## 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 adaptions 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

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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.

> **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 $T_3$ ($tT_3$). Total $T_4$ ($tT_4$) 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 $^{2}H$ 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 $^{18}O{:}^{16}O$ and $^{2}H{:}^{1}H$ 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±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±2.2°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±14.5 MJ day$^{-1}$ versus CG: 61.5±16.3 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±1.1 MJ day$^{-1}$) compared with CG animals (24.6±7.8 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).
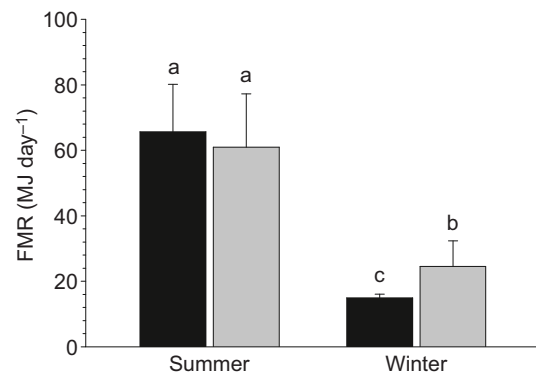


**Fig. 1. Daily energy expenditure measured as field metabolic rate (FMR) in Shetland pony mares during the central European summer and winter.** Black bars indicate animals fed a restricted diet during winter (treatment group, TG; *N*=5) but not during summer; grey bars indicate control animals (control group, CG; *N*=5; see Materials and methods for details). Values are means± 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
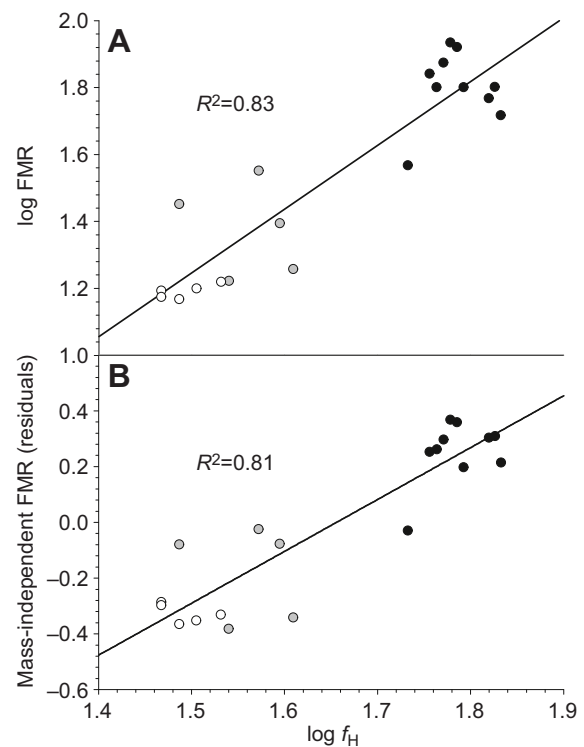


**Fig. 2. Relationship between FMR and resting heart rate ( $f_H$) in Shetland pony mares.** (A,B) Regression between resting $f_H$ (measured in beats min$^{-1}$) and FMR (measured in MJ day$^{-1}$; A) and mass-independent FMR (B). Black dots denote animals measured during summer fed *ad libitum* on 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); tT$_3$, total triiodothyronine; tT$_4$, 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 tT$_3$ (Table 1, Fig. 4A) and negatively correlated with tT$_4$ (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 tT$_3$ over all measurements (Table 1, Fig. 4C) and a negative relationship between locomotor activity and tT$_4$ (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

**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).

seem to increase plasma tT$_3$ and decrease plasma tT$_4$ levels with increased energy expenditure and $f_H$. However, while there were some correlations between THs and FMR, neither tT$_3$ nor tT$_4$ 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 T$_3$ and T$_4$ 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 T$_3$ and free T$_4$ are often claimed to be more relevant and meaningful than tT$_3$ and tT$_4$ 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 T$_3$ and tT$_3$ as well as free T$_4$ and tT$_4$ plasma concentrations are highly correlated (Elliott et al., 2013; Welcker et al., 2013). Furthermore, the correlation between tT$_3$ and BMR was greater than that between free T$_3$ 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 T$_4$ does not provide any additional information about thyroid gland
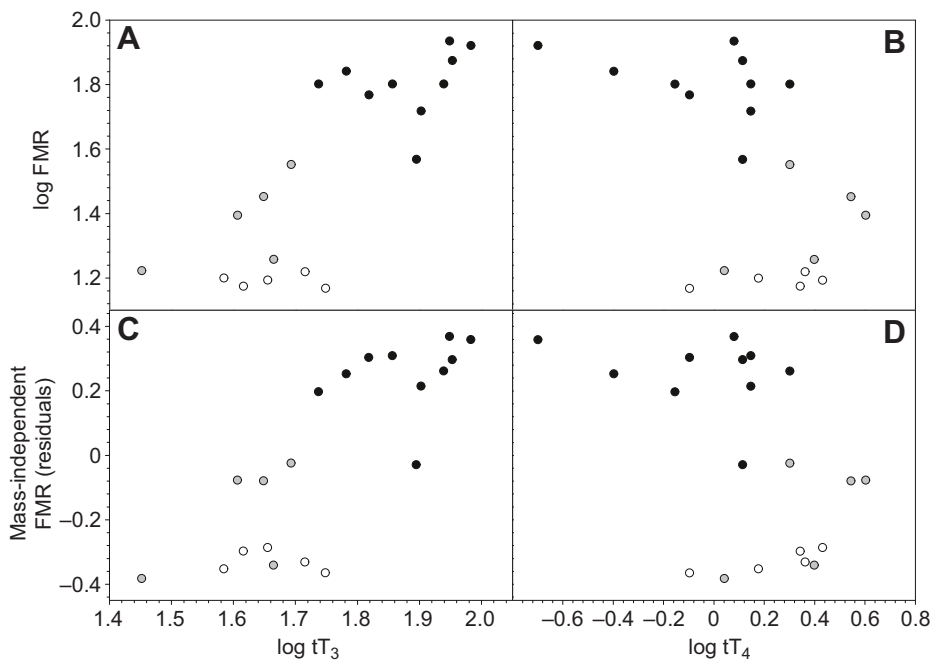
**Fig. 3. Relationship between thyroid hormones (THs) and FMR in Shetland pony mares.** Regression between TH levels [total thyroxine (ng dl$^{-1}$), tT$_3$: A,C; total triiodothyronine (µg dl$^{-1}$), tT$_4$: B,D] and FMR [MJ day$^{-1}$: A,B; mass-independent FMR, residuals of the regression of FMR 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 1.3–4.1 µg 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 µg 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
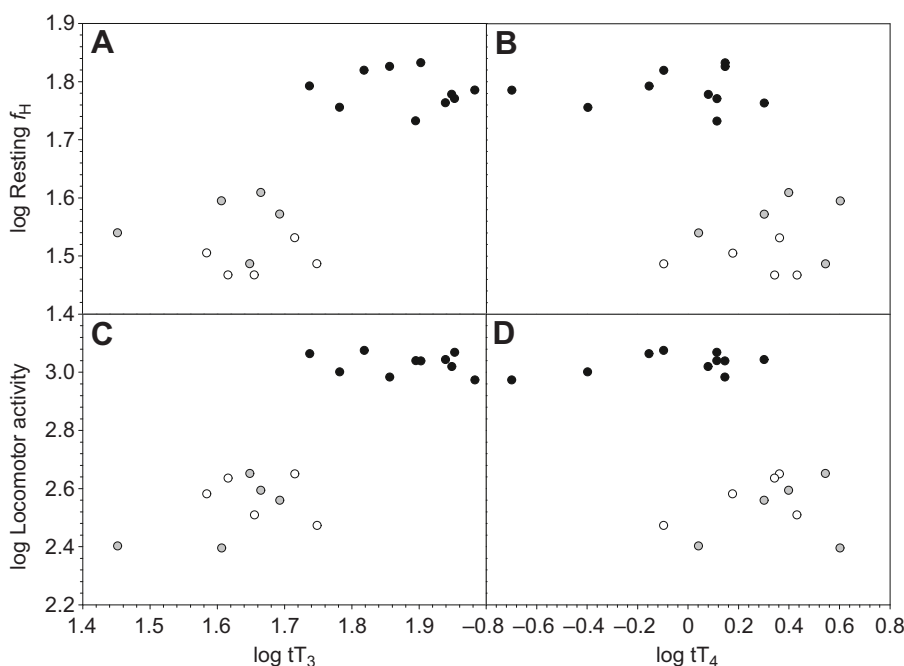


**Fig. 4. Relationship between THs and resting $f_H$ and locomotor activity in Shetland pony mares.** Regression between TH levels [tT$_3$ (ng dl$^{-1}$): A,C; tT$_4$ (µg dl$^{-1}$): B,D] and resting $f_H$ (beats min$^{-1}$; A,B) and locomotor activity (impulses h$^{-1}$; C,D). Black dots denote animals 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 murres 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 Chistoperson, 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.

**Data availability**

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