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Rectal temperature in *Capra hircus*, involvement of the daily rhythm of thyroid hormones, uncoupling protein 1 and clock gene *Per2*

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ABSTRACT

Thermal homeostasis is one of the principal indexes of animal welfare; the circadian rhythm of body temperature is well established and its disruption or alteration are signals of stress. The present study investigated the daily rhythm of 3,5,3'-triiodothyronine (T3), thyroxine (T4), uncoupling protein 1 (UCP1), and clock gene *Per2* in *Capra hircus* to improve the knowledge in this field. Camosciata delle Alpi goats (7 female, 2 years old, 67±2 kg, clinically healthy), were housed in a stable under natural environmental conditions. Blood sample collections were performed every 4 h for a 48-h period. Serum concentrations of T3, T4, UCP1, and clock gene *Per2* were determined. Before the blood sample collection rectal temperature was recorded at all data points. Two-way for repeated measure analysis of variance showed a statistically significant effect of time of day on all studied parameters. T3, T4, *Per2,* and rectal temperature showed a robust daily rhythm. The acrophases observed in the investigated parameters were statistically different. In particular, T3 acrophase was observed between 20:15 and 21:45; T4 acrophase was between 02:41 and 03:35; *Per* 2 acrophase was between 7:18 and 08:11; RT acrophase was between 17:45 and 19:55. *Per2* expression was correlated with T3 and T4 serum levels, and the rectal temperature values were correlated with T3 and T4 serum levels and *Per2* In conclusion, in goats housed in boxes, the rectal temperature daily rhythm was linked to the daily rhythm of thyroid hormones and *Per2* clock gene expression in the peripheral blood. In goats not subjected to thermal stress UCP1 did not show a daily fluctuation.

Introduction

In physiology, the most important concept is homeostasis. It is defined as the relative stability of the physiochemical properties of an organism to maintain the internal environment. Homeostasis applies to various physiological functions; the most intuitive form of homeostasis is the regulation of body temperature (Refinetti, 2006). Body temperature daily rhythm has been extensively investigated in domestic animals; typically, body temperature oscillates around a certain set point daily, it is used as an indicator of the rhythmicity of the biological clock, and a disturbance or unusual rhythm can be a signal of stress (Lowe et al., 2001; Piccione and Refinetti, 2003; Tokizawa et al., 2009). Body temperature homeostasis is ensured by the balance between thermogenesis and thermodispersion (Arfuso et al., 2016). There are two major thermogenic mechanisms responsible for heat production, obligatory thermogenesis, and facultative thermogenesis or no shivering thermogenesis [NST] (Solmonson and Mills, 2016). It is known that the endocrine regulators of these two mechanisms are thyroid hormones (TH). In recent years, the discovery of uncoupling proteins has opened up new insights into the mechanism that involves TH in thermogenesis. The uncoupling protein is the most important protein in this group. It is located in the inner mitochondrial membrane of brown adipose tissue cells', and controls heat production in response to cold or inadequate nutrition through hormonal regulation. Uncoupling protein 1 stimulates the secretion of T3 and it is involved in the response of brown adipose tissue to heat production (Lanni et al., 2003; Sell et al., 2004).

Endocrine factors are of interest in circadian physiology, as they promote adaptation to environmental changes. A daily rhythmicity in serum concentrations of endocrine system components has been identified, including a 24-h fluctuation in hormones controlled by the

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hypothalamic axis (HPA) and the resulting endocrine signals (Gamble et al., 2014).

Thyroid hormones have been investigated in many chronophysiological studies with conflicting results; 3,5,3'-triiodothyronine (T3) and thyroxine (T4) serum concentrations showed a diurnal acrophase in most studies performed in rats. This daily rhythmicity has been attributed to signalling from the SCN via rhythmic TSH secretion (Fahrenkrug et al., 2017).

Clock genes transcribe the temporal information coming from the SCN into the peripheral tissue. In particular, thyroid hormones influence *Per2* expression, in rats (Angelousi et al., 2018).

The factors to which livestock are exposed can influence their circadian physiology. In particular, management conditions can cause the animals to feel uncomfortable and disrupt the daily rhythm of the influenced parameters.

All circadian parameters of body temperature daily rhythm have been shown to exhibit consistent differences between species (Refinetti and Menaker, 1992). The study aimed to improve the knowledge of the role of the circadian system in goats' thermal homeostasis. To this purpose, the daily rhythm of T3 and T4 serum concentrations was investigated in correlation with the daily rhythm of UCP1 and *Per2* in goats kept in stable conditions.

Materials and methods

Animal and experimental design

Seven female Camosciata delle Alpi goats were examined for the present study.

The Camosciata delle Alpi goat breed originates from Switzerland. It is a cosmopolitan breed that is widespread in Europe and is also bred in many non-European countries. It is characterized by an excellent milk yield, and is versatile and adaptable.

The animals were kept in individual pens measuring 2.5×2.5 m, with straw bedding, on the same farm of origin under natural environmental conditions. All pens were equipped with a big opening window to allow natural illumination. The study was conducted during the summer solstice with a light/dark cycle of 15 h of light and 9 h of darkness (sunrise 05:05, sunset 20:55). A data logger (Gemini, Chichester, West Sussex, UK) was used for thermohygrometric recording. Inclusion criteria were clinical health status, absence of internal and external parasites, homogeneous age (2 years) and body weight (67±2 kg), and absence of gestation and lactation to avoid influence of these two physiological conditions on thermal homeostasis (Castro Lima et al., 2022). The clinical health status was evaluated by a complete clinical examination including heamatological, hematochemical, and coprological exams.

During the experimental period, all animals were provided with high-quality hay (Alfalfa hay – Medicago sativa L.) and water *ad libitum*. General animal care was carried out by professional staff who were the same as the farm staff and not associated with the research team. The protocol of the animal study was approved by the Institutional Ethics Committee of the Department of Veterinary Sciences of the University of Messina (protocol code 09/2023_ter).

Before the start of the study, the jugular vein of each animal was cannulated with a 16-gauge catheter (Terumo, Roma, Italy) fixed with a suture (Vicryl, Ethicon, Somerville, USA), after the area had been trimmed and surgically prepared. Blood samples were collected every 4-h over a 48-h period in vacutainer tubes without additives (Terumo Co., Japan), and in PAXgene® Blood RNA Tube (Qiagen).

Prior to blood collection a digital thermometer (model HI92704, Hanna Instruments), with a display resolution of 0.1 °C, a precision of ±0.1 °C and an accuracy of 0.1 °C, was used for rectal temperature recording during all the recording points, with the probe inserted 8 cm into the rectum immediately before the blood collection at each data point. Following the recommendation of the U.S. National Institute of Standards and Technology, we conducted a performance check of the thermometer once a year with a one-point check at the ice melting point (0°C) one a year. All animals tolerate the rectal probes very well and show no signs of stress-induced hyperthermia (Piccione et al., 2002). During the dark phase of the natural L/D cycle, a dim red light (< 3 lux, 15 W Safelight lamp filter 1A, Kodak Spa) was used for data collection. All data collections were performed by the same technician, and the restraint was performed by a staff member who normally takes an operator usually take care of the goats, following the best practice recommended by the European Commission.

Blood samples were allowed to clot for 2 h at room temperature and then centrifuged at 1000 x g for 20 min. The not-hemolyzed sera were stored at −20°C until analysis. The PAXgene® tubes were stored at −80°C.

Serum, T3, T4 and UCP1 concentrations were assessed using an ELISA kit (Goat Tri-iodothyronine, T3 ELISA Kit - MBS700937; Goat Thyroxine, T4 ELISA Kit - MBS703630; Goat Uncoupling Protein 1, Mitochondrial (UCP1) ELISA Kit - MBS094948, MyBioSource, Inc. San Diego, California, USA), according to the manufacturer's instructions.

Blood samples collected in PAXgene® Blood RNA Tube were stored at - 80 ∘C until the clock genes determination. Total RNA was purified directly from whole blood samples, using a PAXgene® Blood RNA kit (Qiagen), according to the manufacturer's instructions and resuspended in 80 µl of Elution Buffer. Reverse Transcription was carried out immediately, using the Superscript VilocDNA Synthesis Kit (Invitrogen), in a final volume of 20 µl, containing 3 µl of total RNA, a 5X Vilo Reaction mix (including random hexamers, MgCl2, and dNTPs) and a 10X SuperScript Enzyme mix. An initial step at 25 ∘C for 10 min was followed by a reverse transcription step at 42 ∘C for 1 h. The resulting cDNA was stored at - 20 ∘C before further analysis by RT-qPCR. Gene-specific primers (Table 1) were designed using Primer 3 software to amplify fragments of Capra hircus clock genes (Per2). All reactions (in triplicate) were performed in a 20 µl of the final volume, containing 2 µl of cDNA product, 1X Buffer Sybr green (Fast Sybr green master mix - Applied Biosystems), and 1 mM of each primer. The thermal profile was: 95 ∘C for 10 min, followed by 40 cycles of 95 ∘C for 30 s, 60 ∘C for 1 min. Melting curve cycles were set as follows: 95 ∘C for 15 s, 60 ∘C for 1 min, and 95 ∘C for 15 s. We verified the efficiency of the primers by doing standard curves for all genes investigated. Moreover, the dissociation curve was used to confirm the specificity of the amplicon. Gene expression levels of selected equine/caprine clock genes were tested together at GADPH (gliceraldeide-3-fosfato deidrogenasi). The relative levels of each RNA were calculated by the 2-ΔΔCT method (CT standing for the cycle number at which the signal reaches the threshold of detection) (Livak and Schmittgen, 2001). Each CT value used for these calculations is the mean of three replicates of the same reaction.

Statistical analysis

The application of Shapiro-Wilk test was used to verify the normal distribution of data ($p>0.05$). To assess statistical differences related to the monitoring day and time of day a two-way for repeated measures analysis of variance (ANOVA) was performed. To describe the periodic phenomenon analytically, by characterizing the main rhythmic parameters according to the single cosinor procedure (Nelson et al., 1979) a trigonometric statistical model of each subject value of each time series was performed (https://www.circadian.org/softwar.html). The program setting included a bin size of 240, 7 bins to use, time of the first bin at 5:00. No outliers were recorded.

To investigate statistical differences in acrophase and robustness based on the monitoring day and the studied variables, two-way ANOVA was applied on the data obtained on the parameters showing a daily rhythm (T3, T4, *Per2*, and rectal temperature).

The correlation coefficient (r) between the investigated parameters was determined. Regression lines with a 95% confidence interval for the different recorded data were determined. $P<0.05$ was considered statistically significant. The data were analyzed using Statistica 7

Table 1
Nucleotide sequences and positions of primers used in RT-qPCR for PER2 assessment in goats.

Gene	*Genbank Number*	*Sequence (5'→3')*	*Length (bp)*	*Primer (μM)*
Goats				
PER2	XM 012108190.1	**for:**CAAGTGAAAGCCAGTGAGGAGTACTAC**rev:**CAATCTCGTGCACGGTGAAG	97	F: 572R: 668
GAPDH	NM 001190390.1	**for:**GGCGCCAAGAGGGTCAT**rev:**GTGGTTCACGCCCATCACA	70	F: 376R: 444

(StatSofts, Inc, USA).

Results

Thermo-hygrometric recording showed ambient temperature between 22 and 26°C, and relative humidity of 65% which falls within the thermoneutrality zone for the caprine species, and aligns with the seasonality of the geographic area.

The application of two-way for repeated measures ANOVA on raw data showed a statistically significant effect of time of day on T3 ($p<0.0001$), T4 ($p<0.0001$), UCP1 ($p<0.0001$), *Per2* ($p<0.0001$) and rectal temperature ($p<0.0001$). Fig. 1 report the trend (mean ± standard deviation) of serum T3, T4, UCP1, *Per2*, and rectal temperature monitored in the 48-hour period, in goats.

A daily rhythm of serum concentrations of T3, T4, *Per2,* and rectal temperature was observed on the two days (Table 2). T3 and rectal temperature acrophase was recorded closest to sunrise, T4 acrophase at about 2 h before sunrise, and *Per2* at about 2 h after sunrise.

The application of the two-way ANOVA on the acrophase and robustness showed statistically significant differences in the acrophase ($p<0.0001$) among T3, T4, *Per2,* and rectal temperature (Table 2). No effect of the monitoring day was observed on the acrophase of all monitored parameters. Robustness did not statistically change among the monitoring parameters, and no effect of the monitoring day was observed (Table 2).

Per2 was negatively correlated with the T3 serum concentration ($r=-0.71$) and rectal temperature ($r=-0.80$) and positively correlated with T4 ($r=0.25$) (Fig. 2). UCP1 was negatively correlated with T4 ($r=-0.32$) (Fig. 3). Rectal temperature was also positively correlated with T3 ($r=0.69$) and negatively correlated with T4 ($r=-0.31$) (Fig. 4).

Discussion

The animals' homeothermic capacity represented an important leap in evolution. The body temperature daily oscillation is, first of all, under homeostatic control and then modulated by the circadian system through a daily oscillation in the thermoregulatory set point (Refinetti, 2006). The circadian timing system has been demonstrated to regulate the variable secretory pattern of the hypothalamic-pituitary-thyroid axis endocrine components (Fahrenkrug et al., 2017).

As expected, rectal temperature showed a nocturnal daily rhythm with a high percentage of robustness. Rectal temperature was significantly linked to T3, T4, and *Per2* peripheral expression

A daily rhythm of T3 and T4 was observed in goats, with a different course of oscillation, and acrophases at different times of the day. In particular, T3 acrophase was observed about six hours before the T4 acrophase. T3 and T4 showed high a robustness of rhythm denoting their strength of oscillation, and stability regarding casual issue.

A daily rhythm of *Per2* expression was observed in association with the thyroid hormones' daily rhythm. In particular, a negative correlation was detected between T3 and *Per2,* and a positive correlation was detected between T4 and *Per2*. Peripherally most tissues respond thermogenically to a thyroid hormone signal with an increase in total animal metabolism (Silva, 2006; Lopez et al., 2013).

In BAT, adaptive thermogenesis is associated with the *Per2* expression. *Per2* has been identified as a potential target of heat shock factors (HSF); the heat shock response (HRS) has been linked to the mechanism of the circadian clock. Additionally, *Per2* has been identified as the

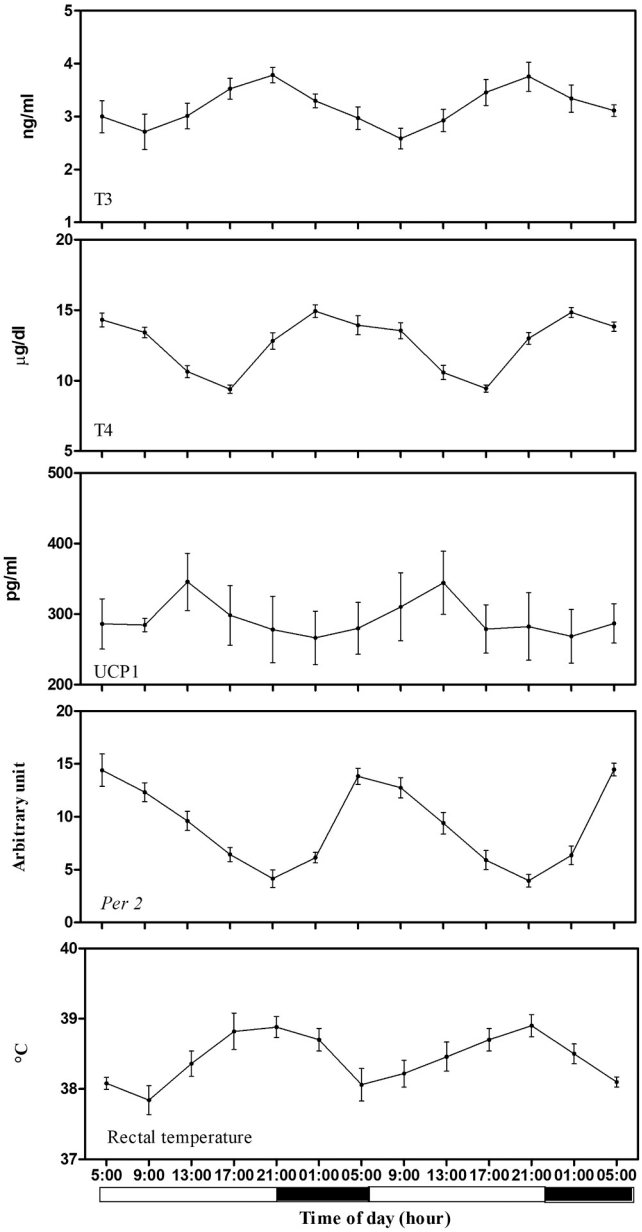

Fig. 1. Trend (mean ± standard deviation) of serum 3,5,3'-triiodothyronine (T3), thyroxine (T4), uncoupling protein 1 (UCP1), PER 2, and rectal temperature recorded in goats during stable conditions for 48 h, expressed in their conventional unit. White and black bars indicate the photophase and the scotophase of the natural photoperiod.

coordinator of the UCP1 production and activation (Chappuis et al., 2013). In adult goats, an auxiliary role in maintaining the body temperature daily rhythmicity was attributed to UCP1, though UCP1 daily rhythmicity has not been found (Arfuso et al., 2017; Giannetto et al., 2017). In cold environmental conditions, the most important mechanism involved in thermal homeostasis is the NST. Brown adipocytes' thermogenic function is under neurohormonal control. Cold-induced

Table 2
Mean ± standard deviation of rhythmic parameters (Mesor, amplitude, acrophase and robustness) observed during the two days of monitoring in 3,5,3'-triiodothyronine (T3), thyroxine (T4), *Per2,* and rectal temperature, expressed in their conventional unit. * indicates the statistical differences ($p<0.0001$) respect to the other two parameters in the same day of monitoring.

Parameters	Day	Mesor	Amplitude	Acrophase (hh:mm)	Robustness (%)
3,5,3'-triiodothyronine (T3)-ng/ml	1	3.22±0.16	0.48±0.19	20:30±15min*	90.30±7.59
	2	3.19±0.19	0.51±0.11	21:15±30min*	89.83±7.58
thyroxine (T4)-µg/dl	1	12.46±0.26	2.45±0.21	03:20±25min*	87.57±3.00
	2	12.44±0.34	2.42±0.29	03:08±27min*	85.00±2.91
Per2-arbitrary unit	1	9.01±0.35	5.08±0.52	07:46±25min*	87.31±4.23
	2	8.93±0.44	5.34±0.45	07:40±22min*	90.99±4.10
Rectal temperature (°C)	1	38.44±0.07	0.54±0.13	19:15±40 min	90.58±4.50
	2	38.47±0.12	0.39±0.09	18:20±35 min	91.40±2.65

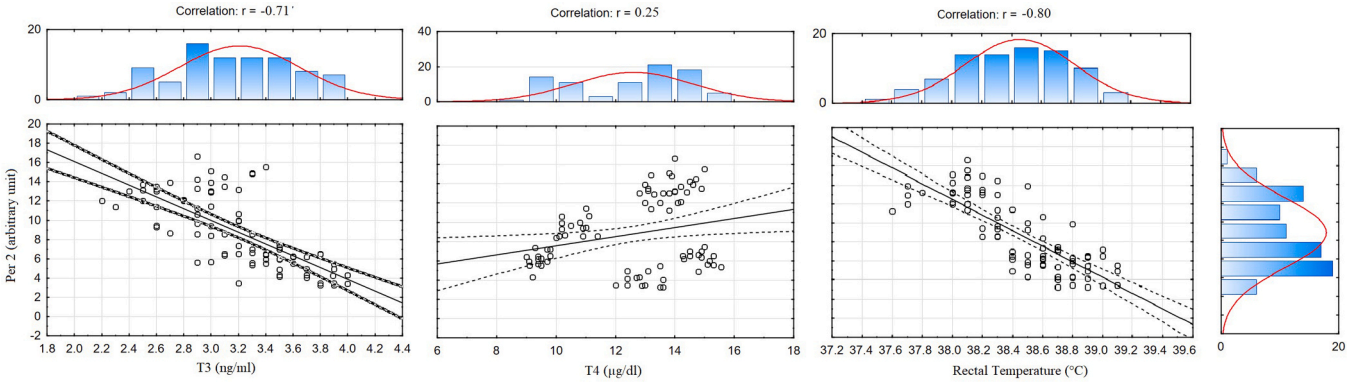

Fig. 2. Correlation between *Per 2* and T3, T4, and rectal temperature.

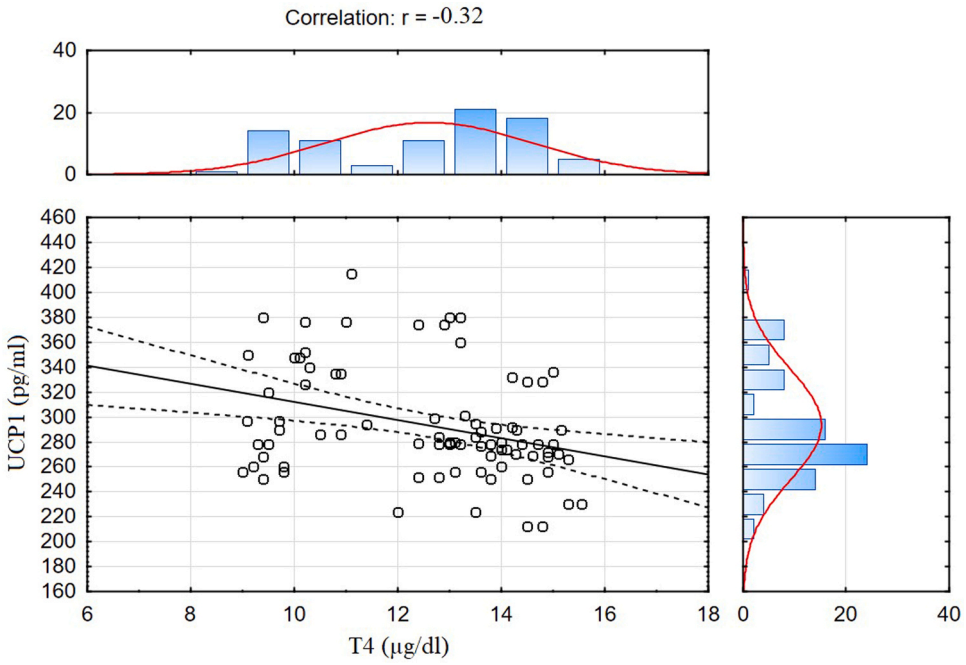

Fig. 3. Correlation between UCP1 and T4.

noradrenergic stimulation of BAT results in the transformation of T4 to T3 (de Jesus et al., 2001). T3 increases the UCP1 expression responsible for the dissipation of heat in BAT's mitochondria (Silva, 2006, 2008). When animals are housed in ambient conditions within the thermoneutral zone, body temperature is maintained constant only by the obligatory thermogenesis, without the involvement of any other temperature homeostatic mechanism (Cannon and Nedergaard, 2004; Silva, 2006). Brown adipose tissue is not always involved in thermoregulation, indeed, there are situations in which its participation is counterproductive by opposing another thermoregulatory effector. Brown adipose tissue heat production is recruited when extra heat is necessary. Therefore, we can suppose that T3-induced thermogenesis and the hypothalamic mechanisms involving BAT were not activated by the living ambient conditions falling within the thermoneutral zone

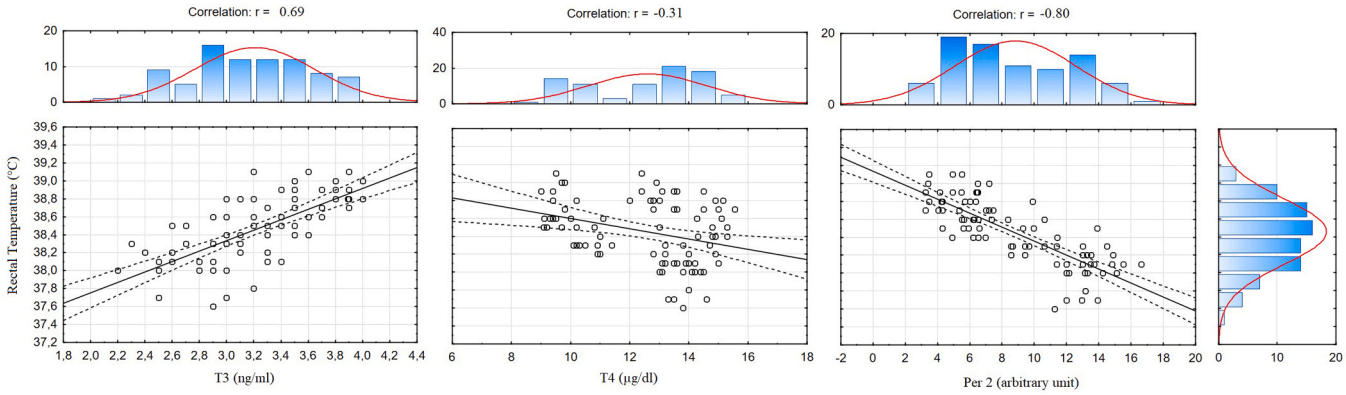

Fig. 4. Correlation between rectal temperature and T3, T4, and *Per 2*.

(Alvarez-Crespo et al., 2016). It has been suggested that BAT is "turned off" by the circadian system when its activity is not necessary (Gerhart-Hines and Lazar, 2015); justifying the absence of the UCP1 serum concentration daily rhythm found in goats. Considering a study on Instagram's role in sustainability outreach (Lamanna et al., 2025) highlights social media's effectiveness in communicating complex topics like animal welfare and metabolic regulation. Such initiatives demonstrate how digital platforms can foster engagement and awareness in chronobiology and farm animal research, particularly, in disseminating new findings on thermal homeostasis and circadian rhythms in goats.

Conclusion

Despite the limited number of animals involved in this study, our data can be considered a preliminary study to identify a clear connection between thermal homeostasis, thyroid hormones, and circadian rhythms in goats. In particular, in goats, T3 and T4 secretion exhibited daily rhythmicity, similar to the principal clock gene involved in thermoregulation, *Per2*. This can be considered a starting point for a better understanding the organization of the goats' thermoregulatory clock. The absence of thermal stress likely accounts for the lack of a UCP1daily rhythm in peripheral blood. Considering in any case that our study was conducted in only one environmental condition, further research is necessary to establish the modification of clock genes related to the thermal homeostasis in the peripheral blood across various environmental conditions. A better understanding of thermal homeostasis in farm animals is important not only for comprehending this process but also for gaining insight into the variability in energy expenditure in livestock.

Ethical statement

The animal study protocol was approved by the Institutional Ethics Committee of the Department of Veterinary Sciences of the University of Messina (protocol code 09/2023_ter).

CRediT authorship contribution statement

Giudice Elisabetta: Data curation. **Guercio Annalisa:** Methodology. **Arfuso Francesca:** Writing – original draft. **Agradi Stella:** Formal analysis. **Piccione Giuseppe:** Writing – review & editing. **Cannella Vincenza:** Methodology. **Perillo Laura:** Investigation. **Giannetto Claudia:** Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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