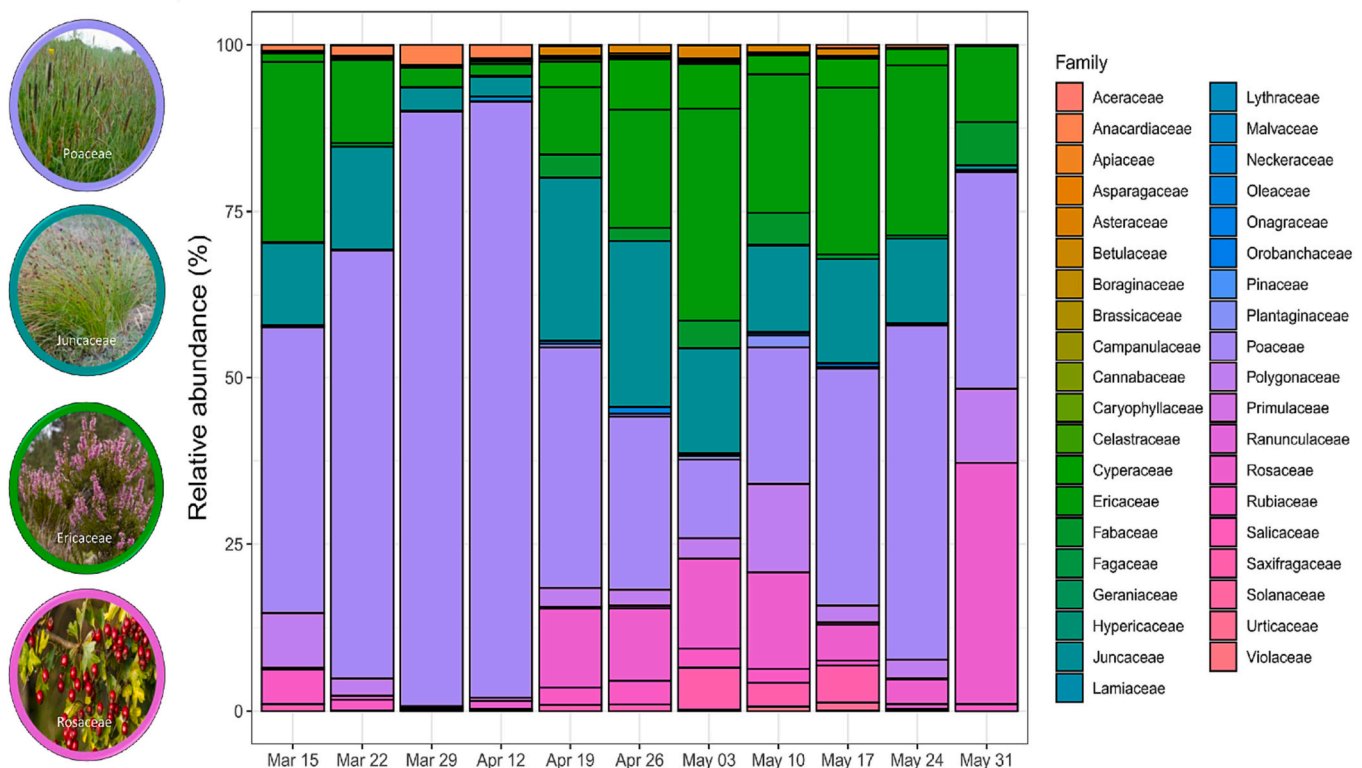


Fig. 2. Descriptive bar-plot of diet composition of the Carneddau ponies across the study period. Diet composition was established from DNA metabarcoding of plant matter found in faecal samples. Week are calendar weeks within a year. RRA is relative read abundancy (RRA) of each plant family – illustrating the proportion of each family with the diet.

fGCM data. The date of sample collection (as a factor) also had a strong effect on log fGCM concentrations ($F_{(1,18)} = 3.408, p\text{-value} < 0.001$), suggesting a strong day to day variation in glucocorticoid levels. Faecal samples collected on the 14th April 2021, in particular, had a higher log fGCM concentrations than the other collection dates (p-value ranged from 0.012 to <0.001; see Supplementary Table 5).

4. Discussion

Carneddau ponies in Wales respond to the dual energetic challenge of low ambient temperature and reduced food availability of winter with ft3M levels decreasing from high concentrations in late winter to low levels in early spring. Both average weekly temperature and food availability (NDVI) decreased throughout the first months of the study year in Snowdonia National Park, and then increased from the second half of April. In line with our prediction, faecal T3Ms tracked NDVI and

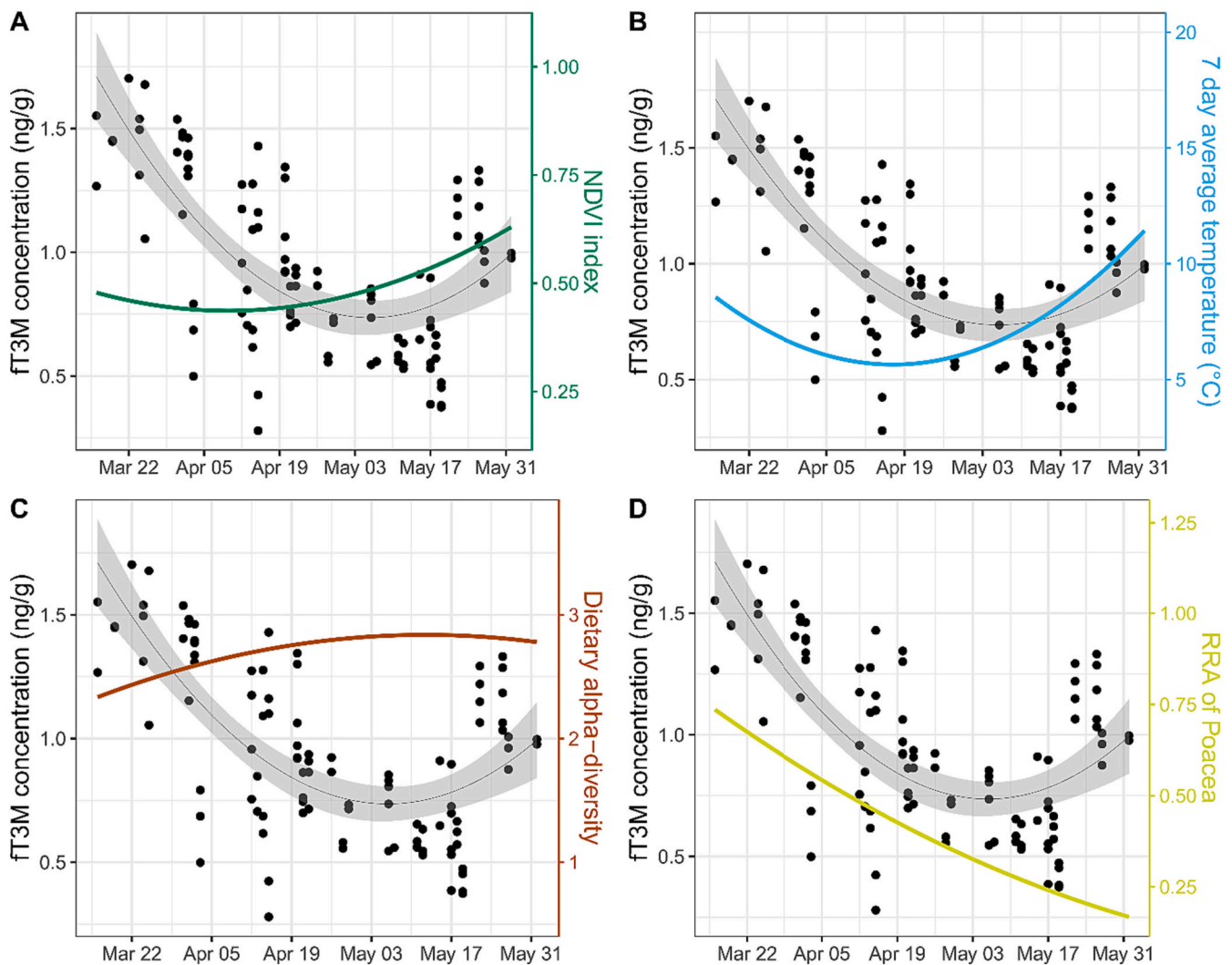


Fig. 3. T3M concentrations (ng/g) measured in faeces of Carneddau free-living ponies and 4 environmental variable: (A) normalised difference vegetation index (NDVI) (green line), (B) 7 day average temperature ($^{\circ}\text{C}$) (blue line), (C) dietary diet diversity (brown line) and (D) relative reads abundance (RRA) of *Poacea* (yellow line), across the study period. Points display raw FT3Ms data with a quadratic line fitted through the raw data (black line). The shaded black area represents the standard error around the quadratic line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

temperature, sharply declining from March until end of April (45 % decline) – suggestive of hypometabolism, and then increasing from mid-May with increasing food availability. This seasonal pattern has also been observed in white-tailed deer, a species that undergoes hypometabolism in winter (Arnold, 2020), with decreased circulating serum T3 levels in free ranging (Hamr and Bubenik, 1990) and penned semi-starved individuals (Bahnak et al., 1981a; Bahnak et al., 1981a; Bahnak et al., 1981b). Similarly, (Brinkmann et al., 2016) found that winter circulating levels of T3 of Shetland ponies in Germany declined 61 % from November to March, this reduction in thyroidal activity being accompanied by a 32 % reduction in field metabolic rate.

Alongside NDVI data, diet DNA metabarcoding enabled us to quantify changes in diet composition which were associated with the decrease in FT3M levels, possible start of hypometabolism, in the Carneddau ponies. Specifically, the ponies ate relatively less grass and their diet had a higher alpha diversity in early spring, suggesting that ponies were switching from a primarily grass-dominated diet to either fallback foods or seasonally available leaf shoots. This same period was associated with lower FT3M levels. Grazing species in other seasonal habitats similarly respond to a reduction in the availability of herbaceous vegetation by shifting to a more diverse non-grass diet (Kartzinel et al., 2015). Here we demonstrate diet shifts associated with reduced thyroid

secretion. Specifically, the early spring decline in FT3M concentrations, suggesting hypometabolism in our study population, was most likely a response to poor nutrient intake and declining energy reserves.

This being said, the spring ‘flush’ associated with increased NDVI was not associated with an immediate switch to higher consumption of *Poacea*, meaning that although food was more plentiful (higher NDVI) the ponies still maintained a diverse diet. One explanation may be that ponies increase consumption of all foods as new vegetation growth increases. As RRA is an index of relative reads it cannot account for changes in total amount consumed. NDVI is a proxy for vegetation biomass as it measure of vegetation greenness (Borowik et al., 2013) and increasing NDVI may be associated with ponies increase consumption of grass and other items. Due to the seasonal flush, grass and other plant families will have nutrient rich new shoots, which the ponies might indiscriminately feed on (Kamstra, 1973). Including a measure of overall biomass ingested could complement diet composition to better indicate changes in caloric intake.

In contrast to FT3Ms, fGCM levels did not vary seasonally and were not related to resource availability or diet composition. Declining resource availability associated with increased GCs in an number of mammalian species (Foerster and Monfort, 2010; Jeanniard du Dot et al., 2009; Pride, 2005) has been interpreted as evidence for

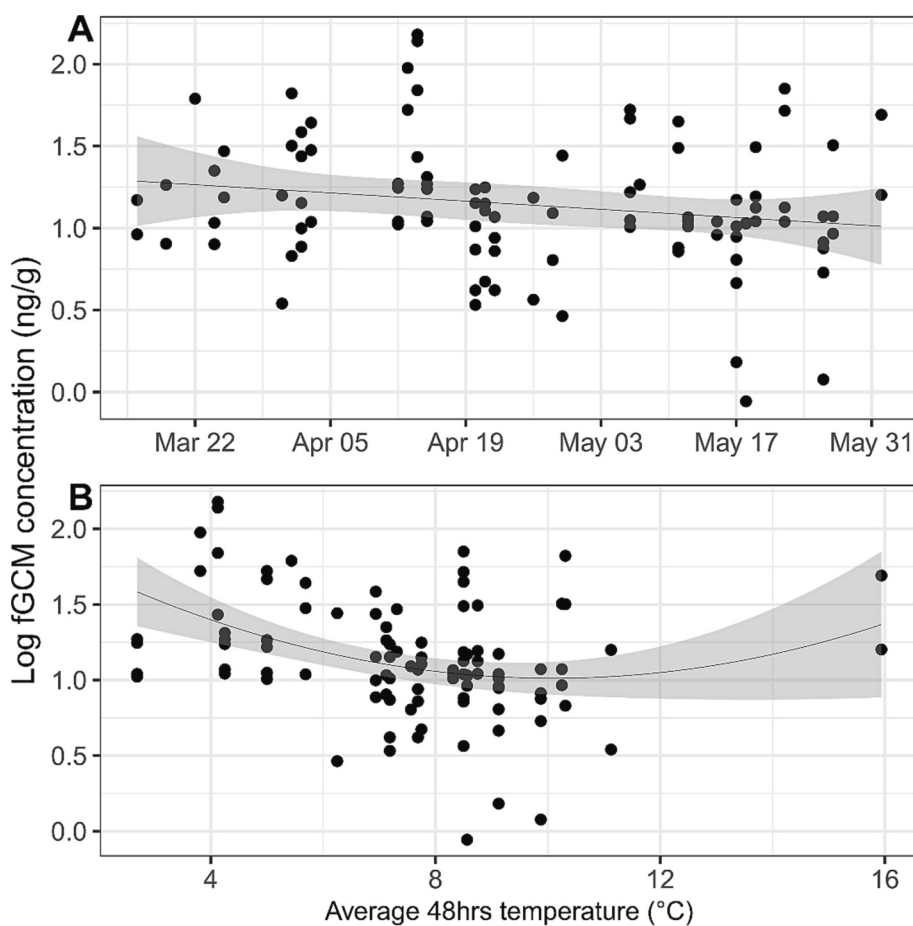


Fig. 4. (A) Log GCM concentrations (ng/g) measured in faeces of Carneddau free-living ponies across the study period, and (B) with average 48 hours temperature. Points display log fGCMs data with a local polynomial regression fit (loess) line fitted through the raw log data (black line). The shaded grey area represents the standard error around the loess line.

individuals mobilizing energy reserves in the absence of adequate food (Kuo et al., 2015). The lack of evidence for such a response in our study population suggests that Carneddau ponies reduce thyroid activity to adaptively manage energy reserves at the end of winter, without requiring the regulatory action of GCs. Faecal GCM concentrations were negatively correlated with average temperature over previous 48 hours, with the highest fGCM concentrations associated with the cold spells. GCs can promote several adaptive thermoregulatory responses to cold temperatures, including vasodilation and heat conduction (de Bruijn and Romero, 2018; Guthrie and Lund, 1998) and so are potentially an adaptive homeostatic response to maintain core body temperature. Elevated faecal GCM concentrations at colder temperatures have also been reported in geladas *Theropithecus gelada* (Beehner and McCann, 2008), red deer (Corlatti et al., 2011; Huber et al., 2003) and arctic ground squirrels *Urocitellus parryi* (Zhang et al., 2020). Huber et al. (2003) also observed this in red deer and noted that the relationship was most pronounced when temperature were $<10^{\circ}\text{C}$. In endotherms, body temperature regulation is least costly within their thermal neutral zone (Bennett, 1988; Morrison et al., 2008), which in domestic horses is between 5°C - 25°C (Morgan, 1998; Snoeks et al., 2015). This is consistent with the relationship we documented, with a strong negative correlation between fGCM concentrations and temperature at low temperatures, but a less clear relationship at higher temperatures.

Elevated fGCM concentrations, but not fT3M concentrations, were found in pregnant and lactating females compared to non-reproductive females and stallions. Desforges et al. (2021) reported that pregnant muskoxen female remained in a hypometabolic state, by maintaining low body temperature, to survive winter conditions. Hypometabolism

experienced by this pony population, including the pregnant and lactating females, may mask any expected fT3Ms signal around parturition and lactation. The observed higher fGCM concentrations in reproductively active females (i.e., pregnant or lactating females) is due to the release of oestrogen and cortisol from the placenta (McLean and Smith, 1999). Higher cortisol levels during late gestation has been reported in mammals (reviewed in (Edwards and Boonstra (2018)). However, there is conflicting evidence in ungulates, with no effect of pregnancy/lactation on cortisol levels in some studies [domestic horses (Aurich et al., 2015; York and Schulte, 2014); sheep (Wintour et al., 1976); cow (Comline et al., 1974); red deer (Huber et al., 2003; Ventrella et al., 2020)], and a positive association between pregnancy and elevated cortisol in others [horses (Nagel et al., 2012); sheep (Brunet and Sebastian, 1991); cow (Braun et al., 2017; Patel et al., 1996); red deer (Pavitt et al., 2016)]. Most of these studies were on domesticated populations, which are cared for to mitigate the extra needs of pregnancy – conditions that are not available to wild populations. In wild ungulates, evidence of elevated GCMs were linked to period of heightened burden during pregnancy, such as the last weeks of pregnancy (Braun et al., 2017; Brunet and Sebastian, 1991; Nagel et al., 2012; Patel et al., 1996), age of the pregnant mother (Pavitt et al., 2016), early lactation (Lang et al., 2012) or sex of the offspring (Pavitt et al., 2016). Here, fGCM levels of reproductively active females were only measured at the end of their pregnancy or the start of lactation, both periods of high metabolic costs. Such conditions may increase allostatic load and trigger a stress response (i.e., GC secretion) allowing these females to temporarily mobilise energy reserves to facilitate the high energy demands of late pregnancy and lactation. This adaptive response could be further

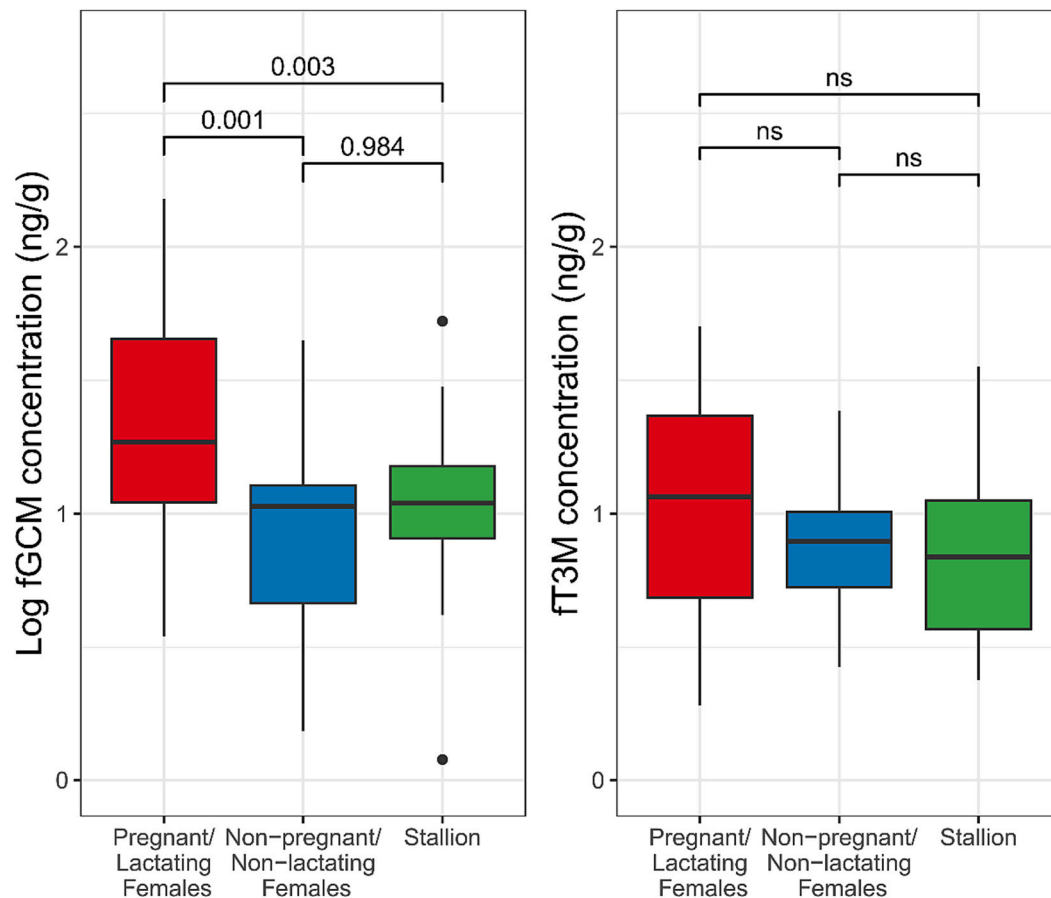


Fig. 5. Boxplot of the log fGCM and FT3M concentrations (ng/g) measured in Carneddau ponies according to reproductive state (Pregnant/Lactating females, non-pregnant/non-lactating females and stallions). P-values from pairwise comparisons are represented. ns = non-significant.

exaggerated by the females having to maintain a hypometabolic state during the high energetic demands of pregnancy.

There is a clear lack of research on endocrine changes during pregnancy in wild ungulates (Pavitt et al., 2016), which cannot be filled by studies on their domestic counterparts (Calisi and Bentley, 2009; Turko et al., 2023). Further research on the reproductive and stress hormones of wild female ungulates around late pregnancy and parturition can highlight trade-offs in relation to intrinsic factors (i.e., of the mother or offspring) and extrinsic factors (i.e., environmental conditions).

5. Summary

This study has investigated the influence of reproductive state, temperature, food availability and diet on the variation of faecal metabolites of two hormones: T3 and cortisol, in Carneddau ponies. Overall, seasonal fluctuation of faecal T3M levels in relation to food availability, diet composition and air temperature in free-living population of Carneddau ponies, coupled with the biological validation of FT3M concentrations in response to dietary restriction in domesticated horses, demonstrates that FT3M is an appropriate biomarker to investigate metabolic rate responses to thermal and nutritional stress in horses, with potential applications to other species (Wasser et al., 2010). On the other hand, fGCMs seem better suited to investigate or highlight adaptive responses to acute challenging periods, with elevated fGCM concentrations during periods of short cold spells and pregnancy/lactation.

Despite the strong potential of biomarkers (i.e., non-invasiveness of sample collection and ease of EIA procedures), caution is still necessary when using hormonal biomarkers, as they do possess some limitations (i.e., stability of metabolites in faeces, cross-species and populations comparisons, assay reactivity) and they require careful interpretation of

their biological and ecological significance (Millsbaugh and Washburn, 2004; Möstl and Palme, 2002; Palme, 2019, 2005; Romero and Beattie, 2022; Sheriff et al., 2011).

Non-invasive biomarkers open an important window for behavioural ecologists to understand the physiological processes involved in responding to environmental pressures and the ability for wild populations to adapt to these pressures. Hypometabolism is commonly reported as an adaptive trait for high latitude ungulates to survive harsh winter conditions (Arnold, 2020). However, low basal metabolic rate, and associated lower body mass, in large animals could be attributed to either the Dehnel effect (i.e., seasonal shrinkage of organs to reduce energy expenditure) or because of nutritional stress (Lovegrove, 2005). The onset of hypometabolism in this pony population coincided with low food availability and poorer diet, supporting Lovegrove (2005)'s second hypothesis for the evolutionary context of hypometabolism.

Glucocorticoids have been used to measure sources of environmental stress in an acute and chronic context (Sheriff et al., 2011), with not always any clear link to a physiological pathway or fitness advantage (Dantzer et al., 2014; Millsbaugh and Washburn, 2004; Romero and Beattie, 2022). Here, elevated fGCMs better represented acute challenges shared across individuals, rather than any long-term survival mechanism. Future studies should focus on exploring if chronic elevated fGCM and serum GC levels are due to repeated acute fGCM or serum GC spikes, or if prolonged GC secretion does confer a survival advantage.

Our study demonstrated the potential of combining behaviour responses, with hormonal biomarkers and detailed diet composition (DNA metabarcoding), to get a fuller picture of how this wild pony population perceive challenges in their environment and what behavioural and physiological mechanisms are put in place to ensure their survival.

CRediT authorship contribution statement

Jessica Granweiler: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Jurgi Cristóbal-Azkarate:** Supervision, Writing – review & editing. **Nathan Morton:** Data curation, Formal analysis, Methodology. **Rupert Palme:** Methodology, Supervision, Writing – review & editing. **Susanne Shultz:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

Data availability

Should the paper be accepted, we will upload the dataset in an online depositary.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yhbeh.2024.105526>.

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