

RESEARCH ARTICLE

Thyroid hormones correlate with field metabolic rate in ponies, *Equus ferus caballus*

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ABSTRACT

During winter, free-living herbivores are often exposed to reduced energy supply at the same time that energy needs for thermoregulation increase. Several wild herbivores as well as robust horse breeds reduce their metabolism during times of low ambient temperature and food shortage. Thyroid hormones (THs) affect metabolic intensity and a positive effect of THs on basal metabolic rate (BMR) has been demonstrated in mammals and birds. As BMR and field metabolic rate (FMR) are often assumed to be intrinsically linked, THs may represent a reliable indicator for FMR. To test this hypothesis, 10 Shetland pony mares were kept under semi-extensive central European conditions. During the winter season, one group was fed 60% and one group 100% of their maintenance energy requirements. We measured FMR, locomotor activity, resting heart rate and TH levels in summer and winter. FMR, locomotor activity, resting heart rate and total T_3 concentrations decreased substantially in winter compared with summer, whereas total T_4 increased. Food restriction led to a reduced FMR and resting heart rate, while THs and locomotor activity were not affected. Across both seasons, FMR, resting heart rate and locomotor activity were positively correlated with total T_3 but negatively and more weakly correlated with total T_4 .

KEY WORDS: Energy expenditure, Food restriction, Resting heart rate, Horses, Locomotor activity, Thyroxine, Triiodothyronine

INTRODUCTION

Animals living in the wild are exposed to changing environmental conditions. When challenged by low ambient temperatures, endothermic animals usually use morphological, physiological, biochemical, metabolic and behavioural adaptations to maintain their body temperature (Schmidt-Nielsen, 1997; Mejdell and Bøe, 2005; Singer, 2007; E. Autio, Loose housing of horses in a cold climate, PhD thesis, Department of Biosciences, University of Kuopio, 2008). Furthermore, by increasing their metabolic rate, heat production is increased (Ekpe and Christopherson, 2000; Young, 1983). In contrast, food restriction and starvation are known to reduce metabolic rate and thus metabolic heat production (Ekpe and Christopherson, 2000; Van Weyenberg et al., 2008). But how do mammals adapt to reduced food availability while being exposed to low temperatures? It is well documented that small mammals often

enter intense forms of hypometabolism, such as hibernation and torpor (Geiser, 1988; Heldmaier et al., 2004) or maintain homeothermy while massively lowering physical activity [e.g. red squirrels (Humphries et al., 2005) and least weasels (Zub et al., 2009)]. Large mammals, with the exception of bears, normally lack the ability to enter torpor (Arnold et al., 2006). However, recent studies on wild Przewalski horses, Alpine ibex, red deer and Shetland ponies all indicate that these species overwinter by employing some form of a hypometabolism (Arnold et al., 2004, 2006; Brinkmann et al., 2014; Signer et al., 2011).

The thyroid hormones (THs) thyroxine (T_4) and triiodothyronine (T_3) are the main hormonal regulators of resting metabolic rate (Hollenberg, 2008; Polat et al., 2014). They increase the rate of metabolism in almost all tissues. Therefore, THs can be used as an indicator of the resting metabolic rate under various climatic and environmental conditions (Fröhli and Blum, 1988; Melesse et al., 2011; Nilssen et al., 1985; Welcker et al., 2013). Cold exposure can lead to an increase of T_3 and T_4 concentrations and metabolic rate (McBride et al., 1985). However, reduced food availability is strongly associated with reduced metabolic rates while the concomitant effects on T_3 and T_4 concentrations are unclear (Beaver et al., 1989; Ekpe and Christopherson, 2000; Fröhli and Blum, 1988; Murphy and Loerch, 1994). There are several studies that have analysed the effect of starvation and restricted feeding on THs in horses (DePew et al., 1994; Sticker et al., 1995a,b). However, these studies mostly examined the effects of short-term food restriction and show inconsistent results regarding T_3 and T_4 concentrations.

The association of T_3 and T_4 with basal metabolic rate (BMR) is well known in homeotherms, while there is a gap of knowledge in the relationship with field metabolic rate (FMR). There are only two studies on birds examining the relationship between THs and FMR (Elliott et al., 2013; Welcker et al., 2013), and they indicate that THs do not correlate well with FMR. These results are contrary to expectations, as there is often a close relationship between BMR and FMR. It is perhaps notable that in the same species in which the absence of a relationship between THs and FMR was reported there was also a disconnection between FMR and BMR. Nie et al. (2015) recently showed that the exceptionally low FMR of the giant panda is correlated with low TH levels, resulting from a panda unique mutation in the dual oxidase 2 (*DUOX2*) gene that is critical for TH synthesis. To our knowledge, however, no studies exploring the link between FMR and THs at the individual level are available for mammals. The aim of the present study was therefore to analyse the effect of changing climatic environmental conditions and prolonged food restriction on metabolic rate and THs in a large mammal, the Shetland pony. Furthermore, we aimed to determine the relationship between THs and FMR. For this purpose, we used previously published (Brinkmann et al., 2014) and new data.

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List of symbols and abbreviations

| | |
|-----------------|------------------------|
| BMR | basal metabolic rate |
| CG | control group |
| FMR | field metabolic rate |
| T ₃ | triiodothyronine |
| T ₄ | thyroxine |
| T _a | ambient temperature |
| TG | treatment group |
| THs | thyroid hormones |
| tT ₃ | total triiodothyronine |
| tT ₄ | total thyroxine |

MATERIALS AND METHODS**Animals, study site and feeding**

Ten non-pregnant Shetland pony mares, *Equus ferus caballus* Linnaeus 1758 (5–13 years), were kept under semi-extensive conditions for 1 year. During the summer period, all ponies were kept on permanent pastures (~2 hectares) partly covered with trees and bushes as natural shelter. In winter, ponies were kept in paddocks at the research stable at the Department of Animal Sciences of the University of Göttingen (Göttingen, Germany) and were allocated to either a treatment group (TG) or a control group (CG) of five animals each. The two groups exhibited a comparable mean body condition score (TG: 4.8±0.4 points, CG: 4.8±0.4 points), body mass (TG: 156±24 kg; CG: 163±33 kg) and age (TG: 9.6±3.1 years; CG: 9.0±2.6 years). Each group was held in a separate rectangular paddock (210 m²) without vegetation. The paddocks each had two adjacent pens measuring 18.9 m² and 9.45 m². The pen floors were covered with a thick layer of wood chips. Additionally, pens were equipped with five feeding stands each (1.35×1.60×0.55 m, height×length×width) to ensure individual feeding of each pony. Pens were unheated to ensure a comparable temperature inside and outside the stable. The light–dark cycle fluctuated according to the natural photoperiod.

In summer on pasture, food consisted of natural vegetation and a mineral supplement provided by a salt lick (Eggersmann Mineral Leckstein, Heinrich Eggersmann GmbH & Co KG, Rinteln, Germany). During paddock housing in winter, only hay and mineral supplements were available to the animals. Water was offered *ad libitum* for all animals at a frost-proof watering place.

During the first 3 weeks of paddock housing in winter, the ponies were allowed to acclimatize to the housing and feeding conditions. The two groups were fed identically with 100% of the recommended energy and protein requirements for Shetland ponies kept outdoors plus 10% energetic demand for slight movement (Kienzle et al., 2010). After the 3 week acclimatization period, the CG animals continued to receive 100% of the recommended energy and protein requirements, while the TG animals were fed a restricted diet to simulate diminishing food availability under natural conditions during winter. The food restriction was intensified stepwise from 90% to 60% of the recommended energy and protein requirements for Shetland ponies. The amount of feed for TG animals was measured to the nearest 0.01 kg and animals were fed individually. Diets were adjusted weekly for each animal according to the animal's body mass.

The ambient temperature (T_a) was recorded every hour throughout the study on the pasture (summer) and paddock (winter) using miniature data loggers (i-Buttons, DS1922L-F5#, resolution: 0.0625°C; Maxim Integrated Products, Sunnyvale, CA, USA).

Procedures performed in our study were in accordance with the German animal ethics regulations and approved by the State Office of Lower Saxony for Consumer Protection and Food Safety (ref. no.: 33.9-42502-04-12/0791).

Resting heart rate, locomotor activity and THs

All analyses were conducted during the first 3 weeks in July (summer) and the last 3 weeks in February (winter). Heart rate (f_H) was recorded for each of the two measurement periods (summer and winter) with a stethoscope. Between 10:00 h and 12:00 h, the heart rate of each pony was determined three times for 60 s and the average was calculated. Before the measurements, ponies were at rest for at least 5 min. The impact of the measuring procedure on the f_H recordings was assumed to be minimal as the ponies were used to being handled.

The locomotor activity for each pony was determined continuously during the FMR measurements using activity pedometers (ALT Pedometer, Engineering Office Holz, Falkenhagen, Germany). Pedometers were tied to the foreleg above the pastern and lined with a silicon pad to avoid pressure marks. The pedometer (125 g mass; 6×5×2 cm, length×width×height) consisted of four acceleration sensors. The locomotor activity was recorded as activity impulses generated by the front leg with a maximum resolution of 2 impulses s⁻¹.

During each measurement period in summer and winter, blood samples (10 ml) for each pony were taken for analyses of TH concentrations. The samples were drawn between 08:00 h and 09:00 h on the sampling day from the vena jugularis and transferred into serum tubes. All samples were centrifuged for 10 min at 3000 rpm and 20°C (centrifugation force: 1620 g). The serum samples were then pipetted into 0.7 ml glass vials and stored at –20°C until analysis. All serum samples were analysed by a commercial laboratory (Laboklin, Bad Kissingen, Germany) that uses appropriate quality-control standards. All samples were run in duplicate. The ADVIA Centaur[®] CP Immunoassay System (Siemens Healthcare, Munich, Germany) was used to analyse total T₃ (tT₃). Total T₄ (tT₄) was determined by an Immulite[®] kit (Siemens Healthcare, Erlangen, Germany).

FMR

FMR was determined for all experimental animals (N=10) for 2 weeks in summer (July) and winter (February) using the doubly labelled water (DLW) method (Lifson and McClintock, 1966; Speakman, 1997). On the first and last day of the FMR measurements, the body mass for each pony was recorded with a mobile scale (Weighing System MP 800, resolution: 0.1 kg; Patura KG, Laudenbach, Germany). On day 1 of the FMR measurement, a blood sample of 5 ml was drawn from the vena jugularis of every animal to estimate the background isotopic enrichment of ²H and ¹⁸O in the body fluids (method D of Speakman and Racey, 1987). Subsequently, each pony was injected intravenously with 0.16 g DLW kg⁻¹ body mass (65% ¹⁸O and 35% ²H; 99.90% purity). The individual dose for each pony was determined prior to the injection according to its body mass. The actual dose given was gravimetrically measured by weighing the syringe before and after administration to the nearest 0.001 g (Sartorius model CW3P1-150IG-1, Sartorius AG, Göttingen, Germany). All animals were then held in the stable without access to food or water for an 8 h equilibration period, after which a further 5 ml blood sample was taken. Further blood samples were taken 3, 5, 7 and 14 days after dosing to estimate the isotope elimination rates. All blood samples were drawn into blood tubes containing sodium citrate. Whole-

blood samples were pipetted into 0.7 ml glass vials and stored at 5°C until determination of ^{18}O and ^2H enrichment. Blood samples were vacuum distilled (Nagy, 1983), and water from the resulting distillate was used to produce CO_2 and H_2 (see Speakman et al., 1990, for CO_2 method; and Speakman and Król, 2005, for H_2 method). The isotope ratios $^{18}\text{O}:^{16}\text{O}$ and $^2\text{H}:^1\text{H}$ were analysed using gas source isotope-ratio mass spectrometry (Isochrom μG and Isoprime, respectively; Micromass Ltd, Manchester, UK). Samples were run alongside five lab standards for each isotope (calibrated to the IAEA International Standards: SMOW and SLAP) to correct delta values to ppm. Isotope enrichment was converted to values of CO_2 production using a two-pool model as recommended for this size of animal (Speakman, 1993). We assumed a fixed evaporation of 25% of the water flux, as this has been shown to minimize error in a range of applications (Van Trigt et al., 2002; Visser and Schekkerman, 1999). Specifically, carbon dioxide production rate per day in moles was calculated using eqn A6 from Schoeller et al. (1986). Daily energy expenditure (i.e. FMR) was calculated from carbon dioxide production by assuming a respiration quotient of 0.85. Isotope analyses and calculations were made blind to the status of the animals or the TH measurements.

Statistical analyses

All statistical analyses were performed using the software package SAS version 9.2 (2008, SAS Institute Inc., Cary, NC, USA). Differences between treatment groups were tested with a Tukey *post hoc* test within a mixed model (PROC MIXED) with treatment group and season as fixed effects and animal as a random factor to account for repeated measurements. During summer, when all animals were kept under the same conditions, no significant differences could be detected between TG and CG animals in any of the variables under investigation (see Results for details). Therefore, most summer data are presented across both groups. All values were log-transformed and body mass was included as a covariate in the mixed model. Data are expressed as adjusted means \pm s.d. The effect of body mass in the equations for FMR was removed by calculating the residuals of the regression of FMR on body mass. Pearson correlation coefficients were calculated between FMR, TH, resting heart rate and locomotor activity over all seasons.

RESULTS

T_a

The mean daily T_a during the summer and winter measurements was 16.4 ± 3.6 and $0.97\pm 2.2^\circ\text{C}$, respectively. T_a ranged from 10.2 to 26.4°C in summer and from -4.7 to 6.9°C in winter.

FMR and THs

The FMR measurement revealed significant differences in the metabolic activity of the ponies in winter and summer ($P<0.001$, $F_{1,9}=95.4$; Fig. 1). The FMR in summer across all animals (63.4 MJ day^{-1}) was about three times higher than that in winter (19.3 MJ day^{-1} ; Brinkmann et al., 2014). In summer, when both groups were fed *ad libitum*, the FMR did not differ between the groups (TG: $65.7\pm 14.5\text{ MJ day}^{-1}$ versus CG: $61.5\pm 16.3\text{ MJ day}^{-1}$, $P=0.72$, $F_{1,8}=0.14$), whereas in winter the restrictive feeding resulted in a significantly lower FMR in TG ($15.0\pm 1.1\text{ MJ day}^{-1}$) compared with CG animals ($24.6\pm 7.8\text{ MJ day}^{-1}$, $P=0.017$, $F_{1,8}=9.6$; Fig. 1; Brinkmann et al., 2014). Furthermore, FMR and mass-independent FMR were highly correlated with resting f_H ($R^2=0.83$, $F_{1,19}=90.57$, $P<0.001$ and $R^2=0.81$, $F_{1,19}=80.95$, $P<0.001$, respectively; Fig. 2A,B, Table 1).

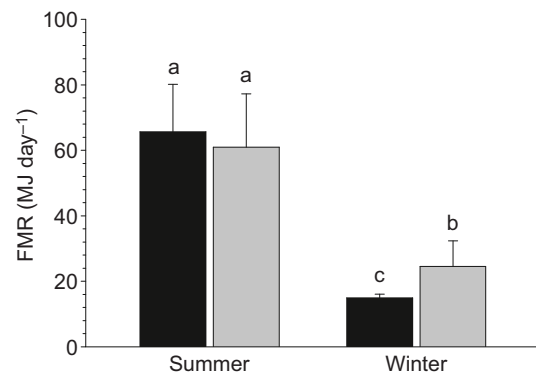


Fig. 1. Daily energy expenditure measured as field metabolic rate (FMR) in Shetland pony mares during the central European summer and winter. Black bars indicate animals fed a restricted diet during winter (treatment group, TG; $N=5$) but not during summer; grey bars indicate control animals (control group, CG; $N=5$; see Materials and methods for details). Values are means \pm s.d. Different letters indicate a significant difference ($P<0.05$).

The tT_3 and tT_4 concentrations also differed between winter and summer measurements (tT_3 : $P<0.001$, $F_{1,9}=135.1$; tT_4 : $P=0.002$, $F_{1,9}=19.4$; Table 2) but not between the two groups (CG versus TG), either in summer (tT_3 : $P=0.24$, $F_{1,8}=1.83$; tT_4 : $P=0.86$, $F_{1,8}=0.03$) or in winter (tT_3 : $P=0.51$, $F_{1,7}=0.49$; tT_4 : $P=0.11$, $F_{1,7}=2.78$). There was a positive relationship between tT_3 and both FMR (Table 1, Fig. 3A) and mass-independent FMR over all measurements (Table 1, Fig. 3C). In contrast, tT_4 concentrations were negatively

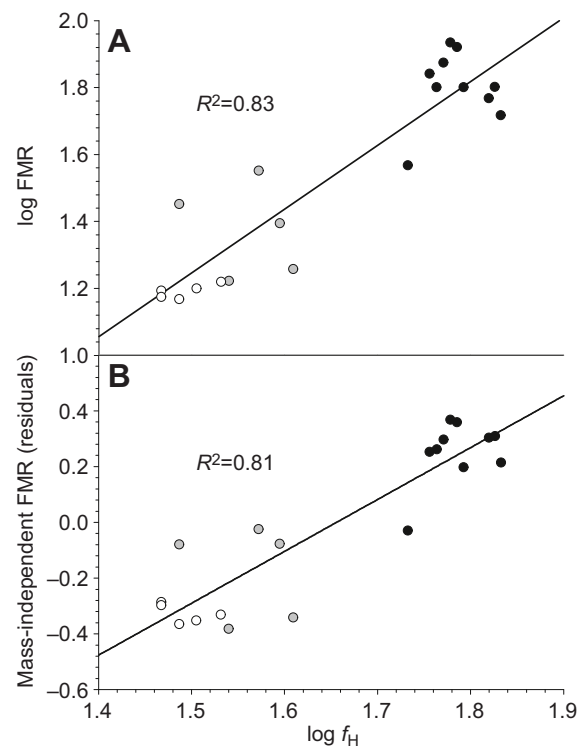


Fig. 2. Relationship between FMR and resting heart rate (f_H) in Shetland pony mares. (A,B) Regression between resting f_H (measured in beats min^{-1}) and FMR (measured in MJ day^{-1} ; A) and mass-independent FMR (B). Black dots denote animals measured during summer fed *ad libitum* on pasture ($N=10$), grey dots denote animals measured during winter fed *ad libitum* ($N=5$) and white dots denote animals measured during winter and fed a restricted diet ($N=5$; see Materials and methods for details).

Table 1. Matrix for Pearson correlation coefficients between energy expenditure, thyroid hormones and physiological parameters in Shetland pony mares

| Variable | FMR (MJ day ⁻¹) | FMR (residuals) | tT ₃ (ng dl ⁻¹) | tT ₄ (µg dl ⁻¹) | f _H (beats min ⁻¹) |
|--|-----------------------------|-----------------|--|--|---|
| FMR (residuals) | 0.94*** | – | – | – | – |
| tT ₃ (ng dl ⁻¹) | 0.83*** | 0.74*** | – | – | – |
| tT ₄ (µg dl ⁻¹) | –0.6*** | –0.63*** | –0.47* | – | – |
| f _H (beats min ⁻¹) | 0.85*** | 0.87*** | 0.79*** | –0.63*** | – |
| Locomotor activity (impulses h ⁻¹) | 0.84*** | 0.86*** | 0.83*** | –0.58*** | 0.93*** |

FMR, field metabolic rate (energy expenditure); tT₃, total triiodothyronine; tT₄, thyroxine; f_H, heart rate.

Data include summer and winter measurements of all animals (N=10).

*P<0.05; ***P<0.001.

related to FMR (Table 1, Fig. 3B) and mass-independent FMR (Table 1, Fig. 3D), respectively. Correlations between TH and FMR were not significant between treatments within seasons.

Resting f_H, locomotor activity and THs

The resting f_H of all animals was higher in summer than in winter (P<0.001, F_{1,9}=196.8; summer: 61.2±15.0 beats min⁻¹, winter: 32.8±4.7 beats min⁻¹; Brinkmann et al., 2014). In summer, the two groups (CG and TG) showed no differences in their resting f_H (P=0.61, F_{1,8}=0.28), whereas in winter, the TG animals had, on average, a lower resting f_H compared with CG animals (P=0.025, F_{1,8}=7.86; Table 2; Brinkmann et al., 2014). Similar to the relationship between FMR and TH over all measurements, resting f_H was positively correlated with tT₃ (Table 1, Fig. 4A) and negatively correlated with tT₄ (Table 1, Fig. 4B).

The average locomotor activity of all study animals was substantially lower in winter compared with summer (P<0.001, F_{1,9}=222.6; winter: 351±102 impulses h⁻¹, summer: 1144±496 impulses h⁻¹; Brinkmann et al., 2014). Locomotor activity did not differ between CG and TG animals either during the summer recording (P=0.12, F_{1,8}=4.28) or during the winter measurement (P=0.45, F_{1,8}=0.63; Table 2; Brinkmann et al., 2014). There was a positive relationship between locomotor activity and tT₃ over all measurements (Table 1, Fig. 4C) and a negative relationship between locomotor activity and tT₄ (Table 1, Fig. 4D).

DISCUSSION

Our study is the first to reveal a correlation between TH and FMR in a semi-free-living ungulate. We demonstrate that Shetland ponies, when kept under semi-natural conditions in winter, reduce their energy expenditure considerably. Additionally, we show that ponies

seem to increase plasma tT₃ and decrease plasma tT₄ levels with increased energy expenditure and f_H. However, while there were some correlations between THs and FMR, neither tT₃ nor tT₄ seems to be a reliable indicator for FMR.

The seasonal FMR variations in our experimental animals demonstrated that ponies kept under semi-natural conditions adapted to harsh winter conditions by an intensive reduction in metabolic activity. Furthermore, ponies receiving less energy during periods of low temperature displayed a hypometabolism (for detailed information, see Brinkmann et al., 2014). Similar reductions of metabolic rate under natural conditions in winter have also been reported for several wild ungulates (red deer, *Cervus elaphus*: Arnold et al., 2004; Przewalski horse, *Equus ferus przewalskii*: Arnold et al., 2006; moose, *Alces alces*: Renecker and Hudson, 1985; Alpine ibex, *Capra ibex*: Signer et al., 2011). The three times higher FMR in summer versus winter may result from increased locomotor activity and altered BMR. Reduced activity levels during low T_a accompanied by a reduction of metabolic rate have also been shown in Alpine ibex (*C. ibex*; Signer et al., 2011), red squirrels (*Tamiasciurus hudsonicus*; Humphries et al., 2005) and least weasels (*Mustela nivalis*; Zub et al., 2009). In parallel to seasonal FMR variations, the resting f_H in our ponies was higher in summer than in winter. Changes in f_H are a response to a change in the body's oxygen demand (Butler et al., 2004) and represent a reliable indicator for changes in metabolic rate (Currie et al., 2014; Hudson and Christopherson, 1985; Woakes et al., 1995). As we measured resting f_H, a parameter that is independent of locomotor activity, the substantially reduced FMR in winter could not only be attributed to reduced activity but probably also to a decrease in BMR.

THs are known to be involved in the regulation of BMR (Duriez et al., 2004; Kim, 2008; Silvestri et al., 2005). In plasma, over 99% of T₃ and T₄ is bound to proteins (Chopra et al., 1975; Evinger and Nelson, 1984; Messer, 1993). According to the free hormone hypothesis, it is the free TH that exerts metabolic effects. Therefore, free T₃ and free T₄ are often claimed to be more relevant and meaningful than tT₃ and tT₄ because the binding proteins albumin and transthyretin are negative acute phase proteins, i.e. their rate of synthesis decreases with adverse conditions such as food restriction (Gruys et al., 2005). However, recent studies showed that free T₃ and tT₃ as well as free T₄ and tT₄ plasma concentrations are highly correlated (Elliott et al., 2013; Welcker et al., 2013). Furthermore, the correlation between tT₃ and BMR was greater than that between free T₃ and BMR, suggesting that metabolic rate is regulated by an elevated deionization rather than by alterations in binding protein levels (Elliott et al., 2013). Free THs in plasma are in equilibration with the protein-bonded proteins in plasma (Barrett et al., 2012). A similar study on horses revealed that the measurement of serum free T₄ does not provide any additional information about thyroid gland

Table 2. Body mass, measured physiological variables and thyroid hormone levels in Shetland pony mares in summer and winter

| Variable | Summer (N=10) | Winter | |
|--|------------------------|------------------------|------------------------|
| | | CG (N=5) | TG (N=5) |
| M _b (kg)* | 160±29 | 165±34 | 140±21 |
| FMR (MJ day ⁻¹)* | 63.4±14.3 ^a | 24.6±7.8 ^b | 15.0±1.1 ^c |
| Locomotor activity (impulses h ⁻¹)* | 1144±496 ^a | 293±88 ^b | 375±151 ^b |
| Resting f _H (beats min ⁻¹)* | 61.2±4.8 ^a | 36.5±4.3 ^b | 31.0±3.2 ^c |
| Total T ₃ (ng dl ⁻¹) | 71.5±15.9 ^a | 43.7±8.2 ^b | 43.0±7.3 ^b |
| Total T ₄ (µg dl ⁻¹) | 1.04±0.63 ^a | 2.52±1.16 ^b | 1.48±0.75 ^b |

M_b, body mass. In winter, animals were allocated to either a control group (CG) or a treatment group (TG) fed a restricted diet; summer data are for all animals combined (see Materials and methods for details).

Data are means±s.d.; different superscript letters indicate a significant difference (P<0.05).

*Data taken from Brinkmann et al. (2014).

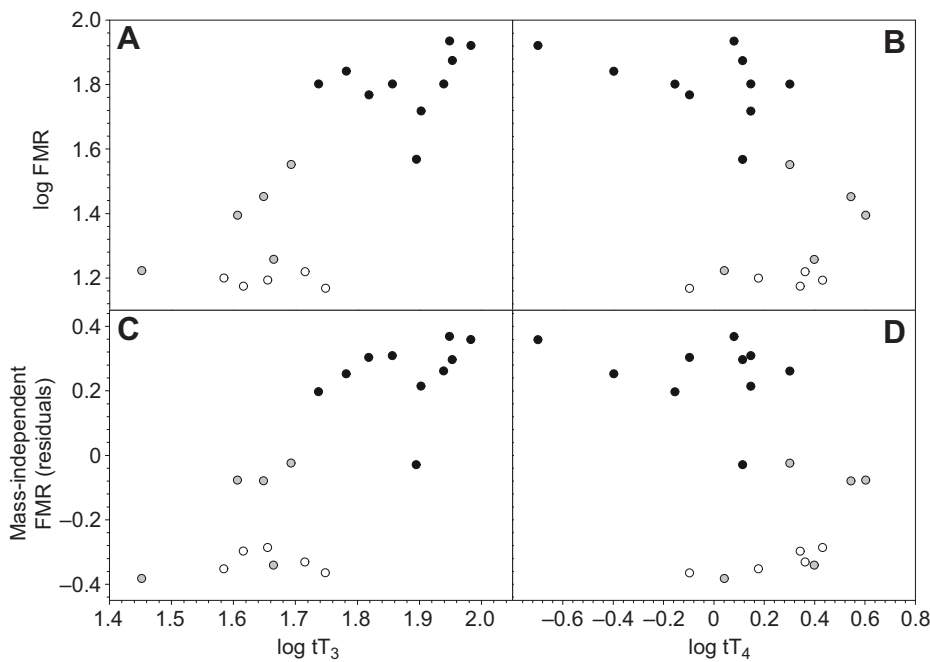


Fig. 3. Relationship between thyroid hormones (THs) and FMR in Shetland pony mares. Regression between TH levels [total thyroxine (ng dl^{-1}), tT_3 : A,C; total triiodothyronine ($\mu\text{g dl}^{-1}$), tT_4 : B,D] and FMR [MJ day^{-1} : A,B; mass-independent FMR, residuals of the regression of FMR on body mass: C,D]. Black dots denote animals measured during summer fed *ad libitum* on pasture ($N=10$), grey dots denote animals measured during winter fed *ad libitum* ($N=5$) and white dots denote animals measured during winter fed a restricted diet ($N=5$; see Materials and methods for details).

function over that gained by measuring tT_4 concentrations (Sojka et al., 1993). Therefore, it is assumed that tT_3 and tT_4 are as relevant and meaningful for analyses of thyroid gland function as free THs.

In order to maintain energy homeostasis, the hypothalamic-pituitary–thyroid axis generally sustains serum TH concentrations, which results in a steady contribution of THs (Andersen et al., 2003). Nevertheless, under natural climatic conditions, animals show a circadian rhythm in thyroid gland activity and TH concentrations are thought to be an important component of cold adaptation in horses (McBride et al., 1985). Generally, cold temperatures stimulate the secretory activity of the thyroid gland and simultaneously increase T_3 and T_4 concentrations (Fregly et al., 1979; Reed et al., 1994), e.g. in sheep (Ekpe and Christopherson,

2000; Kennedy et al., 1977) and horses (McBride et al., 1985), while heat reduces the function of the thyroid gland and reduces TH concentration by up to 41% (Beede and Collier, 1986; Magdub et al., 1982; Melesse et al., 2011). We also found that tT_4 concentrations were lower in summer than in winter. In both experimental groups, summer values were below the reference range of $1.3\text{--}4.1 \mu\text{g dl}^{-1}$, while in winter values were within the range for horses (Laboklin, 2016). This may be attributed to a reduced secretory activity of the thyroid gland to downregulate internal heat production. Other studies on horses found tT_4 values of 0.99 ± 0.43 and $1.76 \mu\text{g dl}^{-1}$ (Breuhaus, 2002; Chen and Riley, 1981). However, tT_3 , the biological active TH in mammals, revealed a contrary trend to that of tT_4 , with higher concentrations in summer

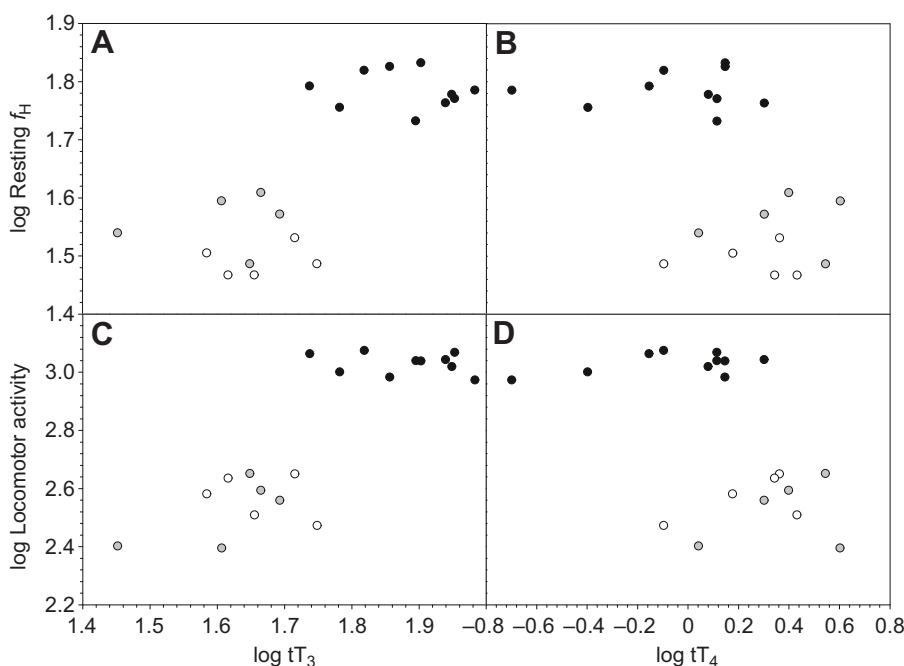


Fig. 4. Relationship between THs and resting f_H and locomotor activity in Shetland pony mares. Regression between TH levels [tT_3 (ng dl^{-1}): A,C; tT_4 ($\mu\text{g dl}^{-1}$): B,D] and resting f_H (beats min^{-1} ; A,B) and locomotor activity (impulses h^{-1} ; C,D). Black dots denote animals measured during summer fed *ad libitum* on pasture ($N=10$), grey dots denote animals measured during winter fed *ad libitum* ($N=5$) and white dots denote animals measured during winter fed a restricted diet ($N=5$; see Materials and methods for details).

than in winter. All measured tT_3 values were within the reference range of 25–180 ng dl⁻¹ for horses (Laboklin, 2016). Similar values (98.7 ng dl⁻¹) for tT_3 were also found by Chen and Riley (1981).

It is well known that the pleiotropic hormones T_4 and T_3 have a stimulating effect on tissue oxygen consumption, thermogenesis and BMR (Kim, 2008; Silvestri et al., 2005). For example, it has been shown in humans that serum tT_3 concentrations increase with an elevated BMR, while no relationship between tT_4 and BMR could be established (Stenlöf et al., 1993). Furthermore, research in birds revealed a positive link between T_3 and BMR (Chastel et al., 2003; Welcker et al., 2013) as well as standard metabolic rate (BMR + thermoregulatory costs; Duriez et al., 2004). When we used resting f_H as a proxy for BMR, our results indicated a relationship between BMR and TH, as seasonal changes in resting f_H were positively correlated with seasonal changes in T_3 and negatively correlated with those of T_4 . However, there is also research indicating that there is no connection between BMR and T_3 . Studies in horses and humans showed no relationship between T_3 and BMR while T_4 concentrations appeared to be negatively correlated with BMR (Johnstone et al., 2005; McBride et al., 1985).

Less is known about the association between TH and FMR, but, as a strong positive relationship between FMR and BMR is often suggested (Daan, 1990; Kim, 2008), FMR should be positively correlated with TH as well. Therefore, THs are often used as an indicator of metabolic activity in animals (Duriez et al., 2004; Pethes et al., 1985; Polat et al., 2014).

A recent study in free-ranging kittiwakes showed that TH concentrations were associated with BMR ($R^2=0.60$) as well as mass-independent BMR ($R^2=0.36$), but not with FMR and mass-independent FMR (Welcker et al., 2013). In another study in murrelets and kittiwakes, a relationship between mass-independent BMR and T_3 has been demonstrated ($R^2=0.48$ and $R^2=0.75$, respectively) but not for FMR (Elliott et al., 2013). However, Nie et al. (2015) recently showed that the exceptionally low FMR of the giant panda was associated with low TH levels. This may indicate that in species where no relationship between TH and FMR could be detected, a disconnect between FMR and BMR exists.

Our study showed that tT_3 concentrations were positively correlated, and tT_4 concentrations negatively correlated, with seasonal changes in FMR, i.e. lower tT_3 concentrations in winter were associated with a decreased metabolic rate, while tT_4 concentrations increased. Furthermore, FMR and resting f_H were highly correlated, indicating a connection between FMR and BMR, which may explain the link between THs and FMR in our study.

In healthy normally fed subjects, 80–90% of T_3 is produced by monodeiodination of about 40% of the circulating T_4 , a reaction catalysed by 5'-monodeiodinases. The remaining 10–20% is directly secreted by the thyroid gland (Economidou et al., 2011). It is well known that the nuclear thyroid receptor in the pituitary gland as well as in the periphery has a much higher affinity for T_3 than for T_4 (Oppenheimer et al., 1987). We could therefore expect a higher correlation between BMR/FMR and T_3 than between BMR/FMR and T_4 . It is therefore suggested that if increased T_3 levels are required for an increase in BMR, T_4 may serve as a precursor for upregulating T_3 concentrations via cellular deiodinases (Elliott et al., 2013). This may explain the contrary course of T_3 and T_4 in our study.

Generally, energy intake below demand is associated with decreased metabolic rates, thyroid activity and serum T_3 concentrations, while T_4 concentrations are not necessarily affected (Blake et al., 1991; Ekpe and Christopherson, 2000; Murphy and Loerch, 1994). Reduced T_3 concentrations were

detected in fasted steers (Fröhli and Blum, 1988; Murphy and Loerch, 1994), sheep (Ekpe and Christopherson, 2000) and horses (Van Weyenberg et al., 2008). Low TH concentrations, as a consequence of scarce feed availability, for example, are known to reduce maintenance energy requirements, possibly enhancing the efficiency of feed conversion (Ekpe and Christopherson, 2000). However, the restrictive feeding of the TG animals in our study did not seem to influence the T_4 and T_3 concentrations, even though the FMR of restrictively fed animals was reduced compared with that of CG animals. Similar results have been found for food-restricted steers (Ellenberger et al., 1989) and horses (Glade et al., 1984; Sticker et al., 1995a,b). Normally, T_4 is converted to T_3 and rT_3 (an inactive metabolite of T_3) in equal parts. During fasting, however, the transformation of T_4 to rT_3 is increased, while the formation of T_3 is decreased because of the inhibition of 5'-deiodase (Hennemann et al., 1986; Peeters et al., 2002). Fasted humans and cattle, for example, had decreased T_3 but increased rT_3 concentrations, while T_4 concentrations remained more or less the same (Danforth et al., 1978; Pethes et al., 1985). Thus, tT_3 concentrations may remain unchanged during fasting while the metabolically active T_3 is reduced. However, as hormone levels are an indicator of the net balance between hormone synthesis and metabolism (McBride et al., 1985), it is also possible that feed restriction in our animals resulted in a change in the TH turnover rate.

In ponies held under natural climatic conditions, changes in ambient temperature and feed quality and quantity resulted in metabolic and TH adaptations. As previously shown for wild horses (Arnold et al., 2006), cold ambient temperatures led to reduced energy expenditure, f_H and activity in our ponies in winter compared with summer. THs, as a proxy for BMR (Elliott et al., 2013; Welcker et al., 2013) and also as an indicator of FMR, were affected by season. In our study, changes in tT_3 concentrations were positively related to changes in FMR, while tT_4 concentrations were negatively related to FMR. However, reduced metabolic activity during fasting was not reflected in changes in THs. Thus, while there were correlations between THs and FMR, neither tT_3 nor tT_4 seems to be a reliable indicator of FMR.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

L.B., M.G. and A.R. contributed to study conception and design; L.B. and A.R. contributed to the execution of the experiments; J.R.S. and C.H. contributed to the field metabolic rate analysis; and all authors contributed to interpretation of the results and drafting of the article.

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Data availability

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