Variation in core body temperature indicates fitness in ruminants, and is related to the potential for reproduction

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Thesis declaration

I, Yuri Kitagawa, certify that:

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Abstract

To achieve food security and health in the world, it is necessary to develop sustainable farming practices. There is a need to produce sufficient food to meet the demands of an increasing population. Animal protein accounts for the one-third of the protein intake, and 17 % of the calorie intake, in the world (OECD - Organization for Economic Cooperation and Development, 2021). Any improvement in the efficiency of the livestock industry would contribute to a sufficient supply of animal protein worldwide. However, the situation for the livestock industries is predicted to become worse, due to climate change and the increased use of intense forms of animal housing. Therefore, a novel approach to solve these problems would be to enhance the capacity for livestock to adapt to the changing environment.

This dissertation focuses on small ruminants, such as sheep and goats. They play an important role in human nutrition, and serve as a useful experimental model for larger ruminants, such as cattle. Ruminants accounts for 25 % of worldwide meat consumption (Ritchie and Roser, 2017). Therefore, it is worth gaining a better understanding of the physiological responses of ruminants to environmental challenges, and the relationship with reproduction, to develop new strategies for the sustainable production of ruminants.

I have focused on the core body temperature (T_c) as an important physiological response in the maintenance of homeostasis in mammals. The main purpose of this dissertation was to assess the hypothesis that variation in the T_c can be used as a new biomarker of the fitness and reproductive performance of animals, especially in ruminants. I analysed the circadian rhythm of the core body temperature (CRT) and stress-induced hyperthermia (SIH) as well-known forms of heterothermy in response to exogenous challenges.

The first aim of my thesis was to understand the effect of genotypes and phenotypes that have a relationship with temperament on the thermal response to psychological stressors, to better understand temperament and its effect on physiological responses. The adequacy of SIH and CRT as biomarkers of psychological stress was assessed is sheep. The second aim of my thesis was to explore the effect of environmental stress that livestock can experience in daily management on the parameters of the CRT. The comprehensive analysis combined several stressors (energy intake, movement restriction, and ambient conditions) in goats.

The third aim of my thesis was to test the validity of the amplitude of the CRT as a marker of reproductive ability, especially in the context of the endocrine control of reproduction. The relationship of the CRT profile and LH secretion was investigated in goats. The fourth aim of my thesis was to explore the possible neural circuitry that is involved in the interaction between energy homeostasis, reproduction, and core body temperature.

In the first experimental chapter (Chapter 2) of my thesis, I assessed the effect of genotypes and phenotypes that are related to temperament. I compared the T_c response to psychological stress during behavioural assessment tests and during exposure to a potential predator, a sheep dog, in groups of sheep with different genotypes and phenotypes. I selected the sheep based on an SNP in the tryptophan 5-hydroxylase (TPH2) gene that codes for an enzyme in the synthesis of serotonin. The activity of the TPH2 enzyme is impacted by the polymorphism, and therefore affects the level of serotonin in the brain. The SNP has been reported to be associated with temperament, as determined by the responses of sheep to an isolation box test (IBT). The responses of sheep were characterised two ways; genetically using groups defined as “genotype AA” (related to calm temperament) and “genotype GG” (related to nervous temperament), and phenotypically using groups defined as “low responder” (with a low IBT score) and “high responder” (with a high IBT score). While genotype had no effect on SIH, phenotype did, with high responders exhibiting larger SIH. In addition, the same sheep didn’t show SIH when they walked the same distance in the absence of a dog, and the behavioural test induced larger SIH with much lower activity increase. In addition, the amplitude of the CRT was increased after the exposure to the dog. The results suggest that strong psychological stress can induce an increase in the amplitude of the CRT, and that SIH would be a good biomarker of psychological stress because it can discriminate the level of stressors and individual reactivity.

In the second experimental chapter (chapter 3), I investigated the T_c response of goats to several potential stressors that ruminants can experience in farming settings. I used ovariectomized (OVX) goats to exclude the effect of gonadal hormones on the Tc and to eliminate other physiological fluctuations that depends on the estrous cycle. I analysed the effect of the ambient temperature, energy intake (EI), and movement restriction, in other words tethering, on the characteristics of the CRT. I conducted the experiment twice, in a

hot/humid season (summer) and in a cold/dry season (autumn) between the summer solstice and winter solstice. The amplitude of the CRT was positively correlated with ambient temperature in summer, but in autumn there was no correlation between ambient temperature and the amplitude of the CRT. These results suggest that the animals' homeostasis might be more affected by the heat stress than by cold stress. The data support the possibility that the amplitude of the CRT can be used as a biomarker of fitness to heat stress in goats. Further, I assessed the effect of restricted feeding and tethering on the characteristics of the CRT. Various kinds of restraint are still used in some forms of farm management, such as housing in tie-stalls. Animals tethered to a stanchion can access food and water, and can sit and stand, but are restricted from other movement such as walking or turning around. Neither restricted feeding or tethering had an effect on the amplitude of the CRT, but it did impact on the mesor, cosinor minimum, and cosinor maximum of the CRT. These results suggest that tethering can attenuate the hypothermia that is induced by low energy intake. My data suggested that housing systems, such as tie-stalls or stanchions, might induce disturbances to the adaptive hypothermic heterothermy of livestock, and induce excess energy expenditure during under nutrition.

In the third experimental chapter (Chapter 4), I investigated the relationship between the characteristics of the CRT and the secretion of luteinizing hormone (LH) as an indicator of the activity of the hypothalamic-pituitary-gonadal (HPG) axis in OVX goats. The characteristics of the CRT were analysed over five consecutive days, and then blood samples were collected one day after and analysed to characterise the parameters of LH secretion, including mean LH, LH pulse frequency, and the inter-pulse interval. The mean and baseline concentrations of plasma LH and the amplitude of the CRT were negatively correlated. These results suggest that the amplitude of the CRT could be used to predict the LH concentration that drives reproductive function in female ruminants. The underlying mechanism connecting the amplitude of the CRT and the LH concentration, and the causal relationship between them, was not investigated in this dissertation. However, variation in the amplitude of the CRT and the LH secretion might be due to variation in the energy status of each goat. If so, it is possible that a common mechanism could control both the T_c and reproduction in response to energy status.

In the fourth experimental chapter (Chapter 5), the role of amylin-calcitonin receptor (CTR) signalling in the control of the activity of the gonadotropin-releasing hormone (GnRH) pulse generator was investigated. I used a multiple unit activity (MUA) recording system in goats to monitor the activity of the GnRH pulse generator. I administrated amylin, an agonist of the CTR, into the lateral ventricle. I observed a facilitatory effect followed by an inhibitory effect on GnRH pulse generator activity in OVX female goats. I showed that the cells expressing *CALCR* (CTR gene) mRNA were widely distributed around the ventricle. The co-expression of *CALCR* (CTR gene) and Kisspeptin was quantified using double *in situ* hybridization for *KISS1* (kisspeptin gene) and *CALCR* (CTR gene), and revealed that only around 1% of the KISS1 expressing cells also expressed *CALCR* in the arcuate nucleus. The results suggest that central amylin-CTR signalling has a biphasic role in the regulation of the GnRH pulse generator by acting on cells other than the kisspeptin neurons of the arcuate nucleus in goats. In addition, amylin-CTR signalling has been reported to be involved in thermoregulation and energy homeostasis. So, the amylin-CTR signalling could be a potential neural circuit that links thermoregulation, energy homeostasis, and reproduction.

My PhD work has added further evidence supporting the concept of the use of the profile of the CRT as a biomarker of an animals' fitness. Variation in T_c is related to the fitness of animals and may reflect the animals' capacity to cope with day-to-day challenges. In the conclusion of my thesis, I suggest a possible mechanism that could integrate the control of energy balance, core body temperature, and reproductive function. I have proposed a new method to estimate an animal's fitness to artificial and natural environments by monitoring the T_c.

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This thesis contains work that has been [published and/or prepared for publication].

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I, Dominique Blache certify that the student's statements regarding their contribution to each of the works listed above are correct.

As all co-authors' signatures could not be obtained, I hereby authorise inclusion of the co-authored work in the thesis.

Coordinating supervisor signature: [redacted] Date: 02/11/2011

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2DG	2-Deoxy-d-glucose
AIC	Akaike information criterion
AMY	Amylin receptor
ARC	Arcuate nucleus
AUC	Area under the curve
BAT	Brown adipose tissue
BMI	Body mass index
BMR	Basal metabolic rate
BoM	Bureau of Metrology
BST	Bed nucleus of the stria terminals
CALCR (Calcr)	Calcitonin receptor gene
CR	Calorie restricted diet
CRT	Circadian rhythm of core body temperature
CTR	Calcitonin receptor
DH31R	Diuretic hormone 31 receptor
DIG	Digoxigenin isothiocyanate
DMH	Dorsomedial hypothalamic nucleus
Dyn	Dynorphin A
E2	17β-estradiol
ECG	Electrocardiography
EI	Energy intake
eNOS	Endothelial nitric oxide synthesis
FITC	Fluorescein isothiocyanate
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GPR	G protein-coupled receptor
HPA axis	Hypothalamic-pituitary-adrenal axis
HPG axis	Hypothalamic-pituitary-gonadal axis
HRV	Heart rate variability
IBT	Isolation body test
IR	Infrared thermal imaging

KISS1	Kisspeptin gene
KO	Knock out
LCT	Lower criteria temperature
LH	Luteinizing hormone
LV	Lateral ventricle
LW	Live weight
MBH	Mediobasal hypothalamus
MD	Maintenance diet
MER	Maintenance energy requirement
MPOA	Medial preoptic area
MUA	Multiple unit activity
NKB	Neurokinin B
NPY	Neuropeptide Y
OVX	Ovariectomized
POA	Preoptic area
POMC	Proopiomelanocortin
PVN	Paraventricular hypothalamic nucleus
RAMP	Receptor activity modifying proteins
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SDGs	Sustainable development goals
SERT	Serotonin transporter
SIH	Stress-induced hyperthermia
SNPs	Single nucleotide polymorphisms
SPZ	Supraventricular zone
T_c	Core body temperature
TNZ	Thermoneutral zone
TPH2	Tryptophan 5-hydroxylase
TPR	Temperature preference rhythm
UCP1	Uncoupling protein 1
UCT	Upper criteria temperature
VMH	Ventromedial hypothalamic nucleus

Chapter 1: General introduction

1.1 General background

1.1.1 World population and food demand

The sustainability of a healthy world population depends on achieving of food security. Six years after the adoption of the "Sustainable development goals" (SDGs) by the United Nations General Assembly, a large proportion of the world population still faces hunger (10%) and food insecurity (30%) (FAO, IFAD, UNICEF, 2021). The COVID-19 pandemic has made the pathway to accomplish the SDG 2 "zero hunger" steeper due to the economic slowdowns and downturns. It has been reported that between 720 and 811 million people faced hunger and the prevalence of undernourishment (PoU) increased 1.5 percent to 9.9 percent in 2020 from 2019 (FAO, IFAD, UNICEF, 2021). The world effort to achieve food security goals and increase food supply needs to include improvements in livestock production (Smith *et al.*, 2013).

1.1.2 Sustainable development of livestock industry

Along with the improvement of living standards, the world's population has increased its consumption of animal products, such as milk, egg, and meat (Godfray *et al.*, 2010; Alexandratos, 2012). Animal proteins are necessary to human health because they abundantly provide essential amino acids (Organization for Economic Cooperation and Development, [OECD] 2021).

An improvement in the efficiency of the livestock industry would contribute to a sufficient supply of animal protein worldwide. Historically, genetic tools, and recently, pharmacological and machinery techniques have been used to improve the efficiency of animal production mainly targeting reproduction efficiency (Terry *et al.*, 2021). Some of these techniques have been criticised because they could compromise the animals' well-being (Thornton, 2010). In addition, the well-being of livestock as well as the efficiency of livestock production could become compromised because of climate change (Henry *et al.*, 2018). A novel approach to solve these two problems, would be to enhance the capacity of livestock to adapt to the changing environment. A better understanding of the physiology and the mechanisms responsible of keeping livestock within their homeostatic limits would

contribute to the development of strategies aiming at a better adaptation of livestock to a challenging environment.

1.1.3 Ruminants

This thesis focuses on small ruminants because sheep and goats have played an important role in human nutrition since being domesticated around 10,000 B.C. (Hirooka, 2013) about 4000 years before the domestication of cattle. Small ruminants have provided human with meat, milk, wool, and labour force. Currently, one-third of the protein intake and the 17 % of calorie intake provided by animal products, and ruminants account for a 25 % of meat consumption, although the ratio is different in the region (OECD, 2021)(Ritchie and Roser, 2017). About 3.6 billion ruminants are farmed worldwide and roughly two billion of them are used for meat and dairy products (Zoupanidou, 2019). The development of strategies to maintain the sustainable production of sheep and goats would contribute to achieving better nutrition for the human population in the face of variations in the climatic conditions. To develop such strategies, it is essential to gain a better understanding of the physiological responses of ruminants to variation of climate and their relationship with reproduction.

1.2 Body temperature control of mammals

Homeostasis is fundamental to the maintenance of life. In mammals and birds, an important homeostatic mechanism is the control of body temperature that result in relatively constant core body temperature in the face of large challenge in environmental temperature. Disturbance of homeostasis are signs of pathology and signal that health is compromised.

1.2.1 Mechanisms controlling core body temperature

1.2.1.1 Heat balance

Mammals generally keep their body temperature constant within a few degrees over a wide range of activities and environmental conditions. The exception is those mammals that enter torpor or hibernation, when normal body temperature control operates at much lower temperature (Geiser, 2013). Apart from episodes of torpor, homeotherms need to keep their core body temperature constant to sustain biochemical activity. To maintain body temperature constant, the rates of heat production and heat loss must be equal. When animals are under normal conditions (ambient temperature is lower than their body

temperature), a certain amount of heat loss exists, and that rate of heat loss is controlled such that it matches metabolic heat production. Usually, more than 75% of the energy consumed by an animal is transformed into heat (Iriki, 2003). Heat is transferred from one site to another within the body and exchanged with the surrounding environment through evaporation, convection, conduction, and radiation (Withers et al., 2016). The heat balance equation explains this equilibrium (Jessen, 2001);

HP = THL

Where:

HP = rate of metabolic heat production

THL = rate of total heat loss

A more detailed equation can be used:

S = MR – (± W) – (± E) – (± C) – (± K) – (± R)

Where: S = rate of storage of heat

MR = rate of metabolic energy transformation

W = rate of external physiological work against the environment

E = rate of evaporative heat transfer

C = rate of convective heat transfer

K = rate of conductive heat transfer

R = rate of radiant heat transfer

In many cases, these terms in the heat transfer equation are positive because normally the ambient temperature is lower than body temperature. However, in the extremely hot condition (in the tropics or in deserts), the ambient temperature is sometimes higher than

body temperature. In that case, these terms become negative. When a homeotherm cannot adjust heat gain to balance heat loss, hyperthermia or hypothermia will result, and those states can compromise the health and productivity of an animal. For example, in the summer hyperthermia can induce lower milk production and conception rate in dairy cattle (Gwazdauskas *et al.*, 1973; Ingraham *et al.*, 1979). Therefore, to improve the productivity of livestock, it is important to understand the mechanisms that animals use to keep their homeostasis and fitness to the environment.

1.2.1.2 Thermoneutral zone (TNZ)

The rate of heat loss increases when ambient temperature decrease. To maintain homeothermy, a mammal will increase metabolic heat production when it is exposed to low ambient temperature. The thermoneutral zone is the ambient temperature range in which the normal adult mammal can maintain their core body temperature without resort to an increase in metabolic heat production. In the thermoneutral zone, the basal rate of heat production is equal to the rate of heat loss to the environment. In the TNZ, mammals are most comfortable, and their production efficiency is highest. The thermoneutral zone differs between species, 5 – 20 °C for adult cattle, 21 – 31 °C for sheep (it highly depends on age and fleece thickness), and 10 – 20 °C for goats (Kerr, 2015). These temperature ranges are highly variable depending on the breed, genetic background, and adaptation of animals.

1.2.1.3 Brain mechanisms controlling the core body temperature

Core body temperature is controlled by a neural circuit, which receive temperature perception information and drives the activity of thermoregulatory effectors. There are two pathways for the perception of thermal information. First, the core body temperature change is perceived by thermosensitive neurons in the preoptic anterior area (POA) of the hypothalamus (Hardy *et al.*, 1961; Imai-Matsumura *et al.*, 1984; Kanosue *et al.*, 1994). Temperature information sensed by skin thermoreceptors is transmitted through the dorsal horn of the spinal cord to the lateral parabrachial nucleus, where it is input to the POA (Nakamura and Morrison, 2008, 2010a; Morrison and Nakamura, 2019). The thermoregulatory effector responses include shivering thermogenesis, non-shivering thermogenesis, such as metabolic heat production by brown adipose tissues, cutaneous vasoconstriction and vasodilation, sweating, and panting (Jessen, 2001). In addition, several

artiodactyls (such as sheep, goats, and cattle) have a specific cooling system for brain, called selective brain cooling (Mitchell *et al.*, 2002). The neural circuit that integrates thermal information and regulates body temperature has been investigated for a number of years and the framework of the neural circuit has been established (Nakamura, 2011), and the central regulator is located in the POA in the hypothalamus (Nakamura and Morrison, 2010b). Although the framework of the temperature control mechanism has been established, the full details of the mechanism of the control of the core body temperature is not fully understood. Thus, the journey to reveal the brain mechanisms controlling the core body temperature remains still ongoing.

1.2.2 Biological adaptation of body temperature to the exogenous challenge

While the strict control of the core body temperature is required for the maintenance of biological activity, animals modulate their core body temperature slightly, or sometimes drastically, to adapt to the environment and survive.

1.2.2.1 Hibernation

Hibernation is a well-known and obvious thermal adaptation of homeotherms (Geiser, 2013). The increased energy cost (associated with a decrease of ambient temperature) and a decrease in energy supply seem to induce hibernation in mammals. By lowering the core body temperature, mammals reduce energy expenditure and can maintain their life until the food supply recovers (Ruf and Geiser, 2015). Recently, Takahashi *et al*. (2020) identified the neural circuit in mice that induce hibernation-like hypothermia, even though mice do not normally enter torpor or hibernation (Takahashi *et al.*, 2020). Thus, even in non-hibernating mammals the brain mechanisms that responsible for torpor are present, raising the potential for those animals to reduce their energy expenditure. Thus, a decreased in the core body temperature might be an adaptation that reduces energy expenditure in all mammals (Geiser, 2013).

1.2.2.2 Core body temperature change for malnutrition

It is known that restriction of energy intake induces lowered core body temperature, even in non-hibernating mammals. A decrease in the core body temperature resulting from calorie intake restriction was first observed in mice (Weindruch *et al.*, 1979), and subsequently in

rodents and other species, such as sheep and goats (Piccione *et al.*, 2002). The lowering of core body temperature has been considered as a mechanism that saves energy and prolongs survival. It has been reported that the low core body temperature that is induced by caloric restriction results in increased lifespan by reducing inflammation and reactive oxygen species (Carrillo and Flouris, 2011). Recently, the brain mechanisms that link core body temperature and energy expenditure have been revealed. Nakamura *et al*. (2017) demonstrated that hypothalamic neuropeptide Y (NPY) signalling inhibits brown adipose tissue thermogenesis together with the stimulation of feeding (Nakamura *et al.*, 2017a). Thus, the control of core body temperature and energy homeostasis have a strong connection and partly share a common regulatory system.

1.2.3 The circadian rhythm of core body temperature (CRT)

In addition to these basal and acute variations of the core body temperature, homeotherms exhibit a daily rhythm of core body temperature. It is called the circadian rhythm of core boy temperature (CRT). Because it is synchronized with the daily activity rhythm, the CRT is sometimes considered to be a by-product of the activity rhythm. But the CRT continues even when there is no activity rhythm, such as in man resting in bed (Gander *et al.*, 1986). Therefore, a biological significance of the endogenous temperature rhythm has been suggested, but it not fully understood. The amplitude of the CRT has been suggested to change in response to various kinds of exogenous stimuli (Maloney *et al.*, 2019). In recent years, there has been interest in the significance and driver of these variations in the amplitude of CRT. Energy balance is a well-known factor that affects the amplitude of the CRT. An increase in the amplitude of the CRT is mainly caused by a decrease in the minimum core body temperature during the resting period (Piccione *et al.*, 2002; Maloney *et al.*, 2013; Goh *et al.*, 2016). As mentioned above, mammals reduce their basal energy expenditure and heat production during food deprivation. Thus, the decrease in the minimum core body temperature could be due to a decrease in the basal metabolic rate during the resting period. These facts suggest that there is strong connection between the control of the CRT and energy balance. The amplitude of the CRT reflects not only the energy balance, but also several aspects of physiological status, such as water balance, reproductive status, age, and disease (Maloney *et al.*, 2019).

1.2.4 Stress-induced hyperthermia (SIH)

Stress-induced hyperthermia is the acute (sometimes chronic) increase in core body temperature in response to stress (Oka, 2015, 2018). The responses to psychological stress, including hyperthermia and hypertension, are thought to increase an animals' performance transiently by promoting energy metabolisms (Bishop, 2014). Therefore, stress-induced hyperthermia has been considered as the response to boost their performance in flight-or-fight situations. The hyperthermia is known to be induced by activation of the sympathetic nerve system (Nakamura, 2015). The activation of the sympathetic nervous system induces an increase in heat production and a decrease in heat dissipation via vasoconstriction, and results in an increase in the core body temperature. Recently, it has been reported that the central master neural pathway that regulates the thermogenic, hyperthermic, and cardiovascular sympathetic responses also mediates avoidance behaviour from the psychological stress (Kataoka *et al.*, 2020). Stress-induced hyperthermia is observed not only humans and rodents, but also in cattle (Lees *et al.*, 2020) and sheep (Cook, 1997). Thus, SIH is a common response in mammals that seems to facilitate coping with stress. Taken together, variations in the core body temperature such as these induced by under-nutrition or exposed to psychological stressors can be recognized as an indicator of animal health and mental status.

1.2.5 Hormones affect the control of core body temperature

1.2.5.1 Gonadal steroid hormone

The body temperature of mammals changes predictably across the oestrous cycle in humans, cattle and sheep (Wrenn *et al.*, 1958; Cagnacci *et al.*, 1997; Suthar *et al.*, 2011; Pinto-Santini and Ungerfeld, 2019) Sex hormones, produced by the gonads in response to stimulation via the hypothalamic-pituitary-gonadal axis (Mori *et al.*, 1987), are well known to influence body temperature in rodents, sheep and cattle, especially in females (Suthar *et al.*, 2011, 2012; Pinto-Santini and Ungerfeld, 2019).

Usually, estrogen tends to promote heat dissipation and progesterone tends to promote heat conservation. 17β-oestradiol (E2) produced by the ovary enhances vasodilation by activating endothelial nitric oxide synthesis (eNOS) which produces nitric oxide, a potent vasodilator (Kim *et al.*, 2008). In addition, central estrogen has an effect that decrease the

threshold temperature that triggers the onset of thermoregulatory heat dissipation responses (Hessemer and Bruck, 1985; Baker *et al.*, 1994). In post-menopausal women, the administration of exogenous hormone replacement therapies that contain estrogens has been associated with a lower body temperature and a lower threshold for the onset of cutaneous vasodilation and sweating during body heating (Brooks *et al.*, 1997).

In contrast to the vasodilator effects of E2, progesterone promotes vasoconstriction of the peripheral microvessels (Wenner et al., 2011). Progesterone also sets the thresholds for thermo effectors higher. During the luteal phase of the menstrual cycle, when progesterone levels are at their highest, the core temperature at which cutaneous vasodilator and sweating responses are initiated is about 0.5 °C higher compared to the early follicular phase when progesterone levels are low in humans and cattle (Sakatani *et al.*, 2016; Charkoudian *et a*l., 2017; Higaki *et al.*, 2019).

It has been reported that the core body temperature in female C57Bl/6J mice, a strain that is often used in the study of longevity, maintain higher core body temperature than do the males (Sanchez-Alavez *et al.*, 2011). Gonadectomy abolishes the sex difference in body temperature (Sanchez-Alavez *et al.*, 2011). Interestingly, gonadal sex hormones influence the effect of calorie restriction on body temperature (Cintron-colon *et al.*, 2019). Gonadectomized male and female mice were more sensitive to the restriction of energy intake restriction and their core body temperature decreased especially in the resting period than intact animals core body temperature decreased (Cintron-Colon *et al.*, 2019). Thus, accumulating evidence suggests that gonadal steroid hormone affects the regulation of body temperature in relevance with energy homeostasis.

1.2.5.2 Other hormones and neurotransmitters

Several neural pathways are involved in thermoregulation. For example, the dopaminergic pathway has a role in T_c control. The oxytocinergic pathway also has a role, in oxytocin reduces T_c and heart rate (Hicks *et al.*, 2014). The injection of dopamine induces an increase of the tail temperature and decrease of T_c in rats (Cox *et al.*, 1981). However, the two receptors, D1 receptor and D2 receptor have different roles in thermoregulation. The administration of dopamine D1 receptor agonist, SK&F38393, induces hyperthermia and the administration of the dopamine D2 receptor agonist, talipexole, induce hypothermia

(Nagashima *et al.*, 1992). The hypothermic effect mediated by the dopamine D2 receptor is mainly due to the heat production in BAT rather than to increased heat loss through sympathetic nervous system (Ootsuka *et al.*, 2007). The administration of the dopamine D2/D3 receptor agonist inhibit the cold induced hyperthermia (Ootsuka *et al.*, 2007) and the activation of the dopamine D2 receptors reduces BAT thermogenesis during stress-induced hyperthermia (Brizuela *et al.*, 2019). Therefore, the role of the dopaminergic pathway in the thermoregulation is still controversial. The involvement of the serotoninergic pathway in the control of body temperature is also controversial. The serotoninergic pathways are difficult to study because the molecule agonises several receptor subtypes and they can have contrasting effects. The administration of serotonin (5-HT) 1A, 5-HT3, and 5-HT7 receptor agonists results in a decrease in body temperature in mice (Naumenko *et al.*, 2011). In mice with ablation of the 5-HT1A receptor, the i.p. administration of a 5-HT agonist ((2R)-(+)-8-Hydroxy-2-(di-n-propylamine) induces a reduction of body temperature compared to wild types that express the 5-HT1A receptor (Nishitani *et al.*, 2019). Another genetically modified mouse, with almost complete ablation of 5-HT neurons, has higher core body temperature and is more active compared to the wild type (Hodges *et al.*, 2008). Therefore, the serotonergic system, especially the 5-HT1A-mediated system, seems to be involved in the regulation of body temperature and activity of the serotoninergic system leads generally to a lower Tc. On the other hand, several reports suggest facilitative effects of serotonin on thermogenesis. Ootsuka *et al.* (2008) suggest that the selective blockade of 5-HT2A receptors attenuates the temperature increase in brown adipose tissue and the transient cutaneous vasoconstriction that is usually seen in response to restraint stress (Ootsuka *et al.*, 2008), and the emotional stress that is observed in response to an intruder (Sinh and Ootsuka, 2019). The role of the serotoninergic pathway in the control of the core body temperature is still not fully understood.

Another endocrine system that contributes to the modulation of circadian body temperature is the calcitonin receptor-mediated pathway. While homeotherms regulate their core body temperature autonomously, insects such as *Drosophila* are poikilotherms and equilibrates with the temperature of their surroundings. But that does not mean that they cannot sense temperature, and *Drosophila* will seek favourable thermal environments. When *Drosophila* are given access to a range of thermal environments, they chose different

temperature at different times of day. This preferred temperature rhythm is called temperature preference rhythm (TPR). In *Drosophila*, the diuretic hormone 31 receptor (DH31R), a homolog of the calcitonin receptor (Calcr) in mammals, mediates the TPR. In mice, Calcr mediated signalling modulates the midnight trough of T_c in the during the active phase in mice (Goda *et al.*, 2018a; Goda and Hamada, 2019). These regulations of body temperature by DH31R and Calcr are not required for coordinating locomotor activity rhythms. So, the authors concluded that Calcr specifically mediates the T_c trough during night. Another report has suggested the DH31R mediates the control of sleep awake rhythm (Kunst *et al.*, 2014). Thus, although *Drosophila* and mice have completely different thermoregulatory systems, they exhibit a daily body temperature rhythm that is mediated by a conserved molecule, DH31R/Calcr. It has been suggested that calcitonin receptors are involved in heat tolerance not only in rodents and flies, but also in cattle (Cheruiyot *et al.*, 2021).

1.3 Temperament

1.3.1 What is temperament?

The behavioural traits that emerge in response to a challenging environment differ between individuals, and these differences are usually referred to as temperament. Temperament has been defined as “nature controlling the way he behaves, feels and thinks” (Cannon, 1927). In addition, it has been recognized that non-human animals also have negative emotions (fear, frustration) and positive (pleasure) emotions (Darwin, 1872). There have been many attempts to assess animal “temperament” on the basis of our criteria that are applied to humans. It has been proposed that temperament represents the emotion of “fearfulness” and reactivity of animals to a challenge, such as human contact, isolation, or exposure to an intruder (Murphy, 1999). Since it is difficult to assess the “well-being” of animals objectively, it is only recently that the study to assess animal temperament has been under investigation (Boissy *et al.*, 2007).

1.3.2 Assessment of the temperament

Several attempts have been made to assess the temperaments of animals, particularly in cattle. The temperament of cattle is mostly measured by their crush behaviour, flight speed, the escape and/or avoidance behaviours (Burrow, 1997). Many different behavioural tests

have been used to assess the reliability of such measures in sheep (Dodd *et al.*, 2012). The isolation box test (IBT) (Blache and Ferguson, 2005) and arena test are the most frequently used tests in sheep (Beausoleil *et al.*, 2008, 2012). However, these behavioural tests to assess temperament takes at least a minute or two to assess each animal, and so are impractical on a large scale. Further investigation is required to find an easier and rapid method, that will reduce time and labour, to measure an indicator of temperament that could be used for the selection of livestock.

1.3.3 Temperament in the livestock industry

Temperament affects sheep productivity (Blache and Bickell, 2010). Phenotypic selection based on temperament not only affects production efficiency but also animal well-being. Calm sheep have better milk production (Lawstuen *et al.*, 1988; Sutherland and Dowling, 2014), growth rate (Voisinet *et al.*, 1997; Burrow, 1998), and better meat quality (Sutherland and Dowling, 2014) than nervous sheep. In addition, temperament can affect reproductive function (Blache and Bickell, 2010). For example, nervous sheep have a lower ovulation rate than calm sheep (Van Lier *et al.*, 2017). It has been proposed that sheep with lower reaction to one particular stressor, used for their phenotypic selection, can better adapt to other stressors (Blache and Ferguson, 2005). Thus, temperament has benefits not only in farm management but also in the productivity of the animals.

1.3.4 The stress response of sheep with different temperaments

Animals with different temperaments display not only different behavioural reactions to stressors but also different physiological responses. More reactive animals exhibit higher hypothalamic-pituitary-adrenal (HPA) axis reactivity (Koolhaas *et al.*, 1999). The secretion of cortisol is higher in nervous sheep in response to isolation stress and others stressors, and in response to the same stressors nervous sheep also have a larger decrease in luteinising hormone (LH) secretion than calm sheep (Hawken *et al.*, 2013a). Heart rate variability (HRV) assesses the relative parasympathetic/sympathetic input to the cardiac pacemaker, and low HRV is associated with sympathetic activity and a strong response to a stressor. Less reactive cattle maintain a higher HRV when they are milked by an unfamiliar human than do more reactive cattle (Kovács *et al.*, 2015).

1.3.5 SNP and serotoninergic pathway

While it has been known that temperament is heritable in sheep from 1990s (Murphy *et al.*, 1994; Blache and Ferguson, 2005; Wolf *et al.*, 2008; Zambra *et al.*, 2015), the underlying genetic differences have only recently been investigated. Several single nucleotide polymorphisms (SNPs) in two genes that are involved in the dopaminergic system show associations with the calm and nervous phenotype (Qiu *et al.*, 2016a). For example, SNPs in the dopamine receptor (DRD_2) and cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17), are associated with temperament and also with ovulation rate (Qiu *et al.*, 2016a). Recently, two SNPs in the serotoninergic pathway, in the enzyme that produces serotonin and in the 5-HT receptor, have been associated with sheep temperament (Ding *et al.*, 2021). Serotonin is a monoamine neurotransmitter and is well known as a bioactive molecule that gives humans feelings of happiness. It is reported that the SNPs in serotoninergic pathway affect the feeling such as fear and anger. It is also involved in thermoregulatory pathways, as described above. In addition, it has been reported that the single nucleotide polymorphisms (SNPs) located close to the genes related to emotional control affect body weight (Garza-Brenner *et al.*, 2020). Therefore, the selection of reliable gene markers that has a relationship with temperament has its potential in the improvement of production efficiency. In this project, I will examine the impact of the newly identified SNPs in the serotoninergic pathways on ultradian and circadian variations in the body temperature of sheep.

1.4 Endocrine control of reproduction

1.4.1 Hypothalamic-pituitary gonadal axis

Reproduction is one of the fundamental factors of the productivity of livestock. The hypothalamic-pituitary-gonadal axis (HPG axis) is the central endocrine regulator of reproductive function (Schillo, 2009). Pulsatile gonadotrophin-releasing hormone (GnRH) secreted from the hypothalamus stimulates the synthesis and secretion of LH and follicle-stimulating hormone (FSH) from the pituitary. The secretion of LH and FSH stimulate follicular development and the synthesis of gonadal steroid hormones. The secretory pattern of LH and FSH is pulsatile, and LH and FSH pulses are synchronised with pulsatile

GnRH secreted into the hypophyseal portal blood in sheep (Moenter *et al.*, 1992), goats (Tanaka *et al.*, 1997), and the median eminence in rhesus monkeys (Terasawa *et al.*, 1988).

The continuous infusion of GnRH induces the hypophysial downregulation of LH and FSH secretion (Belchetz *et al.*, 1978a). On the other hand, pulsatile GnRH injection re-establishes the normal ovulatory menstrual cycle and ovarian steroidogenesis in rhesus monkeys with hypothalamic lesions (Knobil *et al.*, 1980). Thus, the pulsatility of GnRH is necessary for the maintenance of reproductive function in mammals. For successful follicular development and ovarian function, it is important to maintain LH secretion. Sheep that received either pulsatile or constant infusion of LH exhibit an increase of estrogen and inhibin A secretion (Campbell *et al.*, 2007). In ruminants, the frequency of GnRH pulses depends on the reproductive status. During the follicular phase, the interval between pulsatile secretions of LH and FSH are shorter and accelerate the development of follicles, and during the luteal phase, they are longer (Moenter *et al.*, 1991; Tanaka *et al.*, 1995). Since the pulsatile secretion of LH and FSH is controlled by the frequency of GnRH pulses, the frequency of pulsatile secretion of GnRH is one of the key determinants in follicle development.

1.4.2 The GnRH pulse generator

The brain mechanism regulating the pulsatile GnRH secretion is located in the hypothalamus and it is referred to as the "GnRH pulse generator" (Lincoln *et al.*, 1985; Maeda *et al.*, 1995). In rodents, as the deafferentation doesn't affect the pulsatile LH secretion, it has been suggested that the GnRH pulse generator is in the mediobasal hypothalamus (MBH) (Ohkura *et al.*, 1991). However, the GnRH neurones, which produce GnRH, are not located in the MBH. On the other hand, the deafferentation of the anterior arcuate nucleus (ARC) from the MBH (Blake and Sawyer, 1974) disturbed the pulsatile LH secretion (Ohkura *et al.*, 1991). In addition, transplants of fetal MBH into the third ventricle impaired the disruption of LH by the deafferentation of the anterior ARC (Ohkura *et al.*, 1992). From this accumulated evidence, it was suggested that the GnRH pulse generator is in the MBH including the ARC. In addition, since the transplanted tissue did not contain GnRH neurones, the GnRH pulse generator is not consisted with a group of GnRH neurons.

1.4.3 MUA recording

The multiple-unit activity (MUA) recording system is the method that enables us to record the activity of the GnRH pulse generator in the MBH. This is the method that records the multiple neuronal activities close to the electrode. It has been reported that the transient increase of MUA (MUA volley) recorded by the electrode implanted in close to the ARC is synchronized with pulsatile LH secretion in rhesus monkey (Knobil, 1981), rats (Kawakami *et al.*, 1982), and goats (Mori *et al.*, 1991a). MUA recording enables us to monitor the activity directly, and in real-time. In addition, we can conduct chronic investigations into the activity of the GnRH pulse generator, over much longer periods than is feasible with blood sampling for the measurement and detection of LH pulses without adding any stress on animals. Thus, the MUA recording system is a potent tool to investigate the activity of the GnRH pulse generator.

1.4.4 Kisspeptin neurone

Kisspeptin neurones in the ARC in the hypothalamus are suggested to be the central regulator of the HPG axis. Kisspeptin is the endogenous ligand of a G protein-coupled receptor, GPR54, that was known as an orphan receptor (Ohtaki *et al.*, 2001). It was reported that transgenic mice with functional loss of *Kiss1* (De Tassigny *et al.*, 2007) or *Kiss1r* (Funes *et al.*, 2003) present with hypogonadal hypogonadism. It was then shown that *Kiss1* KO also results in hypogonadal hypogonadism because of a lack of pulse and surge in LH secretion (Uenoyama *et al.*, 2015). In addition, kisspeptin- or kisspeptin gene (*KISS1*)-expressing cells are located in the ARC and the POA in sheep (Estrada *et al.*, 2006) and goats (Matsuyama *et al.*, 2011) and contact with GnRH neurones. Further, central or peripheral administration of kisspeptin stimulates LH secretion in rodents, ruminants, and humans (Seminara *et al.*, 2004; Messager *et al.*, 2005; Ohkura *et al.*, 2009). Thus, it has been suggested that kisspeptin is the central regulator of the reproductive function not only in rodents but also in domestic animals (Okamura *et al.*, 2013). Recently, it was reported that the conditional KO of *kiss1* in rats completely suppresses pulsatile LH secretion (Nagae *et al.*, 2021). Therefore, the population of kisspeptin neurons located in the ARC is the most probable candidate for the GnRH pulse generator.

Neurokinin B (NKB) and dynorphin A (Dyn) are co-expressed in kisspeptin neurons and these three neuropeptide form a neural network in the ARC region in sheep (Goodman *et al.*, 2007), mice (Navarro *et al.*, 2009a), goats (Wakabayashi *et al.*, 2010), and cattle (Hassaneen *et al.*, 2016). The neurons in the ARC that contain these three neuropeptides are abbreviated as KNDy neurons. These three neuropeptides are strongly implicated in the feedback regulation of GnRH neurons. While NKB accelerates the activity of the GnRH pulse generator and pulsatile secretion, Dyn suppresses it. The proposed mechanism is that NKB initiates and Dyn terminates the GnRH pulses (Lehman *et al.*, 2010).

1.4.5 Kisspeptin as the integrator of the homeostatic cues

The activity of the GnRH pulse generator is affected by various external factors that are also known to affect fertility. The pulsatile secretion of LH is suppressed by low energy availability (Nagatani *et al.*, 1996) or psychosocial stress (Pierce *et al.*, 2008) in rodents. With respect to energy balance, in ovariectomised (OVX) goats given low doses of estrogen the interval between MUA volleys increases gradually during fasting, and returns slowly to the pre-fasting level after subsequent refeeding (Ichimaru *et al.*, 2001). Such a disorder of the pulsatile secretion of LH and the activity of the GnRH pulse generator represented by the MUA volley interval are one of the causes of reproductive dysfunction. Like in rodents, glucose could be the metabolic signal that might modulate the activity of the GnRH pulse generator in ruminants (Ohkura *et al.*, 2004a). Interestingly, the effect of fasting on the pulsatile LH secretion has a correlation with the energy status represented by the body mass index (BMI) of goats (Tanaka *et al.*, 2002), indicating that the activity of the GnRH pulse generator, and the reproductive function has a strong relationship with individual energy balance.

As well as these energy homeostatic cues, the kisspeptin neuron is suggested to integrate the animal's condition and modulate the activity of HPG axis, and indeed, reproductive ability. Since the downregulation of the activity of the GnRH pulse generator results in infertility, understanding the mechanisms that regulate the activity of the GnRH pulse generator could contribute to the optimization of reproductive function.

1.4.6 Relationship with energy metabolism and core body temperature

Recently, some reports have suggested the involvement of kisspeptin neurons in body temperature control, activity, and energy expenditure (Harter *et al.*, 2018a). In addition to the well-established role in stimulating the reproductive axis, the kisspeptin system also has an important role in body weight, energy balance, and glucose regulation. Kisspeptin receptor gene, *Kiss1r*, knockout mice display dramatically higher body weight compared to wild type (Tolson *et al.*, 2014). *Kiss1r* KO mice expend less energy than wild-type animals and have higher body weight and fat mass even though they have lower food intake (Tolson *et al.*, 2016). As kisspeptin is expressed quite widely outside the brain, especially in metabolic tissues such as the pancreas, liver, and fat, it has been unclear which kisspeptin population has a role to control energy expenditure and body weight (Harter *et al.*, 2018b). However, surprisingly the brown adipose tissue (BAT) conditional *kiss1r* KO results in a decrease of the body weight and higher energy expenditure and body temperature (Tolson *et al.*, 2020). On the other hand, the ARC conditional *kiss1* KO mice has an increase in body weight and decreased locomotor activity and body temperature, especially in the circadian amplitude of body temperature (Padilla *et al.*, 2019). Interestingly these animals also have disrupted rhythms of activity and sleep / wake cycling (Padilla *et al.*, 2019). Thus, the nature of kisspeptin regulation of metabolism is complex, but it must play integrative role connecting reproductive function and energy expenditure, and core body temperature.

1.4.7 The possible brain circuit that is involved in both temperature control and the HPG axis

Amylin-calcitonin receptor (CTR) signalling is a possible candidature that is involved in both core body temperature control and the regulation of the activity of the GnRH pulse generator. Amylin is a peptide hormone and known to have a role to control food intake because it is co-secreted with insulin from pancreatic β-cells (Hay *et al.*, 2015). Amylin is also produced and widely distributed in the brain (Li *et al.*, 2015) and is suggested to have a role in metabolism, memory, and maternal behaviour (Young, 2005). Regarding the control of core body temperature, central amylin administration elicits an increase in sympathetic nerve activity and increased core body temperature (Fernandes-Santos *et al.*, 2013). The increase in core body temperature is suggested to be mediated by proopiomelanocortin (POMC) neurones located in the ARC (Coester *et al.*, 2020). The amylin receptor, AMY, is

composed of the CTR and one of the receptor activity modifying proteins (RAMP) (Morfis *et al.*, 2008). Recently, it was reported that the CTR is expressed in kisspeptin neurons in rats (Assadullah *et al.*, 2018), suggesting that amylin-CTR signalling is also involved in the regulation of the activity of the GnRH pulse generator.

1.5 Purpose of this dissertation

In this dissertation, I aim to assess the hypothesis that variation in the core body temperature can be used as a new biomarker of the fitness and reproductive performance of animals, especially in ruminants. The first aim of this thesis is to understand the effect that genotype and phenotype have on the thermal response to psychological stressors. For the first aim, I used sheep since previous reports have described the specific genotypes that are related to the temperament in sheep. I hope to better understand temperament and its effect on physiological responses. The adequacy of the SIH and CRT as biomarkers of psychological stress was assessed in sheep. The second aim of this thesis was to explore the effect of environmental stress that livestock can experience in daily management on the parameters of the CRT. The comprehensive analysis combining several stressors (energy intake, movement restriction, and ambient condition) was conducted in goats. The third aim of this thesis was to test the validity of the amplitude of the CRT as a marker of reproductive ability, especially in the context of the endocrine control of reproduction. The relationship of CRT profiles to LH secretion was investigated in goats. The fourth aim of this thesis was to explore the possible neural circuit that is involved in energy homeostasis, reproduction, and temperament.

Through this series of chapters, I discuss core body temperature as a biomarker that integrates the animals' status especially energy homeostasis and their productive abilities.

Chapter 2: Stress-induced hyperthermia in sheep with temperaments determined by genotype or phenotype

2.1 Introduction

The temperament of an individual affects the way that individual perceives stressors (Corr and Matthews, 2020) and the behavioural and physiological responses that are associated with exposure to stressors (Romeyer and Bouissou, 1992). Animals with calm temperament are less reactive and express smaller behavioural reactions to a stressor than do animals with nervous temperament (Beausoleil *et al.*, 2012). The level of behavioural reactivity has been often used to define the calmness or nervousness of an individual (Dodd *et al.*, 2012). It is still under investigation if the "calm" or "nervous" phenotype of an animal is because of differences in the perception of or the reactivity to stressors, or both (Dodd *et al.*, 2012).

While behavioural responses have traditionally been used to define animal temperament, physiological responses also differ between individuals with calm and nervous temperament. Sheep selected behaviourally for calm (less reactive) or nervous (more reactive) temperament exhibit different neuroendocrine responses when they are both exposed to the same stressor (Hawken *et al.*, 2013b). More reactive animals exhibit higher hypothalamic-pituitary-adrenal (HPA) axis reactivity and sympathetic reactivity and lower parasympathetic reactivity (Koolhaas *et al.*, 1999). Nervous sheep also had a greater increase in cortisol and bigger decrease in luteinizing hormone secretion in response to a stressor than did calm sheep (Hawken *et al.*, 2013b).

While a number of physiological responses differ between animals with different temperament, little is known about the impact of temperament on the response of core body temperature (T_c) to stressors in sheep. To our knowledge, only one study has reported that the mean daily T_c of nervous sheep decreased more in response to an energy challenge than did that of calm sheep (Henry *et al.*, 2010).

Investigating the impact of temperament on T_c could be interesting because T_c can be affected by exposure to short-term or long-term stressors (Oka, 2018). First, stress-induced hyperthermia (SIH), a rapid and transient elevation of the T_c, is a thermal response that animals express as a response to psychological stress (Oka, 2015). The SIH induced by a stressful situation has been observed in humans and rodents (Nakamura, 2015; Oka, 2015) in response to various types of stressors, such as social defeat and restraint stress (Nakamura, 2015; Watanabe, 2015). It is considered that SIH prepares and boosts physiological performance to fight-or-flight situations (Bishop, 2003). However, the impact of temperament on SIH has never been investigated. In addition to transient changes of T_c during SIH, the pattern of the circadian rhythm of the core body temperature (CRT) has been reported to be affected by physiological challenges (Maloney *et al.*, 2013). Recently, it has been postulated that variations in the pattern of the CRT, mainly in the amplitude of the CRT, could be an indicator of how animals cope with an exogenous challenge (Maloney *et al.*, 2019). Since temperament modulates the biological response of an individual to a challenge, it might reasonably be expected that temperament would affect the pattern of CRT and its response to both acute and chronic psychological stressors.

The temperament of individual animals ,such as sheep, has often been assessed using phenotypic measurements of the level of behavioural reactivity to standardised stressors (Beausoleil *et al.*, 2012). Recently, specific single nucleotide polymorphisms (SNPs) have been associated with different temperament phenotypes. In human and other animals, SNPs in the serotonin, dopamine, and oxytocin pathways have been associated with temperament phenotype (Brown *et al.*, 2005; Qiu *et al.*, 2016b). Lately, SNPs in the tryptophan 5-hydroxylase (TPH2) gene have been shown to be associated with behavioural phenotype in sheep (Ding *et al.*, 2021). The TPH2 is the gene codes for an enzyme in the synthesis of serotonin and affects the level of serotonin in the brain (Liu *et al.*, 2008). Interestingly, the serotonin pathway is also involved in the control of T_c homeostasis (Ray *et al.*, 2011). The administration of antagonists to the 5-HT1A, 5-HT3, and 5-HT7 receptors led to a decrease in body temperature in mice (Naumenko *et al.*, 2011). Mice with genetic ablation of the 5-HT1A receptor have a smaller decrease in T_c when they are injected with selective agonists of the 5-HT1A receptor (Nishitani *et al.*, 2019). Similarly, genetically modified mice with almost complete ablation of 5-HT neurons had higher T_c and activity

than did the wildtypes (Hodges *et al.*, 2008). In addition to the control of T_c, the serotonin pathway is involved in the response of T_c to stressors, such as in the generation and modulation of SIH (Vinkers *et al.*, 2010). Knock out of the serotonin transporter (SERT) attenuates the SIH response, while blockade of the 5-HT1A receptor increases the SIH response (Olivier *et al.*, 2008). The chronic administration of fluoxetine, a serotonin reuptake inhibitor, attenuates the increase in T_c in response to a stressor (Conley and Hutson, 2007). On the other hand, it has been reported that selective blockade of 5-HT2A receptors attenuates the activation of heat production in brown adipose tissue and the transient cutaneous vasoconstriction that occurs in response to restriction stress (Ootsuka *et al.*, 2008) and the emotional stress to an intruder (Sinh and Ootsuka, 2019). Thus, the serotonergic pathway seems to have a role in the thermogenic response to various stressors. Although the role of the serotonergic pathway in the control of T_c is still not fully understood, it could be involved in the effect of temperament on the body temperature response to challenges since the serotoninergic system is common to both temperament expression and the regulation of T_c.

In this study, I hypothesized that the temperament phenotype of sheep will affect the profile of the T_c response to psychological stressors. I hypothesize that an SNP in the serotoninergic pathway will affect T_c, in terms of both SIH and CRT, response to psychological stressors. To test these hypotheses, I selected animals based on an SNP in TPH2 and investigated the reaction of the animals to two kinds of behavioural tests and to herding by a dog. In addition to the groups selected on genotype, I tested the impact of temperament phenotype, measured using standardized behavioural tests, on the response of the T_c to the stressors described above.

2.2 Materials and methods

2.2.1 Experimental design

Sheep were selected from a large commercial flock based on two variants of an SNP located on the TPH2 gene that has been associated with divergent behavioural responses to social isolation (Ding *et al.*, 2021). The behavioural phenotype of the sheep was measured using an arena test and an isolation box test (IBT) during two sessions that were conducted two weeks apart (Session 1 [S1] and Session 2 [S2];Figure 2.2). After the first session, the sheep

were allowed to walk spontaneously from the site of testing back to their home paddock via gates that were left open. After the second session, a dog was used to move the sheep, via the same route, from the site of testing back to their home paddock. The dog was controlled by a shepherd and the pace of movement was kept as close as possible to the spontaneous movement. The T_c of the sheep was measured every 5 minutes using intra-abdominal loggers that had been implanted four weeks before to the start of the first testing session (details below). The profile of T_c was analysed to detect SIH on the days of behavioural testing, and also the pattern of the CRT at specific times during the study.

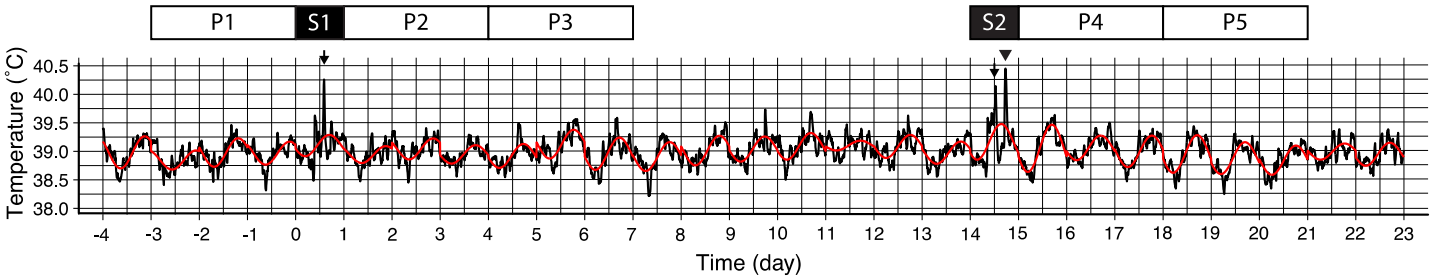


Figure 2.1： Experimental design and representative profile of Tc of one sheep for the duration of the experiment, the plot show the original record of 5-min recordings of Tc (black line), and associated fitted cosinor for each 24 hours (red line). The days when behavioural phenotype was assessed are shown as the black numberd boxes above the plot. The stress induced hypertehrmia (SIH) that was associated with each stressors are indicated by arrows (behavioral test) and triangle (dog exposure). The periods when the characteristic of the CRT were analysed are indicated by open boxes above the trace (P1: three days before S1, P2; three days after S1, P3; three days after P2, P4; three days after S2, P5; three days after P4)

2.2.2 Animals

Three-year-old castrated male sheep were sourced from a commercial flock at the UWA Ridgefield farm (Pingelly, Western Australia). The sheep had not been selected for emotional reactivity and had never experienced the behavioural testing that is described below. All procedures used in this experiment were approved by the Animal Ethics Committee of The University of Western Australia (RA/3/100/1691).

2.2.3 Genotyping to identify the SNPs

From each of the 160 sheep in a commercial flock, a sample of blood was collected via a jugular vein into a vacutainer collection tube with EDTA (Greiner Bio-One, Kremsmunster, Australia). Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden,

Germany) following manufacturer instructions. The genotype of a temperament-related gene (*TPH2*) in each sheep was determined by Sanger sequencing and an Agena Bioscience Mass ARRAY, as described in Ding et al. (2021).

2.2.4 Measurement of core body temperature

The temperature loggers (Bryn O Morgan Industries, Perth, WA) were covered in inert wax (Sasol EXP987, Johannesburg, South Africa). The temperature loggers were equipped with a 3D accelerometer and recorded activity every five minutes by adding the average activity of each of the three axes together. The resolution of these loggers was 0.02°C. The loggers were calibrated against a certified precision platinum thermometer (Center 376, Center Technology Corp., Taipai, Taiwan; certified by the National Association of Testing Authorities, Rhodes, NSW, Australia) between 33°C and 42°C before and after their time in the animals. The loggers recorded T_c every five minutes to an accuracy of better than 0.05°C. The loggers were sterilized by immersion in a solution consist with 0.5 % chlorhexidine (Chlorhex C, Jurox, Rutherford, NSW) and 70 % ethanol for at least 24 h before implantation.

Each sheep was anesthetized by intramuscular injection of a mixture of ketamine (6.3 mg kg^{-1}), xylazine (0.63 mg kg^{-1}) and butorphanol (0.03 mg kg^{-1}). An incision of approximately 10 cm in length was made about 10 cm behind the last rib. The overlying muscle layers were dissected down to the peritoneal wall, and small incision was made, and the datalogger was inserted. A single silk suture was used to anchor the datalogger to the peritoneal wall. The wall and muscle layers were sutured using Vicryl (an absorbable, synthetic, usually braided suture; Ethicon, Raritan, NJ, USA), and then the skin was sutured with absorbable suture (Medtronic, Minneapolis, MN, USA). The animals received a post-operative course of analgesic (4 mg kg^{-1} Carprofen) medication and were given two weeks to recover. The loggers were retrieved from the abdominal cavity at the time of slaughter.

2.2.5 Exposure to challenging events

After the recovery period, the temperament of each sheep was tested twice within 14 days during two sessions of behavioural tests. The same two behavioural tests were used in each session: an arena test and an IBT, as previously described by Beausoleil et al. (2008). During

the arena test, the sheep are placed in a situation of conflict between their desire to be close to their flock mates who are located behind a human, and their reticence to approach the human to be close to their flock mates. If they do not approach the human, they remain isolated (Blache and Bickell, 2010). The arena was divided into four sectors by lines drawn on the floor. Each sheep remained in the arena for three minutes and the behavioural response of each sheep was quantified by the number of bleats (bleats) and the number of sector crosses (crosses) made in that three minutes. The sheep was slowly move toward the next test in a corridor and wait for about one minute. A subjective score on a scale of 0 to 5 (wait) was given to the behaviour of the sheep was recorded with “Zero” describing a sheep not moving during the waiting time and “five” allocated to sheep that jumped the corridor wall (Murphy, 1999). About one minute after the completion of the arena test, each sheep was placed in the isolation box (IBT, H: 1.5 m × L: 1.5 m × W: 0.75 m) for one minute. During the IBT, an agitation metre on the side of the box recorded vibration from the box, caused by movement and vocalisations. The metre was calibrated prior to each use with an electric “sheep” (27 kg) that produced three standardized levels of movement.

2.2.6 Exposure to a dog

Each of the tests was conducted in an enclosed shed and the sheep were brought to the shed at least two hours before the start of testing. At the end of the first session, the gates on the route back to the home paddock of the sheep were left open, and the sheep walked spontaneously back to their home paddock that was located 300 m away from the testing shed. After the second session, the sheep were herded to their home paddock by two dogs and a farmhand. The pace of movement was kept as close as possible to the spontaneous movement after the first testing session.

2.2.7 Grouping of sheep

The experimental data were analysed with the sheep split into two groups, but the two groups were classified in one of two ways, one genotypic and the other phenotypic. The genotype of a single SNP in the TPH2 gene was determined, and two groups of nine sheep were created, one with genotype A/A (homozygous) and one with genotype G/G (homozygous). The A/A and G/G genotypes have been associated with calm and nervous phenotype, respectively (Ding *et al.*, 2021). In A/A genotype, the G > A transition is

identified in both zygotes. Heterozygous A/G or G/A sheep were not used since it has not been reported to be associated with any type of temperament.

A second set of two groups was created based on the behavioural phenotype of each sheep. The agitation value from the IBT was normalised for live weight (LW) by dividing the raw IBT score by the live weight and then multiplied by the weight of the device that was used to calibrate the isolation box (27 kg). The phenotype was defined by the normalized IBT score (IBT/LW) obtained during the IBT during the first session. The nine sheep with the lowest normalized IBT score were classified as “low responders” and the nine sheep with the highest normalized IBT scores were classified as “high responders”.

2.2.8 Climate data

Hourly ambient air temperature and relative humidity were obtained from an official weather station of the Bureau of Meteorology (BoM) located 4.8 km from Ridgefield farm, Pingelly West, Western Australia.

2.2.9 Data analysis

Four of the intra-abdominal loggers, two in AA sheep and two in GG sheep, stopped recording before the start of the behavioural testing sessions. Therefore, the effect of genotype on SIH or patterns of CRT in response to the various psychological stressors was analysed using seven AA and seven GG sheep. Similarly, seven high responders and seven low responders were used to analyse the effect of behavioural phenotype on SIH or patterns of CRT in response to a psychological stressor.

2.2.9.1 Characteristics of the circadian rhythms of core body temperature

The characteristics of the CRT were calculated using a cosinor analysis on 24 hours of data starting at 12 AM each day (Nelson *et al.*, 1979; Maloney *et al.*, 2019). The analysis provided a mesor, amplitude, cosinor minimum, cosinor maximum and the time of the acrophase for each sheep on each day. I compared the characteristics of the CRT between groups by taking an average for each sheep over three days on five occasions; the three days prior to the first behavioural test day (Period 1 ; P1), the three days immediately after the first behavioural test day (Period 2; P2), the three days immediately after that (Period 3 ; P3), the three days immediately after the second behavioural test day (Period 4 ; P4), and finally the

three days after that (Period 5 ; P5). An example of the profile of T_c, fitted cosinor, and the timing of the behavioural testing sessions and the five periods is given in Figure 1.

2.2.9.2 Stress induced hyperthermia

Since it is necessary to distinguish the SIH from the endogenous increase of the Tc (e.g., ultradian rhythms of Tc), we need to have the objective criteria for SIH. The T_c response to specific stressors was calculated in two ways; once by considering only changes in T_c that exceeded, by at least 2SD, the expected T_c for a given time of day, and secondly as the changes in T_c whether or not they differed significantly from the "normal" T_c. For the first method a smoothed value (Ts) was calculated at each timepoint as a running average of 12 h around that time point (6 h prior and 6 h after) during and after the period when the specific stress was given to the animals. For the animals that presented a significant SIH as defined above, the area under the curve (AUC in °C x min) of the T_c response was calculated by summing the difference between T_c and Ts for the period when T_c was 2.0 SD higher than that the baseline Ts. The sum was multiplied by five since T_c was measured every five minutes. The amplitude of the SIH was defined as the maximum value of the difference between T_c and Ts during the period of SIH as defined above. The duration of SIH was defined as the period during which the T_c was higher than Ts.

In the second instance, for all animals, the area under the curve (AUC in °C x min) of the T_c response to specific stressors was calculated by summing the difference between T_c and Ts for 50 minutes after the time that the behavioural test started or after the time point the sheep started moving after the behavioural test finished since the average of the duration of an SIH was about 50 minutes when the sheep were moved with the presence of a dog. The values were multiplied by five since T_c was measured every 5 minutes. In this case, the amplitude was defined as the maximum value of the difference of T_c and Ts in the period used for the calculation of the AUC of SIH.

2.2.9.3 Activity

A relative activity score was calculated for each sheep as the proportion of the maximum activity and minimum activity that was recorded for each intra-ruminal logger during the entire experiment. The total activity score was calculated by summing the relative activity

score for 50 minutes after the time that the behaviour test session stared or after the time point the sheep started moving after the behavioural test finished.

2.2.10 Statistical analyses

A Shapiro-Wilk test and a Bartlett's test were used to assess normality and homogeneity of the data.

The number of sheep presenting a SIH in each genotype or phenotype was compared using Chi-square. The effect of the temperament-related SNP and of the behavioural phenotype on the behavioural and physiological measurements was analysed using Multivariate General Linear Model analysis. The interaction between genotype × session was initially included in the model but was excluded in the final analysis because it was not significant. The difference between groups was analysed with Student's t-Test.

Differences in the characteristics of the CRT over time were analysed using linear mixed-effects models followed by pairwise comparison tests adjusted for multiple comparisons using the Bonferroni method. The effect of genotype and genotype × period interaction was initially included in the model, and then excluded in the final analysis because they were not significant.

The effect of genotype or phenotype on the AUC, amplitude, and duration of the SIH, was analysed using Multivariate General Linear Model analysis. The differences were analysed by pairwise comparison tests adjusted for multiple comparisons using the Bonferroni method.

The pattern of activity over the recording period was analysed using linear mixed-effects models followed by pairwise comparison tests adjusted for multiple comparisons using the Bonferroni method. The effect of genotype and group × treatment interaction was initially included in the model, and then excluded in the final analysis because they were not significant.

The characteristics of ambient temperature in the different periods were analysed using Multivariate General Linear Model analysis. The differences were analysed by pairwise comparison tests adjusted for multiple comparisons using the Bonferroni method.

2.3 Results

2.3.1 PART 1: The effect of TPH2 genotype

From the 160 sheep tested, nine sheep carried genotype (AA) that is associated with calm temperament and 97 sheep carried genotype (GG) that is associated with nervous temperament. Fifty-four sheep carried genotype (AG/GA).

2.3.1.1 Behavioural responses

While there was no significant effect of genotype on the behavioural response to the arena test during either session 1 or session 2 (Table 2.1), the responses to the IBT differed during session 1. The raw IBT score and the IBT/LW were higher in the sheep carrying genotype (GG) than the sheep carrying genotype (AA) during Session 1 ($p = 0.025$), but not during session 2 ($p = 0.355$; Table 2.1).

Table 2.1: Response to behavioural challenges in genotype (AA) and genotype (GG)

First Session	Overall response			Genotype (AA)		Genotype (GG)		AA *vs* GG
	Mean	Max	Min	Mean	SEM	Mean	SEM	Probability
Bleats	0.19	4.00	0.00	0.44	0.44	0.00	0.00	0.347
Crosses	7.42	29.00	2.00	7.78	2.74	8.11	3.89	0.914
Wait	1.88	3.00	1.00	2.44	0.24	1.78	0.22	*0.060*
IBT	39.31	96.00	5.00	25.33	4.91	47.22	6.07	**0.013**
IBT/LW	15.99	40.50	1.63	0.39	0.08	0.69	0.09	**0.025**
Second Session								
Bleats	0.08	1.00	0.00	0.11	0.11	0.11	0.33	1.00
Crosses	5.46	12.00	0.00	4.11	1.11	6.33	3.12	0.164
Wait	2.23	4.00	0.00	3.11	0.35	2.00	1.20	*0.053*
IBT	44.69	100.0	0.00	35.11	6.72	50.22	30.45	0.235
IBT/LW	18.35	46.96	0.00	0.57	0.11	0.76	7.28	0.355

2.3.1.2 Stress induced hyperthermia

The proportion of sheep presenting an SIH that exceeded the 2.0 SD threshold was similar between the sheep that carried genotype AA and the sheep that carried genotype GG during both sessions. One of the genotype AA sheep and none of the genotype GG sheep presented with an SIH when the sheep moved spontaneously back to their home paddock. In contrast, all of the sheep, regardless of their genotype, presented an SIH when the sheep were moved back to the same paddock in the presence of a dog. The effect of genotype on the number of sheep presenting an increase greater than 2 SD was analysed only for session 1 and the presence of dogs, because too few displayed an SIH during session 2 and during spontaneous movement back to their paddock. However, there was no effect of genotype on the AUC, the amplitude, or the duration of SIH (Table 2.2, Figure 2.2)

When all of the animals were included in the analysis, regardless of whether they met the 2 SD criterion, there was still no effect of genotype on the AUC, amplitude, or duration of any peak of T_c within 50 min of the end of the test in either session. During the spontaneous walk back to the paddock, the AUC and the amplitude of any peak of T_c was higher when the dog was present than when the sheep moved spontaneously ($p<0.0001$, Figure 2.3)

Table 2.2: Number of animals exhibit significant SIH

	Genotype AA	Genotype GG	p	High responder	Low responder	p
1st session	6	7	NA	5	5	1.0
2nd session	2	4	0.28	2	2	1.0
Absence of dog	1	0	NA	1	0	NA
Presence of dog	7	7	NA	7	7	NA

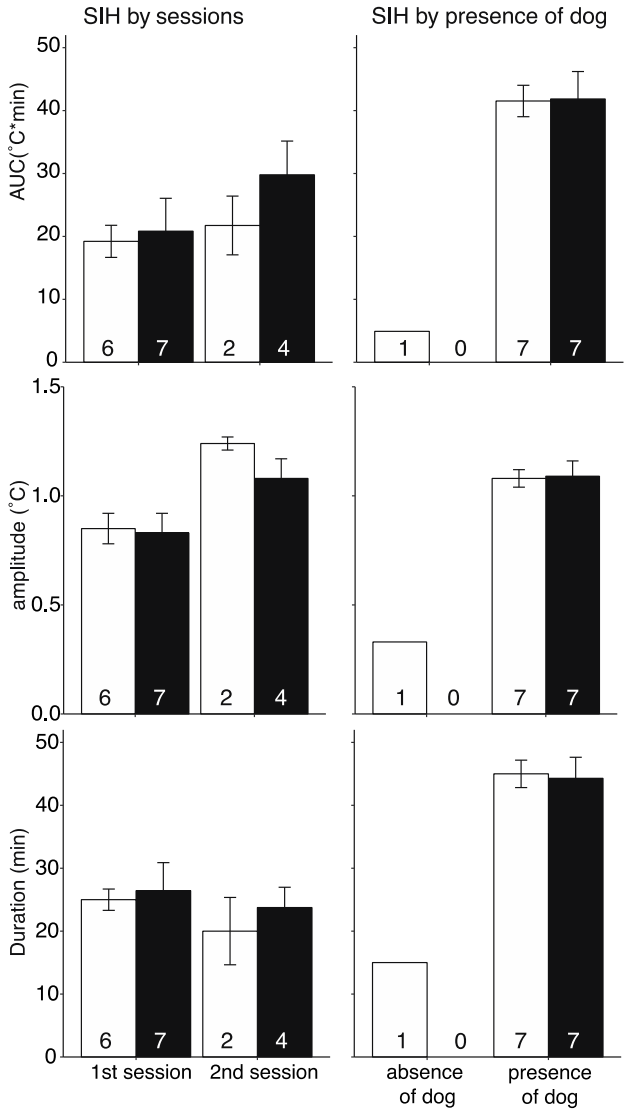


Figure 2.2: Thermal response of those sheep that exhibited a significant increase in T_c in response to stimuli, with groups defined genotypically. Area under the curve (AUC), amplitude, and duration of the stress induced hyperthermia (mean ± SE) in response to behavioural testing during the two sessions and in response to the presence or absence of a dog in sheep carrying genotype AA (white) and sheep carrying genotype GG (black). A SIH response was considered only if increase in T_c was greater than or equal to twice the value of the standard deviation from the baseline (see Methods for details).

When all of the animals were included in the analysis, regardless of whether they met the 2 SD criterion, there was still no effect of genotype on the AUC, amplitude, or duration of any peak of T_c within 50 min of the end of the test in either session. During the spontaneous walk back to the paddock, the AUC and the amplitude of any peak of T_c was higher when the dog was present than when the sheep moved spontaneously ($p<0.0001$; Figure 2.3).

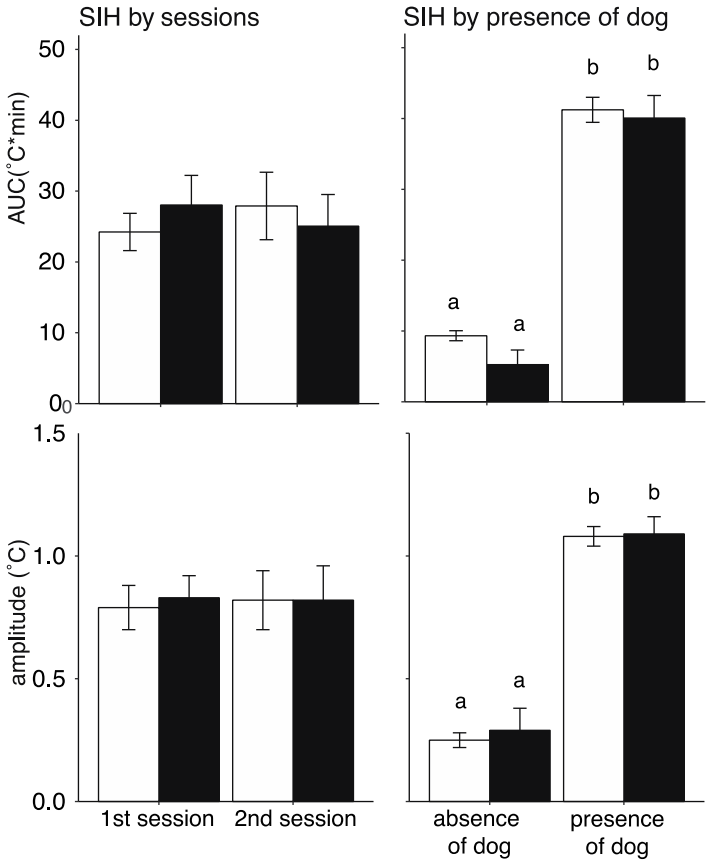


Figure 2.3: Thermal response of all sheep to stimuli, with groups defined genotypically. Area under the curve (AUC) and amplitude of the stress induced hyperthermia (n = 7, mean ± SE) in response to behavioural testing during two sessions and in response to the presence or absence of dogs in sheep carrying genotype AA (white) sheep carrying genotype GG (black).

2.3.1.3 Response of the CRT to specific stressors

There was no significant difference between genotypes (Figure 2.4). in any of the parameters of the CRT. The amplitude of the CRT in period 4 (P4) was higher compared to period 1 (P1) ($p<0.01$). The cosinor maximum of the CRT in P4 was higher than during P1. Mesor, and cosinor minimum were not different between P1 and any of the other periods.

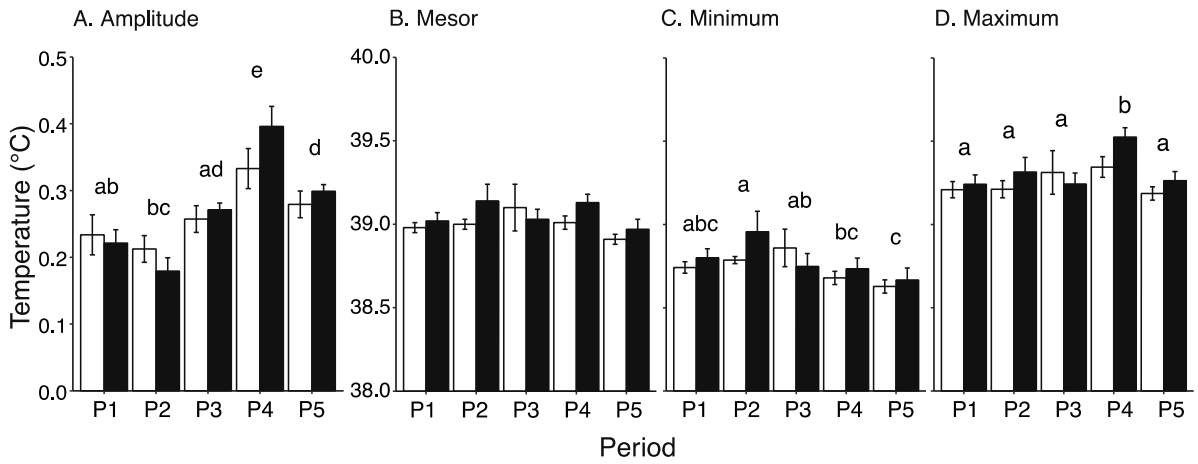


Figure 2.4: The characteristics of circadian rhythm of body temperature in sheep carrying genotype AA (white) and sheep carrying genotype GG (black) (mean ± SE, n=7). amplitude (A), mesor (B), cosinor minimum (C), cosinor maximum (D) over the five periods of three days prior to the first behavioural test day (Period 1 ; P1), the three days of immediately after the first behavioural test day (Period 2; P2), the three days immediately after that (Period 3 ; P3), the three days immediately after the second behavioural test day (Period 4 ; P4), and finally the three days after that (Period 5; P5). Different superscripts within a graph indicates statistical different at $p<0.05$).

2.3.2 PART 2: The effect of phenotype for emotional reactivity

2.3.2.1 Behavioural responses

The wait score was lower in high responders than low responders in session 2 ($p < 0.001$) but not in session 1(Table 2.3). The IBT score and IBT/LW were higher in the high responder than low responders in both Session 1 ($p < 0.001$) and Session 2($p < 0.05$) (Table 2.3).

Table 2.3: Response to behavioural challenges in low and high responder sheep

First Session	Overall response			Low responders		High responders		Low *vs* High
	Mean	Max	Min	Mean	SEM	Mean	SEM	Probability
Bleats	0.19	4.00	0.00	0.00	0.00	0.56	0.44	0.247
Crosses	7.22	29.00	2.00	5.33	1.15	9.56	2.56	0.160
Wait	1.85	3.00	1.00	2.11	0.31	1.56	0.18	0.143
IBT	38.22	96.00	5.00	20.00	3.42	59.89	6.48	**<0.001**
IBT/LW	14.57	36.00	2.03	7.82	1.26	22.61	1.87	**<0.001**
Second Session								
Bleats	0.07	1.00	0.00	0.00	0.00	0.22	0.15	0.169
Crosses	5.70	12.00	0.00	5.67	1.33	6.67	1.09	0.570
Wait	2.28	4.00	0.00	3.33	0.26	1.50	0.29	**<0.001**
IBT	44.04	100.00	0.00	29.89	5.77	56.22	8.73	**0.025**
IBT/LW	17.40	46.96	0.00	11.19	2.69	22.83	4.28	**0.048**

2.3.2.2 Stress induced hyperthermia

The proportion of sheep presenting an SIH above 2.0 SD was similar between the high responders and the low responders during both testing sessions (Table 2.1, Figure 2.5) . One low responder developed an SIH when the sheep moved spontaneously back to their home paddock. No other animal developed SIH that exceeded 2SD. All of the sheep developed an SIH when the sheep were moved back to the home paddock by dogs. There was no effect of phenotype on the AUC, amplitude, or duration of SIH that was induced by behavioural testing during either session 1 or session 2 (Figure 2.5). In the presence of a dog, the AUC and amplitude of SIH were higher in the high responders than in the low responders ($p<0.05$) (Figure 2.5).

When all the animals were included in the analysis, neither the AUC or the amplitude of SIH were affected by the phenotype after either Session 1 or Session 2. In the presence of the

dogs, the AUC was significantly higher in high responders than in the low responders ($p < 0.05$) (Figure 2.6). For both phenotypes, the AUC and amplitude of SIH were higher in the presence of the dogs than when they moved spontaneously back to their paddock ($p<0.0001$) (Figure 2.6).

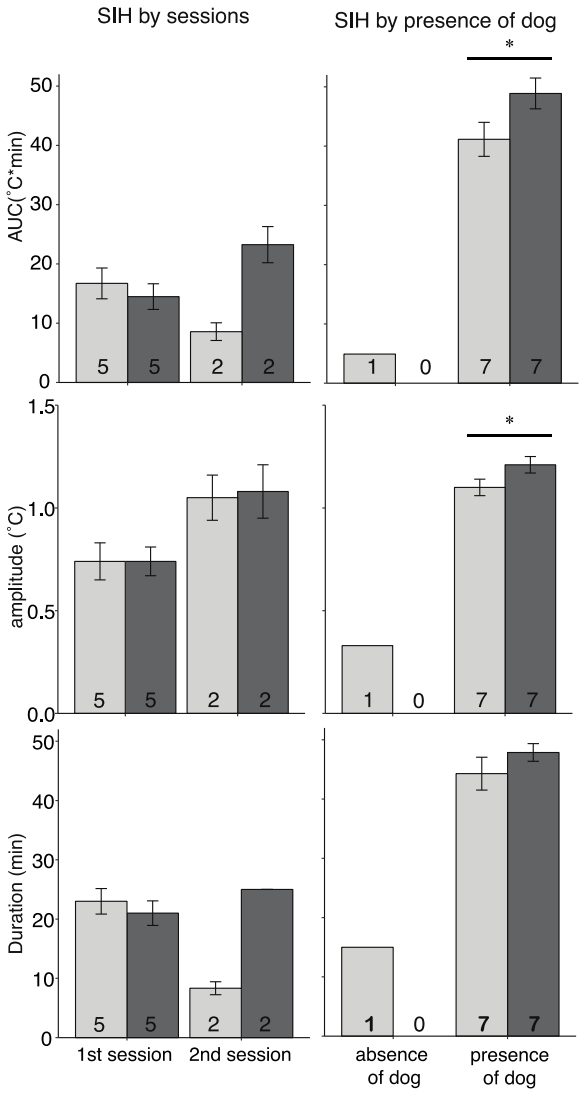


Figure 2.5: Thermal response of those sheep that exhibited a significant increase in T_c in response to stimuli, with groups defined phenotypically.

Area under the curve (AUC), amplitude, and duration of the stress induced hyperthermia (mean ± SE) in the response to behavioural testing during the two sessions and in response to the presence or absence of a dog in sheep classified as low responders (light grey) and high responders (dark grey). A SIH response was considered only if degree of the increase in T_c was greater than or equal to twice the value of the standard deviation from the baseline (see Methods for details). * indicates $p<0.01$ in the student t test of low responders and high responders in moved by dog.

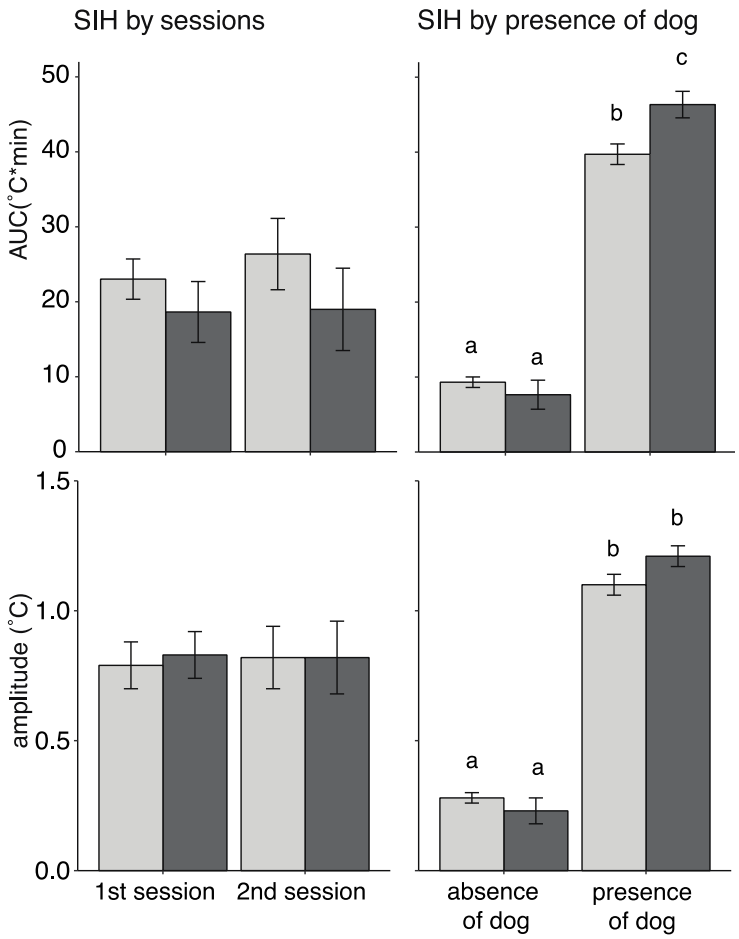


Figure 2.6: Thermal response of all sheep to stimuli, with groups defined phenotypically.

Area under the curve (AUC) and amplitude of the stress induced hyperthermia (n = 7, mean ± SE) in response to behavioural testing during two sessions and in response to the presence or absence of dogs in sheep classified as low responders (light grey) or high responders (dark grey). Different superscripts within a graph indicates statistical different at $p<0.05$).

2.3.2.3 Response of the CRT to specific stressors

There was no significant difference between the high responders and low responders in any of the parameters of the CRT (Figure 2.7). The amplitude of the CRT in period 4 (P4) was higher compared to period 1 (P1) ($p<0.01$). The cosinor maximum of the CRT in P4 was higher than during P1. The mesor, and cosinor minimum was not different between P1 and any of the other periods.

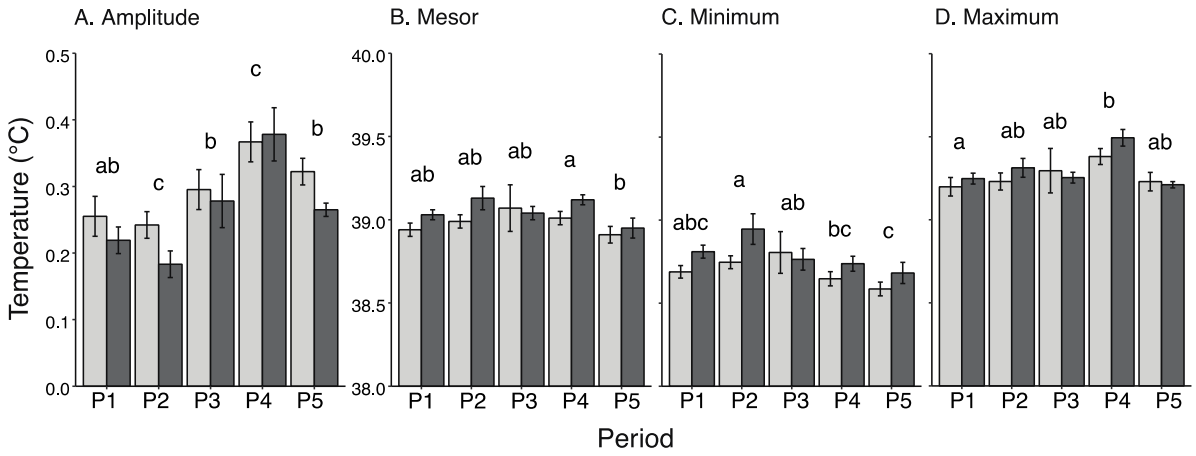


Figure 2.7: The characteristics of circadian rhythm of body temperature in sheep of low responders (light grey) and high responders (dark grey) (mean ± SE, n=7). Amplitude (A), mesor (B), cosinor minimum (C), cosinor maximum (D) over the five periods of three days prior to the first behavioural test day (Period 1; P1), the three days of immediately after the first behavioural test day (Period 2; P2), the three days immediately after that (Period 3 ; P3), the three days immediately after the second behavioural test day (Period 4 ; P4), and finally the three days after that (Period 5 ; P5). Different superscripts within a graph indicates statistical different at $p<0.05$).

2.3.3 Activity data

There was no significant difference between genotype or phenotype in the activity of sheep during the walk to return to the home paddock (Figure 2.8 B). When the sheep moved to their home paddock (whether spontaneously or in the presence of dogs) their activity was about double that recorded during the behavioural testing sessions. The activity of the sheep was higher in the presence of the dogs compared to when they moved spontaneously back to their home paddock (Figure 2.8 B). In addition, the AUC of SIH were larger in both sessions than in the movement with absence of dog in all groups (Figure 2.8 A).

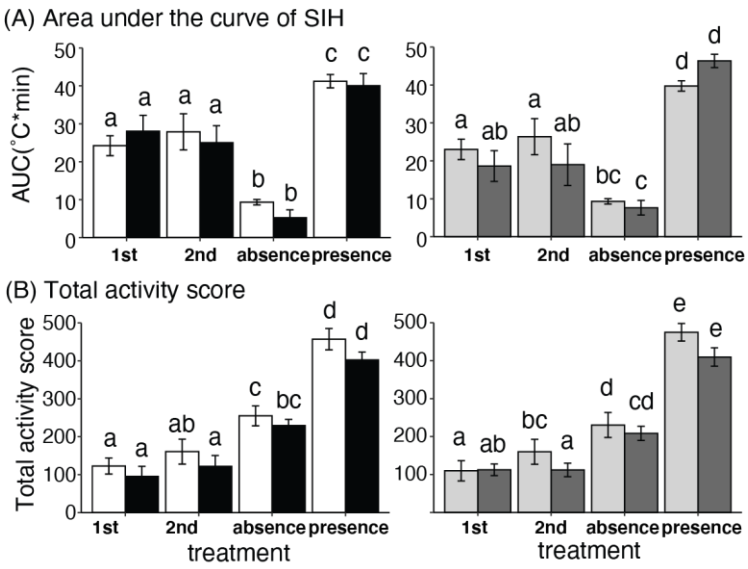


Figure 2.8: (A) The area under the curve of the SIH and (B) the activity score of the sheep carrying genotype AA (white bar) and the sheep carrying genotype GG (black bar) and the sheep of low responder phenotype (grey bar) and of high responder phenotype (dark grey bar) during 1st session (1st), 2nd session (2nd), walk absence of dog (absence), and walk with presence of dog (presence). Different subscripts indicate difference between treatments ($p < 0.05$).

2.3.4 Climate data

The climate conditions did not differ between the periods (Table 2.4).

Table 2.4: Characteristics of the circadian rhythms of ambient temperature during the 5 different periods of the experiment

Period	Mesor	Amplitude	Cosinor maximum	Cosinor minimum
P1	11.78 ± 0.25	1.70 ± 0.59	15.07 ± 0.55	9.61 ± 0.37
P2	10.77 ± 0.80	4.54 ± 0.26	15.60 ±1.26	5.18 ±1.03
P3	10.04 ± 0.19	2.35 ± 0.68	13.58 ± 0.35	6.28 ± 0.94
P4	12.20 ± 1.49	6.15 ± 1.49	20.61 ± 3.02	5.12 ± 0.93
P5	10.10 ± 0.95	5.42 ± 2.09	17.51 ± 2.19	3.70 ± 1.80

2.4 Discussion

In the present study, I demonstrate that emotional reactivity affects the response of T_c to specific stressors. I investigated the effect of a genotype that has been reported to have a relationship with temperament in sheep, as well as the relevance of phenotype as assessed by behavioural test on the T_c response to psychological stressors. I found that the phenotype had relevance to the size of the SIH produced in response to stressors. Our result supports that SIH would be a useful biomarker of psychological stress and the individual perceptivity of stressors in animals. In addition, the amplitude of the CRT was increased for a few days after session 2. The amplitude of the CRT has the potential to be an indicator of psychological stress. Further analyses would be required to elucidate which brain circuits is involved in the different in SIH between high responders and low responders to IBT, and the mechanism that leads to changes in the CRT.

2.4.1 SIH is good biomarker of psychological stress

It seems that SIH can reflect emotional reactivity of individual sheep. In the present study, sheep that responded strongly in the IBT test (high responders) exhibited the strong SIH in response to the dog exposure. The thermogenic pathway in high responders seems to be activated more by psychological stressors than it is in the low responders. On the other hand, the SNP in TPH2 didn't affect the SIH response, even though it affected the IBT store. This is not surprising, because the central pathway that is involved in the determination of emotional reactivity and the activation of SIH involves more than the serotoninergic pathway alone. For example, SNPs in the dopamine receptor (DRD_2) and cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17), are associated with temperament (Qiu *et al.*, 2016b). In fact, the dopaminergic pathway has been suggested to be involved in the modulation of stress induced hyperthermia (Brizuela *et al.*, 2019; Antipov *et al.*, 2020). Also, the oxytocinergic pathway has been associated with the temperament in sheep (Ding *et al.*, 2021) and oxytocin null mice showed larger SIH compared to wildtype in response to psychological stressors (Amico *et al.*, 2004). Further investigations are required to elucidate the relationship between the genotype related to temperament and the thermogenic response on the stress. It might be suggested that brain pathways involved in the perception of the stress outside of the serotoninergic system contribute to differentiate the

SIH response between high responders and low responders. Although further studies are required to elucidate the potential pathways involved in the differentiation of the physiological response, SIH can be considered as a reliable biomarker of psychological stress and animal's emotional reactivity.

2.4.2 The SIH was not only due to exercise, but also to the activation of the sympathetic nervous system in response to psychological stress

In the present study, significant SIH was detected only during psychological challenge, such as IBT and dog exposure, and doesn't seem to be explained by the higher activity of the sheep during the walk. While I observed an increase in activity during the spontaneous walk back to their home paddock, no SIH was detected. In addition, some sheep exhibited a stress induced hyperthermia during the behavioural test session even though the level of activity was lower than during that spontaneous walk. In addition, the phenotype had no effect on activity, but did on the SIH when the sheep were walked by a dog.

The thermogenic pathway that was activated during the SIH in the present study could involve either a decrease in heat dissipation or active heat-generation. At least in rodents, the generation of SIH is controlled by the sympathetic nervous (Nakamura, 2015). An increase in T_c can be the result of a decrease in heat dissipation or an increase in heat generation, or a combination of the two. In rodents, brown adipose tissue thermogenesis and skin vasoconstriction (Umriukhin *et al.*, 2002; Lkhagvasuren *et al.*, 2011) contribute to the increase the T_c. The resting heat production of sheep is 1.6 W kg^{-1} (Hales and Brown, 1974), and the energy cost to walk horizontally is 2.6 kJ kg^{-1} km^{-1} (Graham, 1964). The heat capacity of animal tissue is approximately 3.5 kJ kg^{-1} $°C^{-1}$ (Giering *et al.*, 1995). In the case of a 50 kg sheep that took 30 minutes for T_c to increase by 1°C (this is close to the average of amplitude and duration of SIH when the sheep were moved by dogs), as the total heat capacity of the body is 3.5 * 50 = 175 kJ, the sheep stored 175 kJ in 30 minutes. The sheep also expended 39 kJ to walk 300 m. If they stored all of the metabolic heat and didn't lose any heat to the environment (an impossibility, but used for sake of these calculations), they could store 144 kJ, theoretically. Thus, the sheep spent more than resting metabolic heat to increase the T_c in response to the dogs. Thus, the animals had to increase heat generation in addition to a reduction in the heat dissipation via vasoconstriction. In adult ruminants, there are no reports of substantial existence of brown adipose tissue, but the expression of

uncoupling protein 1 (UCP1) in adipose tissue has been reported in sheep (Henry *et al.*, 2010) as well as the thermogenic capacity of adipose tissue (Henry *et al.*, 2008). It is highly possible that the sympathetic nervous cue that was induced by the psychological stress activated UCP1 in adipose tissue and contributed to the thermogenesis and development of SIH.

2.4.3 SIH is a sensitive and discriminative marker of the stress

In addition to the presence or absence of a significant SIH, SIH also seems to be a sensitive and discriminative marker of the response to stress. The degree of a behavioural reaction seems to be highly dependent on the kind and strength of the stressor (Beausoleil *et al.*, 2008). In the present study, the size (in terms of both AUC and amplitude) of SIH was different between the types of stressors. The exposure to the dog induced larger T_c increase compared to the isolation box. In addition, while some of sheep, especially during test session two, didn't present any significant SIH in the IBT test, all of sheep expressed SIH when they were exposed to dogs. One explanation for that set of result is that a "new" experience is more affective to animals. It is generally considered that SIH is a response that enhances the activity in fight-or-flight situations (Kuwaki and Zhang, 2012). The exposure to a dog might be more potent as a stressor for sheep as the dog is a potential predator that will induce stressor like the situation of fight-or-flight, while the isolation and restriction of the IBT imposes a different type of stress. It has been reported that expose to a dog induced a greater increase in plasma cortisol concentration than did isolation and restrain in ewes (Ralph and Tilbrook, 2016). Our results show that the dog exposure is a stronger stressor for wethers than the IBT test, since more of the sheep exhibited a significant SIH after dog exposure that after isolation. Thus, it is highly possible that the AUC of SIH can reflect the strength of psychological stress that is perceived by a sheep.

There are two well established biomarkers of psychological stress; an increase in the concentration of cortisol in biological media and changes in the characteristics of cardiac activity as measured by electrocardiography (ECG). Concentrations of cortisol in blood or saliva indicate the activity of the hypothalamic-pituitary-adrenal (HPA) axis in response to a stressor (Mormède *et al.*, 2007). The amplitude and the duration of the increase in the concentration of plasma cortisol have been thought to reflect the perceived intensity of the

stressor, with for example the increase in cortisol level lasting minutes after the stressor has gone (Cook, 2004). In the present study, the duration of SIH lasted about 45 minutes (Figure 2 and 5) mirroring the duration of the increase of plasma cortisol previously observed in response to a transient exposure to a dog (Cook, 2002). While the shape of both the SIH and cortisol response might reflect the experience of the situation by the animal, SIH has the advantage to be accessed more rapidly and easily than cortisol. Measuring levels of cortisol in blood or saliva requires sampling and restraint of the animals and the measurement of cortisol in those samples take at least a day. SIH can be measured in real time with some already available apparatus, and could be a close to instantaneous markers of stress in animal once an algorithm is developed to analyse the signal generated by temperature probes (Blache and Maloney, 2017a).

While some characteristics of the ECG can be obtained in real-time, SIH might have some advantages over the measurement of the ECG. Heart rate variability is the variance in time between the beats of the heart, as recorded by the ECG, and has been considered as a biomarker of stress (Thayer *et al.*, 2012). It is a good biomarker of stress because it reflects the relative input of sympathetic to parasympathetic control of the cardiac pacemaker via the autonomic nervous system in response to stressors, and it can be deployed as a non-invasive technique (von Borell *et al.*, 2007). However, the method needs very specific equipment and continuous recording at a very high frequency (1,000 Hz, von Borell *et al.*, 2007). The detection of changes in HRV over long periods requires more storage capacity and a greater source of power than any device that measures core temperature every 1 or 5 minutes. It should be mentioned that, like the other two established biomarkers, the T_c would have some limitations. The T_c has a basal variation in terms of both the CRT as well as ultradian rhythms (Blessing and Ootsuka, 2016; Goh *et al.*, 2019). These limitations are also shared by the other biomarkers of stress, for example as cortisol is secreted in pulses (Rietema *et al.*, 2015), and HRV presents both circadian and ultradian rhythms (Stein *et al.*, 2006). These basal variations make it difficult to discriminate an increase of these biomarkers in response to a stressor from a spontaneous increase. Even though there are a few limitations, the measurement of SIH could be a useful biomarker that reflects the strength of psychological stress that an animal perceives, and the method is relatively easy to apply.

2.4.4 The amplitude of the CRT can be used as another marker of stress

In addition to SIH, the amplitude of the CRT could be an indicator of psychological stress and might be as integrative as SIH but more indicative of a long-term response to a challenging situation. I observed changes in the amplitude of the CRT that lasted for several days after the sheep were exposed to a stressor (For example: Period 4, Figure 2.4 and 2.7). The mechanism by which the amplitude of the CRT was affected over such a long period of time is unknown. The stressful challenge could have affected the amplitude of the CRT either by acting directly on the mechanism that controls the pattern of the CRT or by modifying the energy balance of the sheep. The latter has even shown to modify the amplitude of the CRT. It should be noted that the increase in the amplitude of the CRT was not associated with a change in any parameter of ambient temperature. Therefore, the increase in the amplitude of the CRT that was observed in Period 4 in the present study might have been induced by the strong stress from dog that the sheep experienced on the day of session 2.

2.4.4.1 First possibility: stress affect the amplitude of CRT as itself

In the present study, after the behavioural test and exposure to a dog (Session 2, Period 4), the amplitude of the CRT was higher compared to the period before the sessions (Period 1). It has been shown that the amplitude of CRT changes for a few days in response to different challenges to homeostasis. For example, a decreased energy intake increases the amplitude of the CRT (Maloney *et al.*, 2013; Goh *et al.*, 2016). Changes in the amplitude of CRT are observed within a day after the modification of energy intake, suggesting, like in our study, that a change of amplitude is sensitive, immediate, and integrative. Similarly, water balance (McCarron *et al.*, 2001; Samara *et al.*, 2012), and reproductive status (Coyne *et al.*, 2000; Webster and Smarr, 2020) can affect the amplitude of the CRT. The amplitude of the CRT, like SIH, was not affected by mild challenges such as the behaviour test only. The sensitivity of the amplitude of the CRT as a biomarker of stress will need to be tested using physiological and psychological challenges that are of different relevance to sheep.

2.4.4.2 Second possibility: An indirect effect of psychological stress on the amplitude of the CRT, via negative energy balance

It is possible that a state of the negative energy balance was induced by the stress that then to the increase in the amplitude of the CRT. A state of negative energy balance induces an

increase in the amplitude of the CRT (Maloney *et al.*, 2013; Goh *et al.*, 2016). An emotional stress response costs energy, including an increase in the T_c (Xu *et al.*, 2012). However, to our knowledge, the cost of temperament has not been directly quantified, although studies that have manipulated the feed intake level have suggested that sheep with different temperament might have a different energy requirements (Amdi *et al.*, 2010; Hawken *et al.*, 2012). In this study, since I didn't measure the food intake during the experimental period, it is difficult to assess the actual energy balance in sheep. However, from the previous reports, it is still controversial about the food efficiency and the effect of stressors on food intake in sheep with contrasting temperament phenotype (Amdi *et al.*, 2010). Further study is required to understand the relationship between stress response and energy efficiency. In addition, the existence of an association between stress and energy balance is supported by an overlap between the neuroendocrine mechanisms that control stress response, these that are involved in energy balance, and those that are involved in thermoregulation. For example, Neuropeptide Y (NPY) expressed in the arcuate nucleus (ARC), acts as an orexigenic peptide (Miner *et al.*, 1989), and is also involved in the control of T_c, and metabolic control during malnutrition (Walker and Romsos, 1993; Bouali *et al.*, 1995a; Nakamura *et al.*, 2017b). High levels of NPY diminish the stress response (Heilig *et al.*, 1989) and acute stress increases the expression of NPY in the ARC of rats (Conrad and McEwen, 2000). It is possible that the stress response may affect the amplitude of the CRT via a change in energy balance, or activation of mechanisms that are common to the control of energy balance, the stress response, and thermoregulation.

The mechanism that controls the amplitude of the CRT remains unknown (Maloney *et al.*, 2019). It must be noted that, in the present study, the sheep were exposed to a short psychological challenge (behavioural test and walk back to the paddock with dog), while in the studies that have manipulated level of energy, water, or even reproductive activity, the challenges were applied or present during the response in amplitude of CRT (Maloney *et al.*, 2019). The possibility that psychological stress has a long-term impact (lasting few days) on the amplitude of CRT raises further questions on the mechanism that is involved in the relationship between psychological stress and the CRT and the control of the amplitude of CRT.

2.4.5 Limitations of the study

In the present study, the number of animals possibly limited the power to detect the impact of genotype on the SIH and CRT. In a previous report, Ding *et al.* (2021) described that the proportion of sheep with the calm allele (genotype AA in TPH2 rs10787156) in the commercial flock was about 4 % (Ding *et al.*, 2021). By testing 160 sheep from the commercial flock, I expected to find about six sheep that carried genotype AA. I found that nine of the 160 sheep that I tested from the commercial flock carried genotype AA, about 5 % of the flock. Even though I lost two animals for technical reasons, that left seven animals in each group to be analysed for their T_c data. However, it is possible that larger number of animals would have result in the detection of significant effects of genotype on SIH and the amplitude of the CRT induced by exposure to a stressor.

2.5 Conclusion

In conclusion, I suggest that recording of the T_c can be another good biomarker of animal's emotional reactivity to psychological stressors. It seems that SIH can be a reliable biomarker that can distinguish the strength of the stressors for sheep and it has the relationship with their emotional reactivity. SIH is as sensitive as the other established biomarkers, but more appliable, quick, and precise. The amplitude of the CRT offers a possibility to predict long-term effect of the psychological stress but there is a need for further investigations. In addition, it is possible that SIH is the indicator of the animals' temperament. It seems that the animals with higher emotional reactivity use more energy in response to psychological stressors, and it may induce negative energy compared to animals with lower emotional reactivity. Further investigation is required to elucidate the relationship among energy expenditure, emotional reactivity, and productivity of the animals with different temperament.

Chapter 3: The profile of the circadian rhythm of core body temperature in goats: the effect of ambient temperature, restriction of movement, and modification of energy intake

3.1 Introduction

Farmed livestock face various challenges, such as exposure to extremes of ambient temperature, energy homeostasis, and housing conditions. To improve the welfare of production livestock and their production efficiency, it is important to understand the effect of environmental and other exogenous stressors on the homeostasis of those animals.

In homeotherms including cattle, sheep, and goats, the maintenance of a relatively constant core body temperature (T_c) is an important component of homeostasis. Disturbances to homeostasis, including the dysregulation of T_c, can result in health issues. Large variations of the ambient temperature represent a strong challenge to homeotherms. The thermoneutral zone (TNZ) is the temperature range where normal body temperature is maintained without the animal expending extra energy to warm or cool themselves. Therefore, when an animal is in the TNZ, the energy cost to maintain the homeostasis of T_c is minimal. The exchange of heat between a homeotherm and the environment can be summarised graphically in a heat balance diagram, like that shown in Figure 3.1 (Stanier *et al.*, 1984). The upper criteria temperature (UCT, "D" in Figure 3.1), is the temperature above which a homeotherm needs to increase evaporative heat loss to maintain T_c. On the other hand, when the ambient temperature is below the lower criteria temperature (LCT, "C" in Figure 3.1), a homeotherm needs to increase heat production to maintain their T_c when there is an increase in the non-evaporative heat loss to the environment. Since these thermoregulatory responses require energy expenditure, in extreme hot or cold ambient conditions, homeotherms expend energy to keep their T_c in the homeostatic range. The environmental humidity, as measured as water vapor pressure (kPa) or wet bulb temperature (°C), affects the rate of evaporation of water, and so impacts on the evaporative heat loss (Jessen, 2001). A hot environment is made more stressful if the humidity is also high.

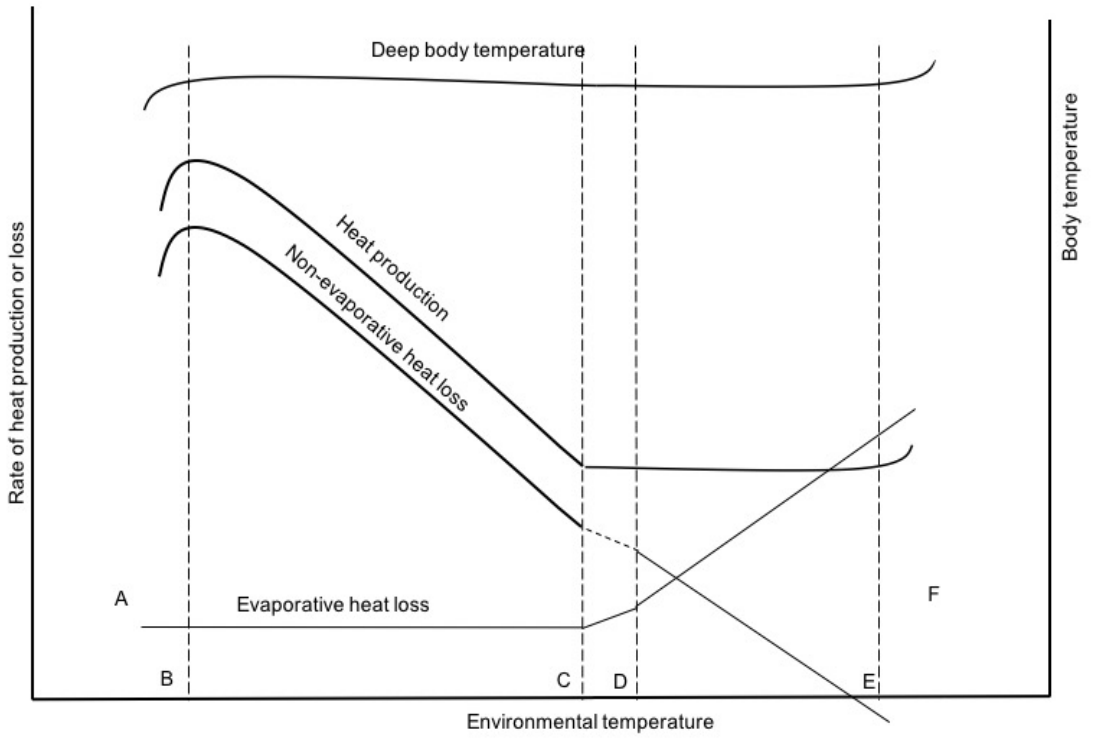


Figure 3.1: Heat balance diagram adapted from Stanier et al. (1984). B; Below the point 'B', the animal will become hypothermic and risks cold injury, C; lower criterial temperature for thermoneutral zone, D; higher criterial temperature for thermoneutral zone, E; above 'E' the animal becomes hyperthermic and is at risk of heat stroke.

While the general level of T_c is homeostatically regulated, it does exhibit an endogenous daily cycle, called the circadian rhythm of core body temperature (CRT). Various kind of exogenous stimuli can affect both the amplitude and the mesor of the CRT (Maloney *et al.*, 2019). While some previous studies have investigated the effect of ambient temperature on T_c control and energy expenditure, there are very few reports that have investigated the relationship between the ambient temperature and the characteristics of the CRT.

Any restriction to energy intake will impact on an animal's productivity and is a challenge to homeostasis. The restriction of energy intake (EI) induces a lower T_c in the inactive phase of the daily activity cycle, and increases the amplitude of the (Piccione *et al.*, 2002, 2003a; Maloney *et al.*, 2013; Goh *et al.*, 2016). Negative energy balance can occur not only in experimental conditions, but also in production systems. For example, cattle reduce their food intake in late pregnancy and early lactation (Ingvartsen *et al.*, 1999). With the combination of the high-energy demands of lactation, a reduction in food intake often induces negative energy balance. In addition, heat stress during the hot and humid season

can affect food intake. Cattle reduce their dry matter intake under heat stress conditions (West *et al.*, 2003). Thus, energy restriction is a stressor that is experienced by livestock and that can be associated with the ambient condition, and T_c.

Other types of stressors are a routine component of some production systems. Various kinds of restraint are still used in some forms of farm management. There is increasing opinion that these tethering systems is not suitable because the system restricts the free movement and social behaviour of the animal, and so compromises the welfare of the animals (Algers *et al.*, 2009; Popescu *et al.*, 2014). Regardless of the increasing criticism, the tie-stall system is still used worldwide for dairy cow management. For example, according to the U.S. Department of Agriculture (2016), 39% of operations for management of the lactating cow use tie-stall or stanchion housing (USDA, 2016). While there are large variations between countries in the use of movement restriction, most farmers tether animals and restrict their movement for at least a part of the day. Arguments for restriction include a reduced risk of lameness, or easier management of some individual animals (Somers *et al.*, 2003; Bouffard *et al.*, 2017). Tethering causes an increase in the level of plasma cortisol in cattle (Odore *et al.*, 2011). The effect of tethering on T_c has not yet been reported.

In this chapter, I examine the T_c response to several relevant challenges to livestock; variation in ambient temperature, restriction of EI, and tethering. I examine the effect of the ambient temperature in two seasons, between the summer solstice and the winter solstice during a period with increasing ambient temperature (summer) and a period with decreasing ambient temperature (autumn). During these two periods, I test the effect of EI restriction and tethering, in combination with the effect of the ambient temperature, on the parameters of the CRT by using a linear mixed effect model.

3.2 Materials and methods

This experiment was approved by the Animal Ethics Committee of The Nagoya University (A210426-001).

3.2.1 Experimental design

Core body temperature was measured in two groups of four goats, as well as ambient temperature during either the hot and humid season in Japan (July to August; referred to as "summer") or during the cold and dry season (October to November; referred to as "autumn"). In each period, each animal was exposed to two stressors; tethering (with two treatments, either tethered or free) and restriction of energy intake (with two treatments, either a maintenance diet (MD) or a calorie restricted diet (CR)). Tethering was conducted by tying a goat to stanchion like poles beside the pen (Figure 3.2). Each goat was exposed to each of the stressors for the same number of days in each period, in a randomized block design where all of the goats were allocated to each treatment combination at least once ('Randomized Block Design', 2008). Thus, each goat was fed both MD and CR while they were tethered and untethered.

Figure 3.2: Goat tethered in stanchion. The goat could sit and stand, and had free access to food and water.

3.2.2 Animals

Adult (4-6 years old) female goats (n = 8; 25 - 35 kg) were sourced from the experimental flock kept at the Togo Field, Nagoya University (Aichi, Japan). The goats were all ovariectomized at least one month before the experiment to remove the influence of gonadal steroids on T_c. They were fed with a mix of dry hay (85 % of maintenance energy requirements) and standard pellets consisting of corn, wheat, and barley (Acemare, Nihonnosan, Yokohama, Japan; 15 % of maintenance energy requirements). The ration was calculated to maintain body weight, using the formula;

$MER\ (kJ\ day^{-1}) = BW^{0,75} * 100\ (kcal\ day^{-1}) * 4.184\ (J\ kg^{-1})$

Where: MER = maintenance energy requirement

BW = body weight (kg)

The animals were weighed at least once a week. The day of the body weight measurement was excluded from the T_c data analyses because of the potential effect of handling on T_c.

3.2.3 Study area and management of animals

During the experiments the goats were housed in individual pens (1.0 × 1.5 m) made of iron pipes on a concrete floor in a shed at the Togo Field, Field Science Center, Graduate School of Bioagricultural Sciences, Nagoya University. The animals were kept in a large shed and not exposed to direct solar radiation, but exposed to the natural light-dark cycle, ambient temperature, and humidity because one side of the shed was open to the environment.

3.2.4 Core body temperature

Each goat was fitted with a temperature logger (Custom made, Bryn Morgan Industries, Perth, Australia) that was covered in inert wax (Sasol EXP987, Johannesburg, South Africa). The resolution of these loggers was 0.02°C. All the temperature sensors and loggers were calibrated against a certified mercury-free thermometer (certified by the Ando Keiki, Rhodes, Tokyo, Japan) between 33°C and 42°C before and after their time in the animals. The loggers recorded T_c every 1 minute to an accuracy of better than 0.05°C. The loggers

were sterilized by immersion in chlorhexidine (Sumitomo Dainippon Pharma, Osaka, Japan) for at least 12 h before implantation.

3.2.5 Surgery

Each goat was anesthetized by intramuscular injection ketamine (1.0 mg kg^{-1}) and xylazine (1.0 mg kg^{-1}) that lasted for the 15-20 minutes that it took to complete the surgical procedure. A logger was inserted into the abdominal cavity via an incision in the right flank and tied with to the skin with nylon thread. After logger implantation, each goat was injected with a long-acting antibiotic (250 mg $head^{-1}$ of dihydrostreptomycin sulfate; Meiji Seika Pharma, Tokyo, Japan). After the experiment, the loggers were retrieved from the abdominal cavity by the same surgery as described above.

3.2.6 Climate data

Climatic data in five minutes interval (air temperature, vapor pressure) were obtained from a climate sensor (ATMOS-41; AINEX, Tokyo, Japan) and data logger (METER; Climatec, Tokyo, Japan) located 150 m meters from the experimental shed and a mini temperature logger (Testo 174H, Testo, Yokohama, Japan) in the shed. To assess the impact of humidity, the wet bulb temperature was calculated using the daily maximum air temperature and the vapor pressure at the time of the daily maximum ambient air temperature.

3.2.7 Data analysis

3.2.7.1 Body weight

To investigate the fluctuation in body weight, I calculate the ratio of the difference between the maximum and minimum body weight during the experimental period. For this analysis, I exclude the 4th and 6th measurement form goat #266 because of technical problems.

3.2.7.2 Temperature data

The characteristics of the CRT (mesor, amplitude, cosinor minimum, cosinor maximum, and acrophase) were calculated using a cosinor analysis on 24 hours of data starting at 12 AM each day (Nelson *et al.*, 1979; Maloney *et al.*, 2019). The relationship between day of experiment and ambient temperature was analysed using a linear regression model within R (R Core Team, 2020).

3.2.8 Statistical analysis

Linear mixed-effect modelling was used to fit a model to examine the relationship between the ambient temperature and the parameters of the CRT (mesor, cosinor minimum, and cosinor maximum). For the analyses of the amplitude of the CRT, I used generalized linear mixed-effect modelling since the distribution of the amplitude of the CRT was not normal. I used R (R Core Team, 2020) and me4 package (Bates *et al.*, 2021) to perform a linear mixed-effects modelling analyses and generalized linear mixed-effects modelling analyses. The "lmerTest" package was used to obtain the p-value of each fixed effect (Kuznetsova *et al.*, 2020). P-values were obtained by a likelihood ratio test of the null hypothesis that the fixed effect had no effect on the parameters of the CRT. The family type used in the generalized linear mixed-effect modelling was a gamma family with link "log" function.

3.2.8.1 Section 1: analysis of the relationship between ambient temperature and core body temperature

For each combination of an ambient parameter and a CRT parameter, all of the models were run to check which model fit the data best. The best model was chosen according to the Akaike information criterion (AIC) value. AIC is a method to select the statistical model with the best fit to the given set of the data (Akaike, 1974). The model that had the lowest AIC value was considered as the best model. When there was no significant difference between models, I chose the simpler model. Four models were built by considering both fixed effect (parameters of ambient temperature, time, tethering, and EI) and random effects (individual difference of intercept and slope for parameter of ambient temperature among animals) (Table 3.1). The result of the model selection is shown in Table 3.2.

For illustrations, I used the *Effects* package and the *ggplot2* package to visualize the relationships between ambient temperature and T_c (Fox *et al.*, 2020; Create *et al.*, 2021). For the calculation of R square, I used *MuMIn* package in R (Barton and Barton, 2020). The effect of ambient temperature on the parameters of the CRT was analysed separately for each season (summer and autumn).

3.2.8.2 Section 2: analysis of the effect of tethering and EI on the profile of the CRT

To maintain the independency of each fixed effect factor, as the cosinor maximum and cosinor minimum are calculated using the mesor and amplitude of the CRT, I compared the

models created by mesor, cosinor maximum, and cosinor minimum (Table 3.3). Several models were built by considering both fixed effects (the mesor of the ambient temperature (ambient_Mesor), the amplitude of the ambient_temperature (ambient_Amplitude), day of treatment (day), tethering, and EI, and random effects (individual differences in the intercept and slope for parameters of ambient temperature between animals (Table 3.3). Model 7 was selected based on the AIC value for further analyses of the effect of tethering and EI. To examine the interaction effect of the tethering and EI, I further compared Models 7 and 10 for each parameter of the CRT (Table 3.4). The selected models are shown in Table 3.5. The data from summer and autumn were combined to examine the effect of tethering and EI on the parameters of the CRT of the eight animals in total. The difference between the treatments of tethering or EI on the amplitude, mesor, cosinor minimum, and cosinor maximum of the CRT was analysed by pairwise comparison tests adjusted for multiple comparisons using the Bonferroni method.

Table 3.1: Parametrisation of the mixed-effect models to examine the relationship between parameters of the CRT and parameters of the ambient temperature

Model 1	CRT_parameter ~ ambient_parameter + time + tethering + EI + (1|animalID)
Model 2	CRT_parameter ~ ambient_parameter + time + tethering + EI + (1+ambient_parameter|animalID)
Model 3	CRT_parameter ~ ambient_parameter + tethering + EI + (1|animalID)
Model 4	CRT_parameter ~ ambient_parameter + tethering + EI + (1 + ambient_parameter|animalID)

Table 3.2: Models selected for each combination of the parameters of the CRT and the parameters of the ambient temperature

CRT parameter	**ambient parameter**	**summer**	**autumn**
amplitude	Amplitude	Model 2	Model 2
	Mesor	Model 3	Model 1
	Cosinor Minimum	Model 1	Model 2
	Cosinor Maximum	Model 4	Model 1
meosr	Amplitude	Model 2	Model 4
	Mesor	Model 2	Model 2
	Cosinor Minimum	Model 2	Model 2
	Cosinor Maximum	Model 2	Model 4
cosinor minimum	Amplitude	Model 2	Model 4
	Mesor	Model 2	Model 2
	Cosinor Minimum	Model 2	Model 2
	Cosinor Maximum	Model 2	Model 3
Cosior maximum	Amplitude	Model 1	Model 1
	Mesor	Model 1	Model 2
	Cosinor Minimum	Model 1	Model 4
	Cosinor Maximum	Model 2	Model 4

Table 3.3: Parametrisation of the mixed-effect models to examine the effect of tethering and EI on the different parameters of the CRT

Model 1: CRT_parameter ~ ambient_Mesor + ambient_Amplitude + tethering + EI + day + (1 | animalID)
Model 2: CRT_parameter ~ ambient_CosMin + ambient_Amplitude + tethering + EI + day + (1 | animalID)
Model 3: CRT_parameter ~ ambient_CosMax + ambient_Amplitude + tethering + EI + day + (1 | animalID)
Model 4: CRT_parameter ~ ambient_Mesor + ambient_Amplitude + tethering + EI + day + (1 + ambient_Mesor | animalID)
Model 5: CRT_parameter ~ ambient_CosMin + ambient_Amplitude + tethering + EI + day + (1 + ambient_Mesor | animalID)
Model 6: CRT_parameter ~ ambient_CosMax + ambient_Amplitude + tethering + EI + day + (1 + ambient_Mesor | animalID)
Model 7: CRT_parameter ~ ambient_Mesor + ambient_Amplitude + tethering + EI + day + (1 + ambient_Mesor + ambient_Amplitude | animalID)
Model 8: CRT_parameter ~ ambient_CosMin + ambient_Amplitude + tethering + EI + day + (1 + ambient_Mesor + ambient_Amplitude | animalID)
Model 9: CRT_parameter ~ ambient_CosMax + ambient_Amplitude + tethering + EI + day + (1 + ambient_Mesor + ambient_Amplitude | animalID)

Table 3.4: Models selected to examine the effect of tethering and EI

Model 7: CRT_parameter ~ ambient_Mesor + ambient_Amplitude + tethering + EI + day + (1 + ambient_Mesor + ambient_Amplitude | animalID)
Model 10: CRT_parameter ~ ambient_Mesor + ambient_Amplitude + tethering * EI + day + (1 + ambient_Mesor + ambient_Amplitude | animalID)

Table 3.5: Models selected to test the effect of tethering and EI on each parameter of the CRT

CRT parameter	**Model selected**
Amplitude	Model 7
Mesor	Model 10
Cosinor Minimum	Model 10
Cosinor Maximum	Model 10

3.3 Results

3.3.1 Body weight

The body weight during the experimental periods (summer and autumn) is shown in Figure 3.3. The maximum and minimum body weight and the ratio of the fluctuation of average body weight is shown in Table 3.6.

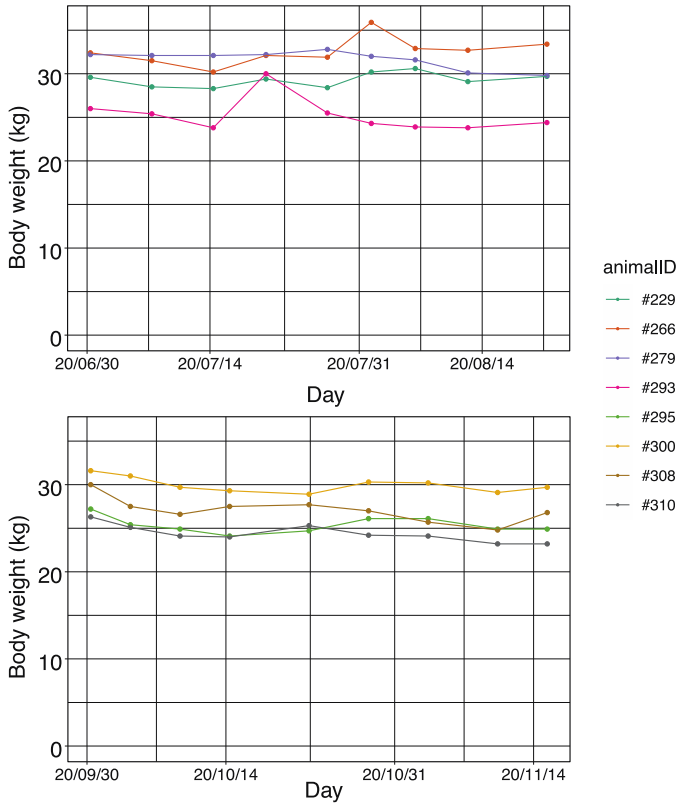


Figure 3.3: Profile of body weight of individual goats during the two experiments: goats #229, #266, #279, and #293 in summer (upper panel), and goats #295, #300, #308, and #310 in autumn (lower panel). The days are notated as YY/MM/DD.

Table 3.6: Profile of body weight for each goat during the study: goats #229, #266, #279, and #293 in summer, and goats #295, #300, #308, and #310 in autumn.

	#229	#266	#279	#293	#295	#300	#308	#310
Maximum (kg)	30.6	33.4	32.8	26.0	27.2	31.6	30.0	26.3
Minimum (kg)	28.3	30.2	29.8	23.8	24.1	28.9	24.8	23.2
Maximum-Minimum (kg)	2.3	3.2	3.0	2.2	3.1	2.7	5.2	3.1
Average (kg)	29.3	32.1	31.7	24.6	25.4	30.0	27.1	24.4
Ratio (%)	**7.8**	10.0	9.5	8.9	12.2	9.0	**19.2**	12.7

3.3.2 Climatic data for the two periods

In summer, all of the parameters of the ambient temperature were positively correlated with the day of experiment (Figure 3.4, Table 3.7). In autumn, the amplitude of ambient temperature was positively correlated with the day of experiment. The other parameters of ambient temperature were negatively correlated with the day of experiment (Figure 3.4, Table 3.7). The frequency of days with each wet bulb temperature (1°C categories) is shown in Figure 3.5.

Table 3.7: Equations depicting the relationships between the day of experiment (x) and the different characteristics of the profile of ambient temperature during the summer experiment (A) and during the autumn experiment (B)

A. Summer	**Equation**	**p-value**
Amplitude	y = 1.21 + 0.072 x	p < 0.001
Mesor	y = 22.3 + 0.166 x	p < 0.001
Minimum	y = 20.5 + 0.104 x	p < 0.001
Maximum	y = 24.9 + 0.239 x	p < 0.001
Cosinor minimum	y = 21.1 + 0.0941 x	p < 0.001
Cosinor maximum	y = 23.5 + 0.238 x	p < 0.001

B. Autumn	**formula**	**p-value**
Amplitude	y = 2.69 + 0.0476 x	p = 0.017
Mesor	y = 20.5 - 0.241 x	p < 0.001
Minimum	y = 18.4 - 0.296 x	p < 0.001
Maximum	y = 23.4 - 0.126 x	p = 0.0012
Cosinor minimum	y = 18.6 -0.283 x	p < 0.001
Cosinor maximum	y = 22.4 - 0.145 x	p < 0.001

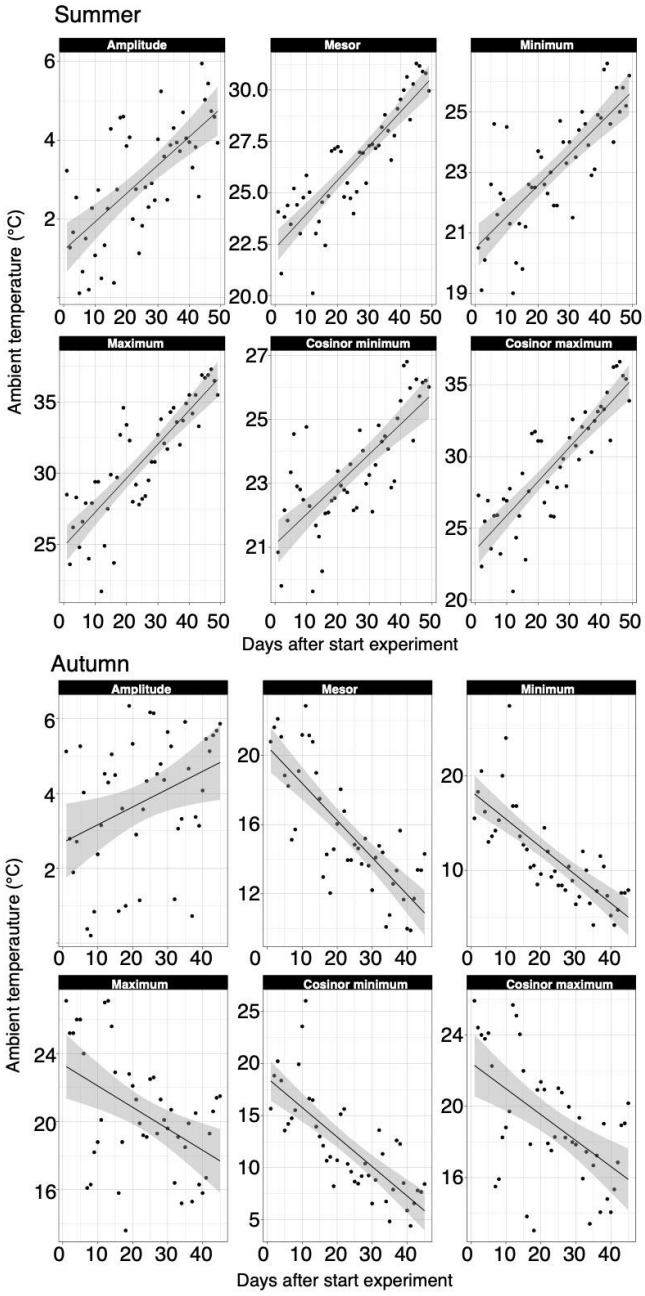


Figure 3.4: The relationship between the number of days after start of experiment and the parameters of ambient temperature (amplitude, mesor, minimum, maximum, cosinor minimum, and cosinor maximum) during the experiment conducted in summer (upper two rows) and the experiment conducted in autumn (lower two rows)

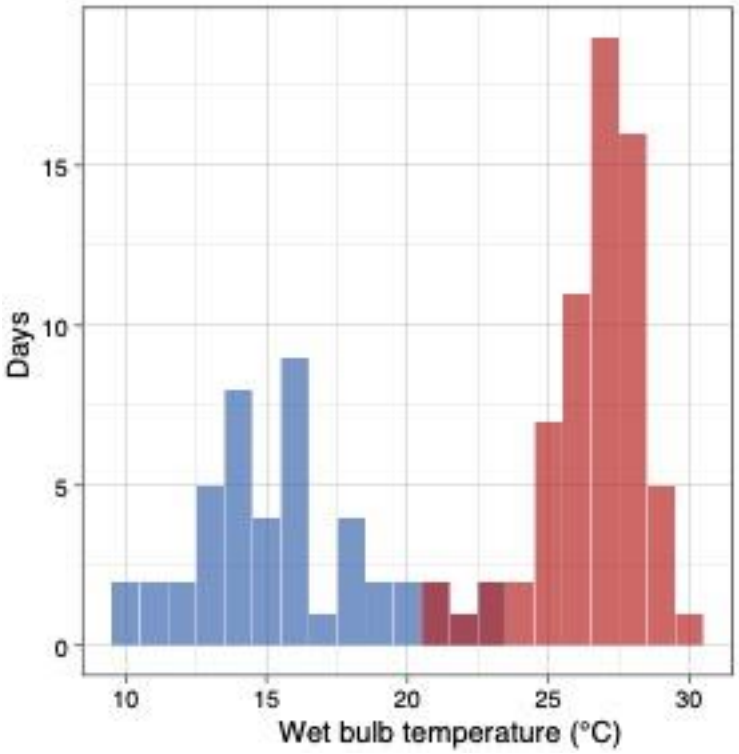


Figure 3.5: Number of days with a given wet bulb temperature in autumn (blue) and in summer (red).

3.3.3 Section 1: the relationship between ambient temperature and core body temperature

The relationships between the parameters of the ambient temperature and the characteristics of the CRT in summer and autumn are shown in Figure 3.6 and Figure 3.7. Figure 3.8 shows two seasons together. The regression lines between the ambient temperature and the CRT for the individual animals are shown in Figure 3.9.

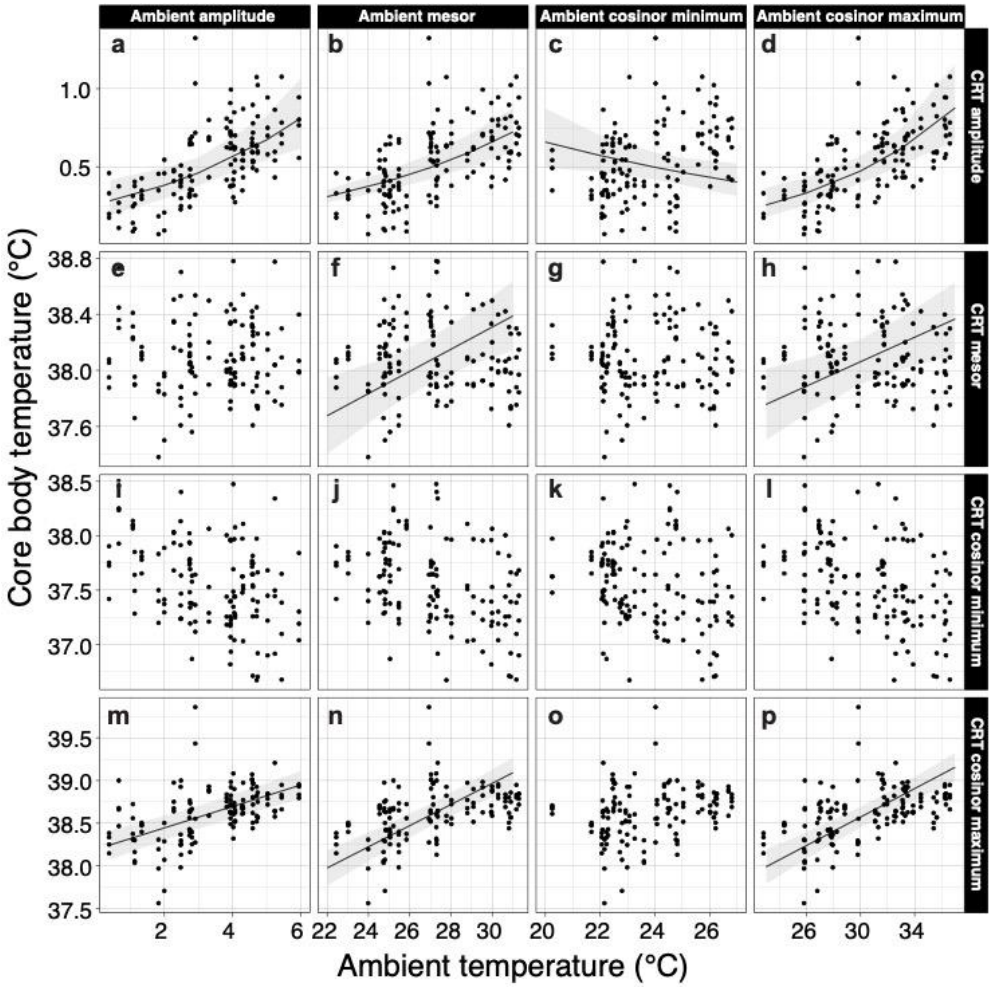


Figure 3.6: The relationship between the characteristics of the CRT (amplitude, mesor, cosinor minimum, and cosinor maximum) and the same characteristics of the ambient temperature rhythm (amplitude, mesor, cosinor minimum, and cosinor maximum) in two groups of goats (n= 4) exposed to natural climatic conditions during the summer period (July to August). The shaded regions correspond to 95% predictive intervals using the estimated parameters.

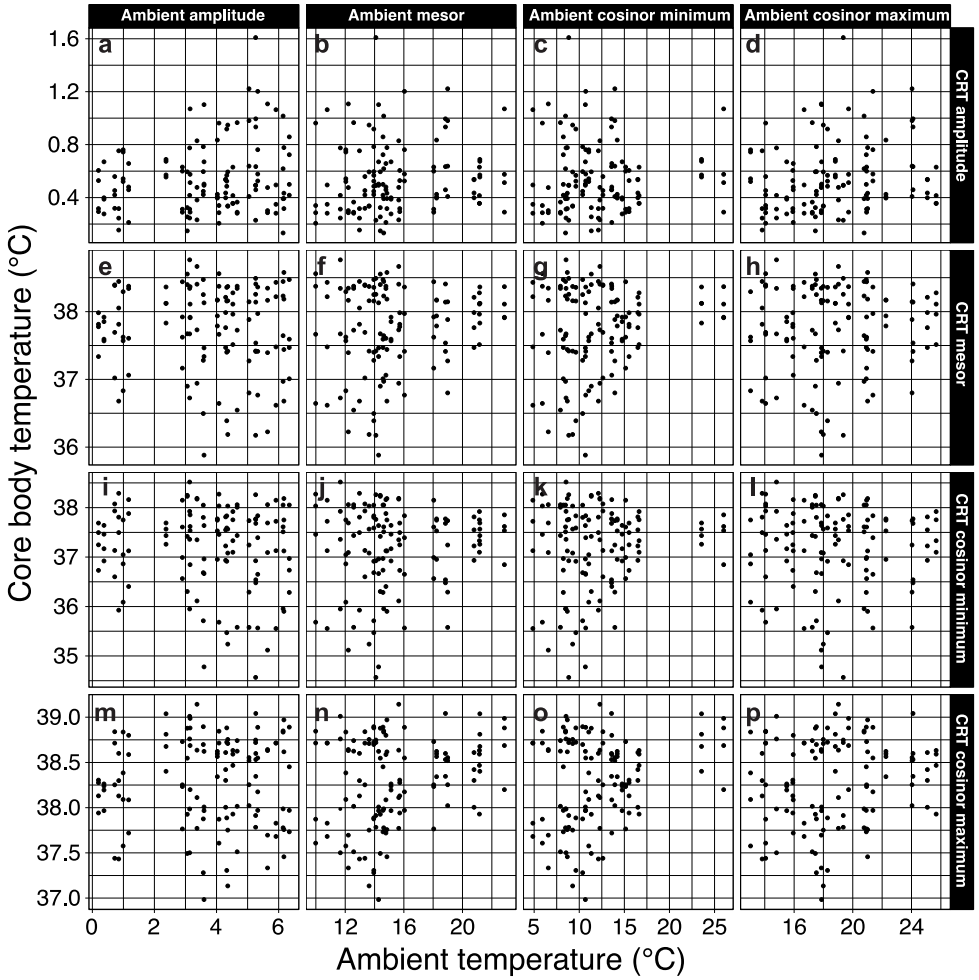


Figure 3.7: The relationship between the characteristics of the CRT (amplitude, mesor, cosinor minimum, and cosinor maximum) and the same characteristics of the ambient temperature rhythm (amplitude, mesor, cosinor minimum, and cosinor maximum) in two groups of goats (n= 4) exposed to natural climatic conditions during the autumn period (October to November).

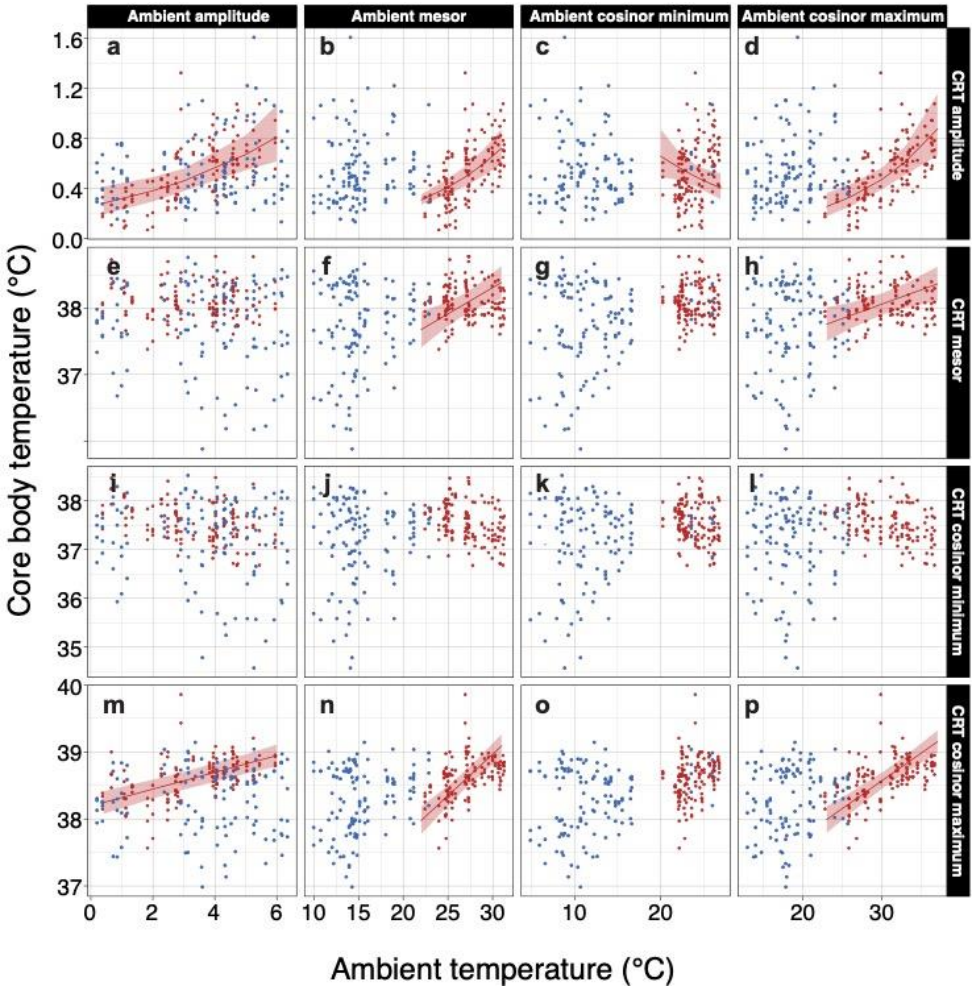


Figure 3.8: The relationship between the characteristics of the CRT (amplitude, mesor, cosinor minimum, and cosinor maximum) and the characteristics of the ambient temperature rhythm (amplitude, mesor, cosnior minimum, and cosinor maximum) during the summer period (red symbols) and the autumn period (blue symbols). The shaded regions correspond to 95% predictive intervals using the estimated parameters.

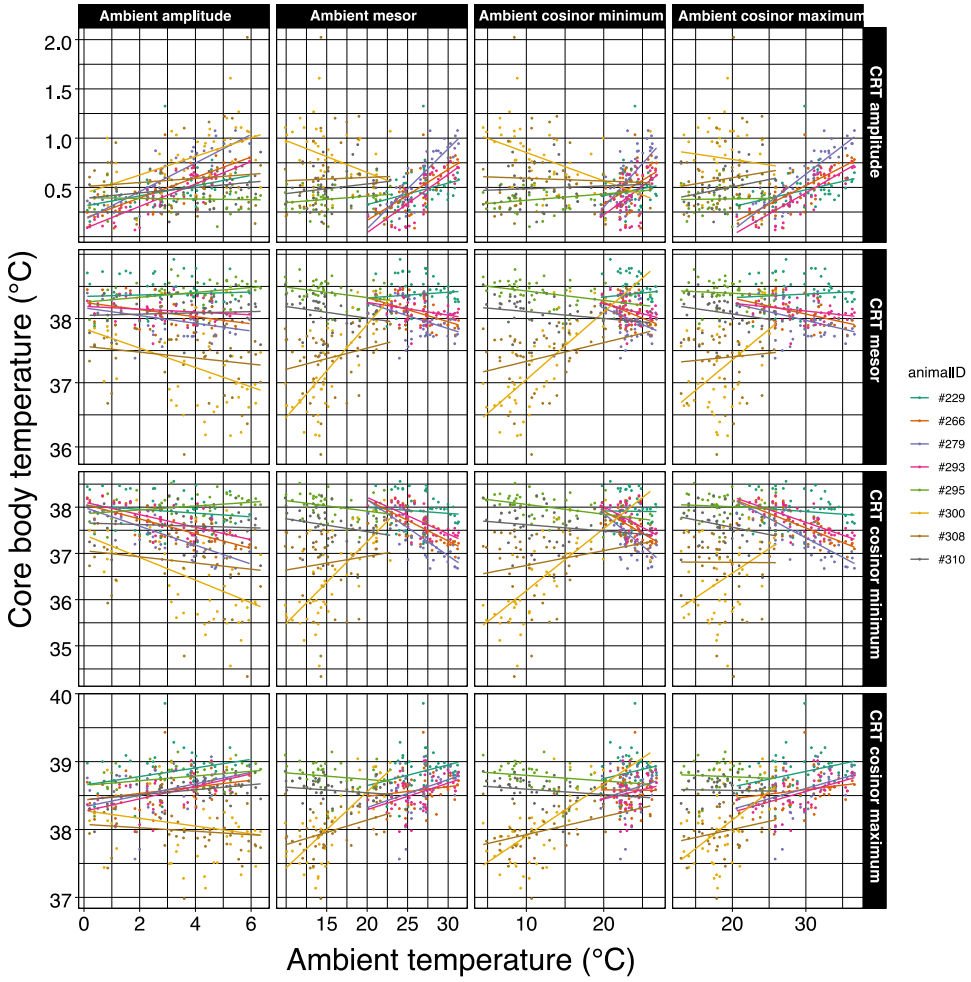


Figure 3.9: The relationship between the characteristics of the CRT (amplitude, mesor, cosnior minimum, and cosinor maximum) and the characteristics of the ambient temperature rhythm (amplitude, mesor, cosinor minimum, and cosinor maximum) for each individual goat. Linear regression models are fitted for individual goats (n = 4 for the summer period and n= 4 for the autumn period).

3.3.3.1 CRT amplitude

In summer, the amplitude of the CRT was positively correlated with the daily amplitude of ambient temperature (Figure 3.6A-a, Table 3.8A), the daily mesor of ambient temperature (Figure 3.6A-b, Table 3.8B), and the daily cosinor maximum of ambient temperature (Figure 3.6A-d, Table 3.8D). The amplitude of the CRT was negatively correlated with the daily cosinor minimum of ambient temperature (Figure 3.6A-c, Table 3.8C). In autumn, the amplitude of the CRT had no relationship with the parameters of ambient temperature (Figure 3.7B.a to d, Table 3.9A to D).

Table 3.8: The effect of amplitude (A), mesor (B), cosinor maximum (C) and cosinor minimum (D) of the daily profile of ambient temperature, tethering, and restriction of energy intake on the amplitude of the CRT in summer. n = 4.

A. Amplitude

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	4.98×10^{-2}	2.23×10^{-1}	
ambient_Amplitude		2.76×10^{-3}	5.26×10^{-2}	-0.90
Residual		1.16×10^{-1}	3.41×10^{-1}	

Fixed effects:

	Estimate Std.	Error	t value	Pr(>|z|)
(Intercept)	-1.75	2.17×10^{-1}	-8.055	10^{-15} ***
ambient_Amplitude	1.89×10^{-1}	5.00×10^{-2}	3.776	1.59×10^{-4} ***
time	8.87×10^{-3}	3.05×10^{-3}	2.909	3.63×10^{-3} **
Tethering tether	1.80×10^{-1}	6.38×10^{-2}	2.830	4.66×10^{-3} **
EI MD	9.90×10^{-2}	6.08×10^{-2}	1.629	1.03×10^{-1}

B. Mesor

Random effects:

Groups	Name	Variance	Std.Dev.
animalID	(Intercept)	8.66×10^{-3}	9.31×10^{-2}
Residual		1.32×10^{-1}	3.64×10^{-1}

Fixed effects:

	Estimate Std.	Error	t value	Pr(>|z|)
(Intercept)	-3.59	1.18×10^{-2}	-304.703	10^{-16} ***
ambient_Mesor	9.48×10^{-2}	4.65×10^{-3}	20.382	10^{-16} ***
time	5.29×10^{-3}	2.95×10^{-3}	1.794	7.28×10^{-2}
Tethering tether	2.21×10^{-1}	1.15×10^{-2}	19.194	10^{-16} ***
EI MD	9.29×10^{-2}	1.17×10^{-2}	7.934	10^{-14} ***

C. Cosinor minimum

Random effects:

Groups	Name	Variance	Std.Dev.
animalID	(Intercept)	8.57×10^{-3}	9.26×10^{-2}
Residual		1.38×10^{-1}	3.71×10^{-1}

Fixed effects:

	Estimate Std.	Error	t value	Pr(>|z|)
(Intercept)	-7.19×10^{-2}	6.36×10^{-1}	-0.113	9.10×10^{-1}
ambient_CosMin	-7.02×10^{-2}	3.02×10^{-2}	-2.326	2.04×10^{-2} *
time	2.99×10^{-2}	4.29×10^{-3}	6.981	10^{-11} ***
Tethering tether	2.06×10^{-1}	6.38×10^{-2}	3.230	1.24×10^{-3} **
EI MD	1.49×10^{-1}	7.26×10^{-2}	2.056	3.98×10^{-2} *

D. Cosinor maximum

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	4.29×10^{-1}	6.55×10^{-1}	
ambient_CosMax		4.33×10^{-4}	2.08×10^{-2}	-0.99
Residual		1.19×10^{-1}	3.44×10^{-1}	

Fixed effects:

	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-3.52	6.03×10^{-1}	-5.843	10^{-8} ***
ambient_CosMax	8.83×10^{-2}	1.89×10^{-2}	4.671	10^{-5} ***
Tethering tether	1.61×10^{-1}	7.98×10^{-2}	2.020	4.34×10^{-2} *
EI MD	7.10×10^{-2}	6.03×10^{-2}	1.179	2.39×10^{-1}

Table 3.9: The effect of amplitude (A), mesor (B), cosinor maximum (C) and cosinor minimum (D) of the daily profile of ambient temperature, tethering, and restriction of energy intake on the amplitude of the CRT in autumn. n = 4.

A. Amplitude

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	1.41×10^{-2}	1.19×10^{-1}	
ambient_Amplitude		1.50×10^{-3}	3.87×10^{-2}	-0.49
Residual		1.15×10^{-1}	3.40×10^{-1}	

Fixed effects:

	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-4.02×10^{-1}	1.46×10^{-1}	-2.750	5.96×10^{-3} **
ambient_Amplitude	5.69×10^{-2}	3.96×10^{-2}	1.436	1.51×10^{-1}
time	-1.25×10^{-2}	3.20×10^{-3}	-3.893	10^{-4} ***
Tethering tether	-2.93×10^{-1}	6.18×10^{-2}	-4.742	10^{-5} ***
EI MD	-8.94×10^{-2}	6.88×10^{-2}	-1.300	1.93×10^{-1}

B. Mesor

Random effects:

Groups	Name	Variance	Std.Dev.
animalID	(Intercept)	2.04×10^{-2}	1.43×10^{-1}
Residual		1.32×10^{-1}	3.64×10^{-1}

Fixed effects:

	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-3.93×10^{-1}	3.39×10^{-1}	-1.158	2.47×10^{-1}
ambient_Mesor	7.85×10^{-3}	1.54×10^{-2}	0.510	6.10×10^{-1}
Time	-8.77×10^{-3}	4.51×10^{-3}	-1.945	5.18×10^{-2}
Tethering tether	-3.12×10^{-1}	6.60×10^{-2}	-4.721	10^{-5} ***
EI MD	-5.93×10^{-2}	7.62×10^{-2}	-0.778	

C. Cosinor minimum

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	7.66×10^{-2}	2.77×10^{-1}	
ambient_CosMin		2.04×10^{-4}	1.43×10^{-2}	-0.90
Residual		1.26×10^{-1}	3.56×10^{-1}	

Fixed effects:

	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-1.07×10^{-1}	3.28×10^{-1}	-0.325	7.45×10^{-1}
ambient_CosMin	-6.88×10^{-3}	1.52×10^{-2}	-0.451	6.52×10^{-1}
Time	-1.23×10^{-2}	4.01×10^{-3}	-3.072	2.13×10^{-3} **
Tethering tether	-3.47×10^{-1}	8.33×10^{-2}	-4.160	10^{-4} ***
EI MD	-4.48×10^{-2}	7.16×10^{-2}	-0.625	5.32×10^{-1}

D. Cosinor maximum

Random effects:

Groups	Name	Variance	Std.Dev.
animalID	(Intercept)	2.00×10^{-2}	1.42×10^{-1}
Residual		1.30×10^{-1}	3.60×10^{-1}

Fixed effects:

	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-6.84×10^{-1}	2.96×10^{-1}	-2.310	2.09×10^{-2} *
ambient_CosMax	2.14×10^{-2}	1.17×10^{-2}	1.836	6.64×10^{-2}
Time	-7.70×10^{-3}	3.63×10^{-3}	-2.123	3.38×10^{-2} *
Tethering tether	-3.09×10^{-1}	6.51×10^{-2}	-4.747	10^{-5} ***
EI MD	-8.54×10^{-2}	7.50×10^{-2}	-1.139	2.55×10^{-1}

3.3.3.2 CRT mesor

In summer, the mesor of the CRT was positively correlated with the mesor of the ambient temperature (Figure 3.6A~f, Table 3.10B), and the cosinor maximum of the ambient temperature (Figure 3.6A~h, Table 3.10D). On the other hand, the mesor of the CRT had no relationship with the amplitude of the ambient temperature (Figure 3.6A-e,g, Table 3.10A, C). In autumn, the mesor of the CRT had no relationship with the parameters of ambient temperature (Figure 3.7B-e~h, Table 3.11A~D).

Table 3.10: The effect of amplitude (A), mesor (B), cosinor maximum (C) and cosinor minimum (D) of the daily profile of the ambient temperature, tethering and restriction of energy intake on the mesor of the CRT in summer. n = 4.

A. Amplitude

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	3.10×10^{-2}	1.76×10^{-1}	
ambient_Amplitude		1.66×10^{-3}	4.07×10^{-2}	-0.48
Residual		2.80×10^{-2}	1.67×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.81 x 10	1.03×10^{-1}	3.60	369.084	10^{-8} ***
ambient_Amplitude	4.75×10^{-2}	2.45×10^{-2}	3.73	1.938	1.30×10^{-1}
time	-6.02×10^{-3}	1.64×10^{-3}	1.25×10^{2}	-3.681	3.45×10^{-4} ***
Tethering tether	-1.47×10^{-1}	3.39×10^{-2}	9.60 x 10	-4.345	10^{-4} ***
EI MD	1.80×10^{-1}	3.25×10^{-2}	1.25×10^{2}	5.548	10^{-6} ***

B. Mesor

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	1.26	1.12	
ambient_Mesor		1.73×10^{-3}	4.16×10^{-2}	-0.99
Residual		1.80×10^{-2}	1.34×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.64 x 10	6.02×10^{-1}	3.36	60.538	10^{-5} ***
ambient_Mesor	7.90×10^{-2}	2.29×10^{-2}	3.87	3.445	2.75×10^{-2} *
time	-1.65×10^{-2}	2.01×10^{-3}	1.24×10^{2}	-8.223	10^{-12} ***
Tethering tether	-2.23×10^{-1}	3.67×10^{-2}	1.14×10^{2}	-6.080	10^{-7} ***
EI MD	1.51×10^{-1}	2.64×10^{-2}	1.25×10^{2}	5.706	10^{-7} ***

C. Cosinor minimum

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	8.15×10^{-1}	9.03×10^{-1}	
ambient_CosMin		1.61×10^{-3}	4.02×10^{-2}	-0.99
Residual		2.69×10^{-2}	1.64×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.71 x 10	5.23×10^{-1}	3.36	71.023	10^{-5} ***
ambient_CosMin	4.61×10^{-2}	2.37×10^{-2}	3.89	1.951	1.25×10^{-1}
time	-6.91×10^{-3}	1.75×10^{-3}	1.25×10^{2}	-3.959	1.26×10^{-4} ***
Tethering tether	-1.50×10^{-1}	3.59×10^{-2}	8.22 x 10	-4.164	10^{-4} ***
EI MD	1.80×10^{-1}	3.19×10^{-2}	1.25×10^{2}	5.649	10^{-6} ***

D. Cosinor maximum

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	6.24×10^{-1}	7.90×10^{-1}	
ambient_CosMax		6.76×10^{-4}	2.60×10^{-2}	-0.98
Residual		2.03×10^{-2}	1.43×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.71 x 10	4.22×10^{-1}	3.05	87.987	10^{-5} ***
ambient_CosMax	4.35×10^{-2}	1.44×10^{-2}	3.68	3.011	4.4×10^{-2} *
time	-1.36×10^{-2}	1.94×10^{-3}	1.25×10^{2}	-6.975	10^{-9} ***
Tethering tether	-2.07×10^{-1}	3.66×10^{-2}	1.10×10^{2}	-5.652	10^{-6} ***
EI MD	1.58×10^{-1}	2.80×10^{-2}	1.25×10^{2}	5.651	10^{-6} ***

Table 3.11: The effect of amplitude (A), mesor (B), cosinor maximum (C) and cosinor minimum (D) of the daily profile of the ambient temperature, tethering and restriction of energy intake on the mesor of the CRT in autumn. n = 4.

A. Amplitude

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	1.16×10^{-1}	3.40×10^{-1}	
ambient_Amplitude		3.05×10^{-3}	5.52×10^{-2}	1.00
Residual		1.31×10^{-1}	3.62×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.76 x 10	1.92×10^{-1}	3.49	195.591	10^{-7} ***
ambient_Amplitude	-2.30×10^{-2}	3.32×10^{-2}	3.31	-0.694	5.33×10^{-1}
Tethering tether	4.24×10^{-1}	6.62×10^{-2}	1.13×10^{2}	6.409	10^{-8} ***
EI MD	7.65×10^{-2}	6.78×10^{-2}	1.13×10^{2}	1.129	2.61×10^{-1}

B. Mesor

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	2.18	1.48	
ambient_Mesor		3.97×10^{-3}	6.30×10^{-2}	-1.00
Residual		9.34×10^{-2}	3.06×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.66 x 10	7.81×10^{-1}	4.21	46.895	10^{-6} ***
ambient_Mesor	4.73×10^{-2}	3.39×10^{-2}	4.18	1.395	2.33×10^{-1}
Tethering tether	4.50×10^{-1}	5.94×10^{-2}	2.17 x 10	7.573	10^{-6} ***
EI MD	-7.80×10^{-3}	6.54×10^{-2}	1.09×10^{2}	-0.119	9.05×10^{-1}
time	7.86×10^{-3}	3.65×10^{-3}	108.588887	2.149	*

C. Cosinor minimum

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	8.53×10^{-1}	9.24×10^{-1}	
ambient_CosMin		1.59×10^{-3}	3.99×10^{-2}	-0.99
Residual		8.80×10^{-2}	2.97×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.69 x 10	4.90×10^{-1}	6.17	75.425	10^{-9} ***
ambient_CosMin	3.41×10^{-2}	2.14×10^{-2}	5.69	1.595	1.65×10^{-1}
Tethering tether	4.56×10^{-1}	5.90×10^{-2}	3.26 x 10	7.726	10^{-8} ***
EI MD	2.51×10^{-2}	6.14×10^{-2}	1.08×10^{2}	0.408	6.84×10^{-1}
time	7.94×10^{-3}	3.40×10^{-3}	1.08×10^{2}	2.339	2.12×10^{-2} *

D. Cosinor maximum

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	1.30	1.14	
ambient_CosMax		1.02×10^{-3}	3.20×10^{-2}	-1.00
Residual		1.31×10^{-1}	3.62×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.74 x 10	6.03×10^{-1}	3.08	62.003	10^{-5} ***
ambient_CosMax	5.59×10^{-3}	1.93×10^{-2}	3.62	0.289	7.88×10^{-1}
Tethering tether	4.26×10^{-1}	6.63×10^{-2}	1.13×10^{2}	6.431	3.16×10^{-9} ***
EI MD	5.27×10^{-2}	7.43×10^{-2}	1.13×10^{2}	0.709	4.80×10^{-1}

3.3.3.3 CRT cosinor minimum

In summer, the cosinor minimum of the CRT had no relationship with the parameters of ambient temperature (Figure 3.6A-i~l, Table 3.12A-D). In autumn, the cosinor minimum of the CRT had no relationship with the daily parameters of ambient temperature (Figure 3.7B-i~l, A-D).

Table 3.12: The effect of amplitude (A), mesor (B), cosinor maximum (C) and cosinor minimum (D) of the daily profile of the ambient temperature, tethering and restriction of energy intake on the cosinor minimum of the CRT in summer. n = 4.

A. Amplitude

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	2.14×10^{-2}	1.46×10^{-1}	
ambient_Amplitude		4.17×10^{-3}	6.46×10^{-2}	-0.30
Residual		4.94×10^{-2}	2.22×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.80 x 10	1.02×10^{-1}	4.54	372.701	10^{-10} ***
ambient_Amplitude	-3.05×10^{-2}	3.70×10^{-2}	3.75	-0.824	4.59×10^{-1}
time	-1.11×10^{-2}	2.17×10^{-3}	1.25×10^{2}	-5.084	10^{-5} ***
Tethering tether	-2.32×10^{-1}	4.43×10^{-2}	7.90 x 10	-5.229	10^{-5} ***
EI MD	1.51×10^{-1}	4.32×10^{-2}	1.25×10^{2}	3.493	6.61×10^{-4} ***

B. Mesor

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	9.90×10^{-1}	9.95×10^{-1}	
ambient_Mesor		1.66×10^{-3}	4.07×10^{-2}	-0.98
Residual		4.36×10^{-2}	2.09×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.72 x 10	5.99×10^{-1}	7.85	62.207	10^{-11} ***
ambient_Mesor	3.41×10^{-2}	2.53×10^{-2}	9.68	1.345	2.09×10^{-1}
time	-1.96×10^{-2}	3.13×10^{-3}	1.24×10^{2}	-6.282	10^{-8} ***
Tethering tether	-2.67×10^{-1}	5.28×10^{-2}	6.50 x 10	-5.055	10^{-5} ***
EI MD	1.25×10^{-1}	4.11×10^{-2}	1.24×10^{2}	3.036	2.92×10^{-3} **

C. Cosinor minimum

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	1.80	1.34	
ambient_CosMin		3.84×10^{-3}	6.20×10^{-2}	-0.99
Residual		4.33×10^{-2}	2.08×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.66 x 10	7.50×10^{-1}	3.49	48.828	10^{-5} ***
ambient_CosMin	6.45×10^{-2}	3.48×10^{-2}	3.77	1.855	1.42×10^{-1}
time	-2.00×10^{-2}	2.21×10^{-3}	1.25×10^{2}	-9.038	10^{-14} ***
Tethering tether	-2.50×10^{-1}	4.57×10^{-2}	8.79 x 10	-5.476	10^{-6} ***
EI MD	1.25×10^{-1}	4.04×10^{-2}	1.25×10^{2}	3.104	2.37×10^{-3} **

D. Cosinor maximum

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
Animal ID	(Intercept)	7.79×10^{-1}	8.82×10^{-1}	
ambient_CosMax		1.10×10^{-3}	3.32×10^{-2}	-0.98
Residual		4.56×10^{-2}	2.14×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.79 x 10	4.95×10^{-1}	3.62	76.624	10^{-6} ***
ambient_CosMax	3.75×10^{-3}	1.91×10^{-2}	4.38	0.197	8.53×10^{-1}
time	-1.45×10^{-2}	2.91×10^{-3}	1.25×10^{2}	-4.964	10^{-5} ***
Tethering tether	-2.62×10^{-1}	5.27×10^{-2}	6.85 x 10	-4.968	10^{-5} ***
EI MD	1.40×10^{-1}	4.19×10^{-2}	1.25×10^{2}	3.338	1.11×10^{-3} **

Table 3.13: The effect of amplitude (A), mesor (B), cosinor maximum (C) and cosinor minimum (D) of the daily profile of the ambient temperature, tethering and restriction of energy intake on the cosinor minimum of the CRT in autumn. n = 4.

A. Amplitude

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	1.46×10^{-1}	3.82×10^{-1}	
ambient_Amplitude		7.04×10^{-3}	8.39×10^{-2}	0.94
Residual		2.47×10^{-1}	4.97×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.71 x 10	2.27×10^{-1}	3.57	163.181	10^{-7} ***
ambient_Amplitude	-5.18×10^{-2}	4.90×10^{-2}	3.01	-1.058	3.67×10^{-1}
Tethering tether	6.08×10^{-1}	9.11×10^{-2}	1.11×10^{2}	6.671	10^{-8} ***
EI MD	6.82×10^{-2}	9.31×10^{-2}	1.10×10^{2}	0.733	4.65×10^{-1}

B. Mesor

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
Animal ID	(Intercept)	3.52	1.88	
ambient_Mesor		6.28×10^{-3}	7.92×10^{-2}	-1.00
Residual		2.13×10^{-1}	4.61×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.59 x 10	1.01	3.89	35.456	10^{-5} ***
ambient_Mesor	4.51×10^{-2}	4.40×10^{-2}	3.81	1.025	3.66×10^{-1}
Tethering tether	6.52×10^{-1}	9.17×10^{-2}	2.14 x 10	7.101	10^{-6} ***
EI MD	1.10×10^{-2}	9.88×10^{-2}	1.08×10^{2}	0.111	9.12×10^{-1}
time	1.11×10^{-2}	5.52×10^{-2}	1.08×10^{2}	2.011	4.69×10^{-2} *

C. Cosinor minimum

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	2.00	1.41	
ambient_CosMin		3.83×10^{-3}	6.19×10^{-2}	-0.99
Residual		1.91×10^{-1}	4.37×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.61 x 10	7.46×10^{-1}	3.54	48.316	10^{-5} ***
ambient_CosMin	4.09×10^{-2}	3.30×10^{-2}	3.29	1.240	2.96×10^{-1}
Tethering tether	6.67×10^{-1}	8.94×10^{-2}	3.93 x 10	7.458	10^{-8} ***
EI MD	3.30×10^{-2}	9.06×10^{-2}	1.08×10^{2}	0.365	7.16×10^{-1}
time	1.33×10^{-2}	5.01×10^{-3}	1.08×10^{2}	2.658	9.06×10^{-3} **

D. Cosinor maximum

Random effects:

Groups	Name	Variance	Std.Dev.
animalID	(Intercept)	4.64×10^{-1}	6.81×10^{-1}
Residual		2.74×10^{-1}	5.23×10^{-1}

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.70 x 10	4.41×10^{-1}	8.03	83.979	10^{-12} ***
ambient_CosMax	-9.65×10^{-3}	1.56×10^{-2}	1.13×10^{2}	-0.617	5.39×10^{-1}
Tethering tether	6.08×10^{-1}	9.56×10^{-2}	1.13×10^{2}	6.361	10^{-8} ***
EI MD	7.82×10^{-2}	1.07×10^{-1}	1.13×10^{2}	0.729	4.68×10^{-1}

3.3.3.4 CRT cosinor maximum

In summer, the amplitude of the CRT was positively correlated with the daily amplitude of ambient temperature (Figure 3.6 m, Table 3.14 A), the daily mesor of ambient temperature (Figure 3.6 n, Table 3.14B), and the daily cosinor maximum of ambient temperature (Figure 3.6A-p. Table 3.14 D). On the other hand, the cosinor minimum of the CRT was not correlated with the cosinor minimum of ambient temperature (Figure 3.6 o, Table 3.14 C). In autumn, the amplitude of the CRT had no relationship with the daily parameters of ambient temperature (Figure 3.7 m~p, Table 3.15 A-D).

Table 3.14: The effect of amplitude (A), mesor (B), cosinor maximum (C) and cosinor minimum (D) of the daily profile of the ambient temperature, tethering and restriction of energy intake on the cosinor maximum of the CRT in summer. n = 4.

A. Amplitude

Random effects:

Groups	Name	Variance	Std.Dev.
animalID	(Intercept)	1.65×10^{-2}	1.28×10^{-1}
Residual		5.07×10^{-2}	2.25×10^{-1}

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.81 x 10	9.57×10^{-2}	1.22 x 10	397.906	10^{-16} ***
ambient_Amplitude	1.25×10^{-1}	1.83×10^{-2}	1.28×10^{2}	6.848	10^{-9} ***
time	-9.89×10^{-4}	2.20×10^{-3}	1.28×10^{2}	-0.449	6.54×10^{-1}
Tethering tether	-4.18×10^{-3}	3.87×10^{-2}	1.28×10^{2}	-0.108	9.14×10^{-1}
EI MD	2.10×10^{-1}	4.37×10^{-2}	1.28×10^{2}	4.795	10^{-5} ***

B. Mesor

Random effects:

Groups	Name	Variance	Std.Dev.
animalID	(Intercept)	1.66×10^{-2}	1.29×10^{-1}
Residual		4.63×10^{-2}	2.15×10^{-1}

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.55 x 10	3.49 x 10-1	1.30×10^{2}	101.869	10^{-16} ***
ambient_Mesor	1.24×10^{-1}	1.55×10^{-2}	1.28×10^{2}	7.970	10^{-12} ***
time	-1.34×10^{-2}	3.22×10^{-3}	1.28×10^{2}	-4.158	10^{-4} ***
Tethering tether	-4.12×10^{-3}	3.69×10^{-2}	1.28×10^{2}	-0.112	9.11×10^{-1}
EI MD	1.77×10^{-1}	4.23×10^{-2}	1.28×10^{2}	4.169	10^{-4} ***

C. Cosinor minimum

Random effects:

Groups	Name	Variance	Std.Dev.
animalID	(Intercept)	1.59×10^{-2}	1.26×10^{-1}
Residual		6.82×10^{-2}	2.61×10^{-1}

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.76 x 10	4.25×10^{-1}	1.31×10^{2}	88.633	10^{-16} ***
ambient_CosMin	2.77×10^{-2}	1.99×10^{-2}	1.28×10^{2}	1.394	1.66×10^{-1}
time	6.18×10^{-3}	2.78×10^{-3}	1.28×10^{2}	2.223	2.79×10^{-2} *
Tethering tether	-4.43×10^{-3}	4.48×10^{-2}	1.28×10^{2}	-0.099	9.22×10^{-1}
EI MD	2.35×10^{-1}	5.07×10^{-2}	1.28×10^{2}	4.630	10^{-5} ***

D. Cosinor maximum

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	1.16×10^{-1}	3.40×10^{-1}	
ambient_CosMax		9.60×10^{-5}	9.80×10^{-3}	-0.94
Residual		4.01×10^{-2}	2.00×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.64 x 10	2.69×10^{-1}	5.95	134.932	10^{-10} ***
ambient_CosMax	8.33×10^{-2}	1.01×10^{-2}	13.4	8.235	10^{-5} ***
time	-1.27×10^{-2}	2.73×10^{-3}	1.13×10^{2}	-4.643	10^{-5} ***
Tethering tether	-5.46×10^{-2}	4.12×10^{-2}	16.6	-1.326	2.03×10^{-1}
EI MD	1.76×10^{-1}	3.93×10^{-2}	1.13×10^{2}	4.491	10^{-4} ***

Table 3.15: The effect of amplitude (A), mesor (B), cosinor maximum (C) and cosinor minimum (D) of the daily profile of the ambient temperature, tethering and restriction of energy intake on the cosinor maximum of the CRT in autumn. n = 4.

A. Amplitude

Random effects:

Groups	Name	Variance	Std.Dev.
animalID	(Intercept)	1.71×10^{-1}	4.14×10^{-1}
Residual		9.58×10^{-2}	3.10×10^{-1}

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.82 x 10	2.31×10^{-1}	4.53	165.104	10^{-9} ***
ambient_Amplitude	1.15×10^{-2}	1.61×10^{-2}	1.12×10^{2}	0.712	4.78×10^{-1}
Tethering tether	2.35×10^{-1}	5.65×10^{-2}	1.12×10^{2}	4.160	10^{-4} ***
EI MD	3.90×10^{-2}	6.45×10^{-2}	1.12×10^{2}	0.605	5.47×10^{-1}
time	-4.78×10^{-3}	2.97×10^{-3}	1.12×10^{2}	-1.612	1.10×10^{-1}

B. Mesor

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
Animal ID	(Intercept)	1.53	1.24	
ambient_Mesor		2.91×10^{-3}	5.40×10^{-2}	-1.00
Residual		5.88×10^{-2}	2.42×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.73 x 10	6.50×10^{-1}	3.62	57.442	10^{-5} ***
ambient_Mesor	4.96×10^{-2}	2.88×10^{-2}	3.59	1.724	1.68×10^{-1}
Tethering tether	2.60×10^{-1}	4.75×10^{-2}	2.75 x 10	5.472	10^{-5} ***
EI MD	-2.66×10^{-2}	5.19×10^{-2}	1.09×10^{2}	-0.513	6.09×10^{-1}
time	4.61×10^{-3}	2.90×10^{-3}	1.09×10^{2}	1.591	1.14×10^{-1}

C. Cosinor minimum

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	7.38×10^{-1}	8.59×10^{-1}	
ambient_CosMin		1.36×10^{-3}	3.69×10^{-2}	-1.00
Residual		6.36×10^{-2}	2.52×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.79 x 10	4.35×10^{-1}	3.02	87.063	10^{-5} ***
ambient_CosMin	2.40×10^{-2}	1.92×10^{-2}	3.07	1.248	2.99×10^{-1}
Tethering tether	2.61×10^{-1}	4.73×10^{-2}	1.12×10^{2}	5.509	10^{-6} ***
EI MD	5.06×10^{-3}	5.05×10^{-2}	1.13×10^{2}	0.100	9.20×10^{-1}

D. Cosinor maximum

Random effects:

Groups	Name	Variance	Std.Dev.	Corr
animalID	(Intercept)	9.54	9.77×10^{-1}	
ambient_CosMax		9.09×10^{-4}	3.02×10^{-2}	-1.00
Residual		8.47×10^{-2}	2.91×10^{-1}	

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.78 x 10	5.13×10^{-1}	3.09	73.672	10^{-5} ***
ambient_CosMax	2.08×10^{-2}	1.74×10^{-2}	3.45	1.196	3.07×10^{-1}
Tethering tether	2.44×10^{-1}	5.33×10^{-2}	1.12×10^{2}	4.574	10^{-4} ***
EI MD	2.71×10^{-2}	5.97×10^{-2}	1.13×10^{2}	0.454	6.50×10^{-1}

3.3.4 Section 2: the effect of tethering and EI on the profile of the CRT

Neither tethering or the level of EI had any effect the amplitude of the CRT (Figure 3.10, Table 3.16). There was an interaction between tethering and EI on the mesor, cosinor minimum and cosinor maximum of the CRT (Table 3.17, Table 3.18, Table 3.19). The mesor, cosinor minimum, and cosinor maximum of the CRT were lower when the untethered goats were fed a calorie restricted diet (CR) than when fed a maintenance diet (MD) (Figure 3.10 B-D, Table 3.17, Table 3.18, Table 3.19). The mesor, cosinor minimum, and cosinor maximum of the CRT were larger when the goats were tethered than when they were untethered when the goats were fed the calorie restricted diet, but not when the goats were fed the maintenance diet (Figure 3.10B-D, Table 3.17, Table 3.18, Table 3.19). All plots of treatment group (tethering and EI) for the experimental period of eight goats are shown in Figure 3.11 and Figure 3.12.

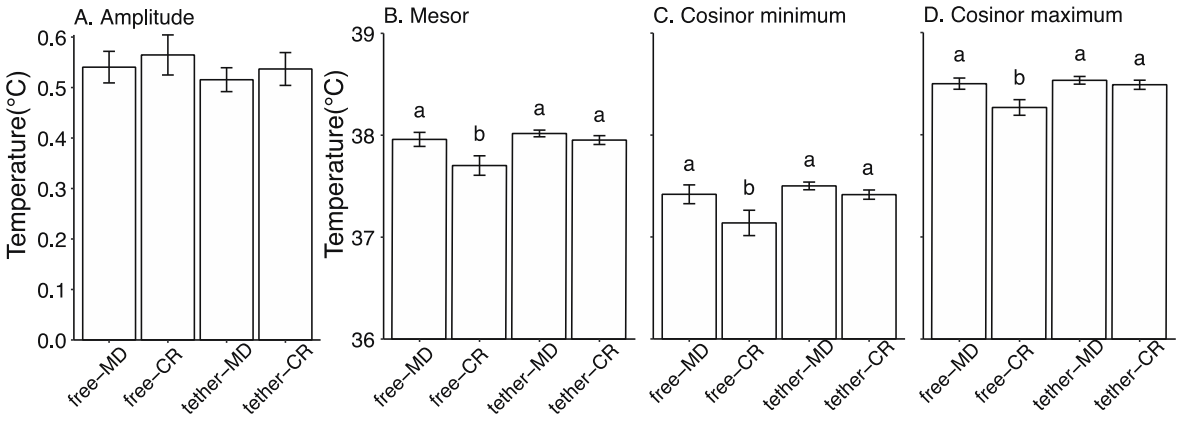


Figure 3.10: The amplitude (A), mesor (B), cosinor minimum (C), and cosinor maximum (D) of daily core body temperature during each treatment for the goats. MD; maintenance diet, CR; calorie restricted. Bars with different superscripts within a graph indicate statistical difference between means at $p<0.05$

Table 3.16: The effect of energy intake (EI) and tethering (Tethering), involving the daily profile of the ambient temperature on the amplitude of the CRT in autumn. n = 4.

Random effects:

Groups	Name	Variance	Std.Dev.	Corr	
animalID	(Intercept)	2.68×10^{-1}	5.18×10^{-1}		
ambient_Mesor		2.95×10^{-4}	1.72×10^{-2}	-0.81	
ambient_Amplitude		3.91×10^{-3}	6.25×10^{-2}	-0.56	-0.01
Residual		1.33×10^{-1}	3.64×10^{-1}		

Fixed effects:

	Estimate	Std. Error	t value	Pr(>|z|)
(Intercept)	-1.79	4.42×10^{-1}	-4.053	10^{-4} ***
ambient_Mesor	3.15×10^{-2}	1.62×10^{-2}	1.947	5.15×10^{-2}
ambient_Amplitude	1.22×10^{-1}	4.42×10^{-2}	2.765	5.69×10^{-3} **
Tethering tether	-9.35×10^{-2}	5.40×10^{-2}	-1.732	8.32×10^{-2}
EI MD	-2.09×10^{-3}	5.04×10^{-2}	-0.041	9.67×10^{-1}
day	2.26×10^{-3}	8.44×10^{-3}	0.268	7.89×10^{-1}

Table 3.17: The effect of energy intake (EI) and tethering (Tethering), involving the daily profile of the ambient temperature on the mesor of the CRT in autumn. n = 4.

Random effects:

Groups	Name	Variance	Std.Dev.	Corr	
animalID	(Intercept)	1.47	1.21		
ambient_Mesor		3.07×10^{-3}	5.54×10^{-2}	-0.99	
ambient_Amplitude		1.48×10^{-3}	3.85×10^{-2}	0.90	-0.94
Residual		7.16×10^{-2}	2.68 x 1		

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	3.73 x 10	4.50×10^{-1}	8.36	82.947	10^{-12} ***

ambient_Mesor	2.13×10^{-2}	2.03×10^{-2}	8.53	1.050	3.23×10^{-1}
ambient_Amplitude	-1.88×10^{-2}	1.84×10^{-2}	1.06×10	-1.023	3.29×10^{-1}
Tethering tether	3.31×10^{-1}	6.04×10^{-2}	1.82×10^{2}	5.482	10^{-6} ***
EI MD	2.39×10^{-1}	5.24×10^{-2}	2.37×10^{2}	4.566	10^{-5} ***
day	-6.43×10^{-3}	6.66×10^{-3}	2.34×10^{2}	-0.965	3.36×10^{-1}
Tethering tether:EI MD	-2.24×10^{-1}	6.99×10^{-2}	2.38×10^{2}	-3.210	1.51×10^{-3} **

Table 3.18: The effect of energy intake (EI) and tethering (Tethering), involving the daily profile of the ambient temperature on the cosinor minimum of the CRT in autumn. n = 4.

Random effects:

Groups	Name	Variance	Std.Dev.	Corr	
animalID	(Intercept)	4.1	2.03		
ambient_Mesor		$7.\ 5 \times 10^{-3}$	8.66×10^{-2}	-0.99	
ambient_Amplitude		4.24×10^{-3}	6.51×10^{-2}	0.47	-0.61
Residual		1.46×10^{-1}	3.83×10^{-1}		

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	37.3	7.44×10^{-1}	7.45	50.110	10^{-9} ***
ambient_Mesor	5.87×10^{-3}	3.14×10^{-2}	7.60	0.187	8.57×10^{-1}
ambient_Amplitude	-6.79×10^{-2}	2.91×10^{-2}	9.21	-2.333	4.39×10^{-2} *
Tethering tether	4.66×10^{-1}	8.92×10^{-2}	2.30×10^{2}	5.221	10^{-6} ***
EI MD	2.71×10^{-1}	7.50×10^{-2}	2.36×10^{2}	3.611	3.72×10^{-4} ***
day	-7.35×10^{-3}	9.55×10^{-3}	2.34×10^{2}	-0.770	4.42×10^{-1}
Tethering tether:EI MD	-2.79×10^{-1}	1.00×10^{-1}	2.37×10^{2}	-2.776	5.94×10^{-3} **

Table 3.19: The effect of energy intake (EI) and tethering (Tethering), involving the daily profile of the ambient temperature on the cosinor maximum of the CRT in autumn. n = 4.

Random effects:

Groups	Name	Variance	Std.Dev.	Corr	
animalID	(Intercept)	6.53×10^{-1}	8.08×10^{-1}		
ambient_Mesor		1.48×10^{-2}	3.8×10^{-2}	-0.99	
ambient_Amplitude		1.44×10^{-3}	3.79×10^{-2}	0.60	-0.70
Residual		5.83×10^{-2}	2.41×10^{-1}		

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	37.5	3.09×10^{-1}	8.03	121.200	10^{-13} ***
ambient_Mesor	3.20×10^{-2}	1.44×10^{-2}	7.53	2.227	5.86×10^{-2}
ambient_Amplitude	3.47×10^{-2}	1.75×10^{-2}	5.97	1.987	9.43×10^{-2}
Tethering tether	2.46×10^{-1}	5.36×10^{-2}	1.59×10^{2}	4.581	10^{-5} ***
EI MD	2.14×10^{-1}	4.73×10^{-2}	2.39×10^{2}	4.523	10^{-5} ***
day	-4.74×10^{-3}	6.01×10^{-3}	2.30×10^{2}	-0.789	4.31×10^{-1}
Tethering tether:EI MD	-1.91×10^{-1}	6.31×10^{-2}	2.34×10^{2}	-3.032	2.7×10^{-3} **

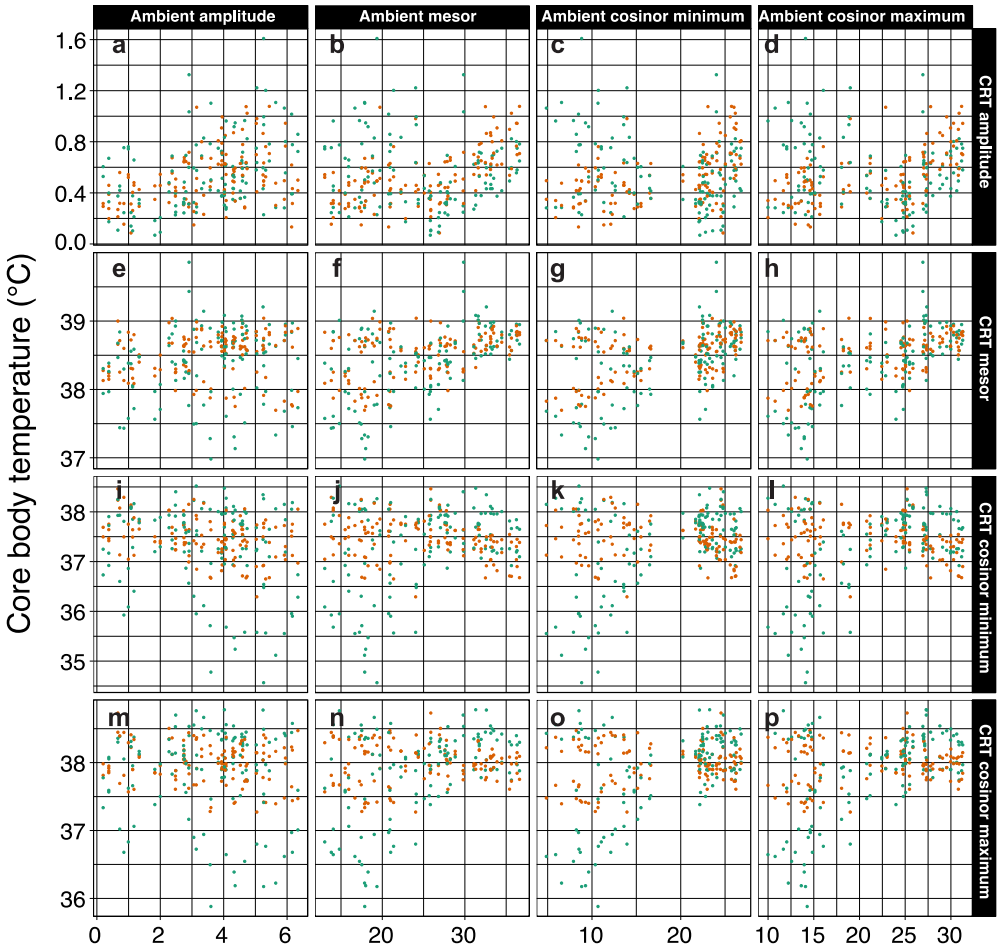


Figure 3.11: The relationship between the characteristics of the CRT (amplitude, mesor, cosinor minimum, and cosinor maximum) and the characteristics of the ambient temperature rhythm when the goats were tethered (orange dot) or not tethered (green dot). The data from both experiment periods (summer and winter) are included.

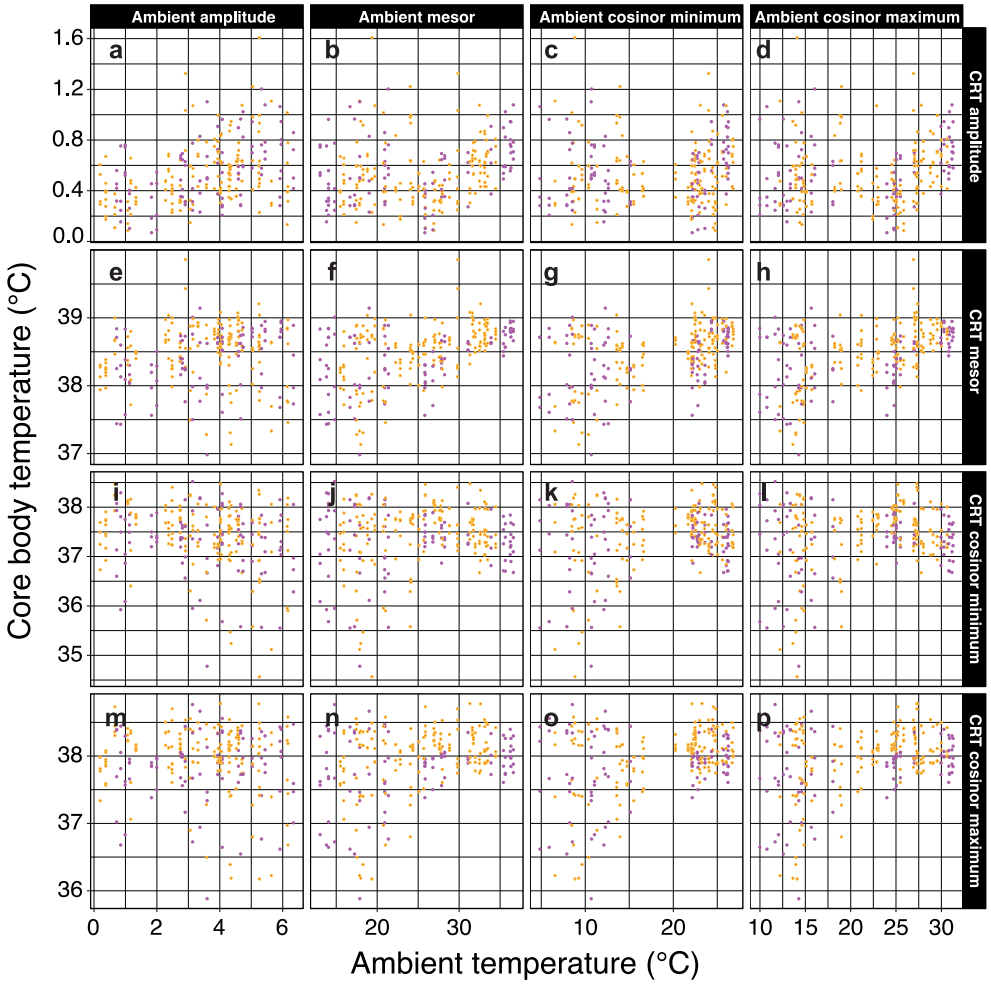


Figure 3.12: The relationship between the characteristics of the CRT (amplitude, mesor, cosinor minimum, and cosinor maximum) and the characteristics of the ambient temperature rhythm (amplitude, mesor, cosinor minimum, and cosinor maximum) in goats fed a maintenance diet (yellow dot) or fed a calorie restricted diet (purple dot). The data from both experiment periods (summer and autumn) are included.

3.4 Discussion

In the present study, I found that the T_c was affected by the ambient temperature in summer, when it was hot and humid. The amplitude of the CRT was the most sensitive parameter that reflected the thermal challenge on goats in summer. In addition, energy intake restriction decreased the mesor, cosinor minimum, and cosinor maximum of the CRT when the goats were untethered but had no significant effect on the amplitude of the CRT. On the other hand, when the goats were tethered, there were no effect of energy intake restriction. Tethering seemed to attenuate the decrease of the mesor, cosinor minimum and, cosinor maximum of the CRT that occurred in response to energy restriction, possibly due to restraint stress while energy restriction decreases the T_c.

3.4.1 Effect of ambient temperature on CRT profile

In summer, the amplitude of the CRT was the most reliable and evident marker of changes in T_c with changes in ambient temperature. An increase in the amplitude of the CRT on hot days was due to an increase in the cosinor maximum of the CRT, not to a decrease in the cosinor minimum of the CRT. The most well-known factor that induces a hyperthermia in mammals during exposure to heat stress is water deficiency (Hetem *et al.*, 2016). Dehydration induces a reduction in the rate of evaporative water loss (Taylor, 1970a), leading to an increase in the cosinor maximum body temperature during heat exposure (Taylor, 1970a, b). However, in the current study, water was offered *ad libitum* throughout the experimental period, making it very unlikely that the changes in cosinor maximum T_c were due to dehydration. One possible explanation for the hyperthermic heterothermy, in the absence of dehydration, was the humidity. When an animal activates evaporative heat loss, the rate of the evaporation depends on the water vapor gradient between the evaporating surfaces of the animal and the surrounding air (Edwards and Haines, 1978; van Dyk *et al.*, 2019). In Japan, the humidity is quite high in the summer season, which will hinder the power of evaporative heat loss to limit a rise in the T_c. In fact, in the present study, days with high wet bulb temperature were frequently observed in the summer period. Thus, even though the water supply was sufficient, the hyperthermic heterothermy might be due to a combination of the excess heat load from the ambient temperature and a disturbance to the power of evaporative heat loss. Although the mesor of the CRT also had an association with ambient temperature, the amplitude of the CRT seems more reliable to assess the response because for mammals in general the mean T_c has a negative correlation with body mass while the amplitude of the CRT is independent of body mass (Hetem *et al.*, 2016).

3.4.2 Effect of energy restriction on CRT profile

A negative energy balance is another factor known to induce an increase in the amplitude of the CRT. In sheep and rodents a decrease in energy intake induces an increase in the amplitude of the CRT (Maloney *et al.*, 2013; Goh *et al.*, 2016). An increase in the amplitude of the CRT is mainly due to a decrease in the cosinor minimum of the CRT during the resting period. It is thought that this adaptive hypothermic heterothermy is induced by a negative energy balance via decreasing metabolic heat (Nakamura *et al.*, 2017b). In goats, there are

several ways that high ambient temperature might induce negative energy balance, via either excess energy expenditure when the activity of effectors that enhance evaporative heat loss are activated, such as panting and sweating, or that the higher T_c increases an increase in metabolic heat production via the Q_{10} effect, or because the high T_c might induce inappetence. However, there was no significant relationship between the ambient temperature and the cosinor minimum of the CRT, and the goats didn't decrease food intake on the hot days; they ate all the offered feed every day. Therefore, it is unlikely that the increased amplitude of the CRT was induced by negative energy balance.

On the other hand, in autumn, there were no relationship between any of the parameters of the CRT and the ambient temperature. That finding is consistent with the fact that there are no reports that low ambient temperature is associate with the lowered T_c within the range above 'B' in figure 3.1. It must be noted that one goat (#308) showed a very different relationship to ambient conditions than the others, and its T_c varied widely with variation in the ambient temperature. The body weight of that goat (#308) varied by up to 19.2 % of its average body weight during the study. Those results suggest that that individual had some underlying illness or pathology that resulted in very different results compared to the other goats. When I excluded that single goat (#308) from the analysis, the other goats maintained the T_c constant regardless of the variation of the ambient temperature in autumn. Goats seem to thermoregulate better against the temperature range in autumn than in autumn. It would be interesting to test much colder ambient temperatures to confirm the fitness of the goats to cold environment.

3.4.3 Effect of tethering on CRT profile

Tethering induced hyperthermia and seemed to disrupt the mechanism that led to a reduction in T_c during food deprivation, therefore probably cancelling the energy savings that are usually achieved by lower T_c. Consistent with previous reports in goats and sheep (Piccione *et al.*, 2002) calorie restriction induced hypothermia. The mesor, cosinor minimum, and cosinor maximum decreased in the goats when they were calorie restricted while they were untethered. But when they were tethered, an effect of calorie restriction was not seen.

Tethering, a kind of the restraint stress that is common in some animal industries, has the potential to induce stress-induced hyperthermia (SIH). While SIH is a well-studied response to acute psychological stressors such as “novel object” or “intruder” challenges, there are few reports on the response of T_c to chronic movement restriction, especially in large mammals such as ruminants. Restraint stress has been used as an experimental treatment to induce the psychological stress for the investigation of SIH in rodents (Hyden *et al.*, 1997; Hashimoto *et al.*, 2001) and pigs (Parrott and Lloyd, 1995). There are few reports on the thermal response to restraint stress in ruminants, but it has been reported that restraint stress induces an increase in plasma cortisol and the number of cells co-expressing Fos and corticotrophin-releasing hormone in the paraventricular nucleus (Nozu *et al.*, 1992). Thus, I hypothesized that chronic tethering (about two weeks for each animal) would induce restraint derived hyperthermia in goats.

In previous studies in rodents, four to five weeks of immobilization for three hours daily enhanced the increase in T_c and the increase in temperature in brown adipose tissue (BAT) in response to noradrenaline administration (Nozu *et al.*, 1992). Even though the restraint was not chronic, repeated restraint stress altered the noradrenergic signaling that is involved in metabolic heat production in BAT. The enhanced sensitivity of thermogenic signaling via a noradrenergic pathway might disturb mechanisms that reduce the metabolic rate in BAT in response to negative energy balance (Nakamura *et al.*, 2017a). Although the physiological mechanisms are still unclear, tethering stress could attenuate the energy restriction induced hypothermia potentially because a form of SIH, that necessitates an increase in energy expenditure, overrides the normal energy savings that presumably are made when hypothermia is induced by a decrease in energy intake.

In the present study, even though there was a slight trend, there was no significant effect of calorie restriction (CR) on the amplitude of the CRT. This result is contrary to previous studies in sheep (Maloney *et al.*, 2013) and rats (Goh *et al.*, 2016) that have reported an increased amplitude during CR. In general, the increased amplitude of the CRT is due to a decrease in the daily cosinor minimum T_c (Hetem *et al.*, 2016). In the present study, the cosinor minimum of the CRT was lower, consistent with those previous studies, but the cosinor maximum of the CRT also decreased during CR when the goats were untethered. In addition, as mentioned above, when the goats were tethered, there was no decrease in the

cosinor minimum of the CRT or the cosinor maximum of the CRT. Therefore, I didn't find any significant change in the amplitude of the CRT during CR.

3.4.4 Limitations of the study

One possible reason why I didn't see results that were consistent with previous reports on the effect of CR on the amplitude of the CRT is that Maloney et al. (2013) conducted their experiments in an indoor facility with controlled temperature. Even though I chose a model that included the ambient temperature as a covariate, there is the possibility that some other environmental factors, not measured in the present experiment, could have influenced the CRT. For example, the results may have been different if the same experiment at constant ambient temperature and light:dark cycle.

Considering that there were only four animals in each season, and one of them displayed quite different responses of the CRT to the ambient temperature, further investigation with higher numbers is required, especially for the autumn season with cold/dry ambient conditions.

3.5 Conclusion

This study has shown that; (1) the amplitude of the CRT is strongly correlated with ambient temperature in summer, and (2) tethering disturbs the adaptive hypothermia response to energy deficiency. The current data support the possibility that the amplitude of the CRT can be used as a biomarker of adaptation of livestock to heat stress. In addition, the housing system, such as tie-stall or stanchion, might induce disturbances to the adaptive hypothermic heterothermy of livestock and induce excess energy expenditure during under nutrition. Overall, the present study explored the potential stressors that livestock can experience and need to adapt to in the farm management and, obtained data to further support the idea that the heterothermy in T_c can be a new biomarker of the fitness of animals to a production system.

Chapter 4: The relationship between luteinizing hormone secretion and the circadian rhythm of core body temperature in goats

4.1 Introduction

Core body temperature (T_c) and reproduction have a strong connection in homeotherms. The T_c varies through the oestrous cycle (Piccione *et al.*, 2003b; Baker *et al.*, 2020), and the temperature gradient in the reproductive organs (such as ovary, uterus in the female, testes in male) is important to maintain reproductive function (Hunter *et al.*, 2017). In male mammals, the temperature of the testis needs to be lower than T_c for spermatogenesis (Morgentaler *et al.*, 1999). In female mammals, there is a temperature gradient between the ovary and uterus, such that the ovary is cooler than the uterus, is important for successful pregnancy (Hunter, 2012; Hunter *et al.*, 2017). In addition, it is known that exposure to high ambient temperature can lead to a decline in conception rate, especially in summer (Gwazdauskas *et al.*, 1973). Hot and especially hot and humid conditions induce a physiological state called heat stress, when the heat load on the body exceeds the capacity of the animal for heat loss. The loss of capacity to regulate T_c means that the T_c, including that of reproductive organs, reaches levels that exceed the physiological range. Thus, the control of the T_c within the normal range is very important for successful reproduction.

The circadian rhythm of T_c (CRT) varies with reproductive events (Maloney *et al.*, 2019). For example, in humans, the amplitude of the CRT is larger in the follicular phase compared to the luteal phase (Lee, 1988; Parry *et al.*, 1997; Coyne *et al.*, 2000). Recently, there have been attempts to use the CRT to predict the timing of the window of fertility (Webster and Smarr, 2020). In wild rabbits, there was a significant correlation between the amplitude of the CRT and the number of pregnancies during the breeding season (Maloney *et al.*, 2017). While the interaction between the menstrual cycle and the T_c seems due to the effect of gonadal steroids from the ovary on the hypothalamic neural circuits involved in the control of T_c (Webster and Smarr, 2020), the mechanism(s) that connect the CRT profile with reproductive ability is still poorly understood.

Reproductive function is controlled by the hypothalamic-pituitary-gonadal (HPG) axis. Luteinizing hormone (LH), secreted in a pulsatile manner from the pituitary, is indispensable for the development of follicles in the ovary in females (Dierschke *et al.*, 1970). A decline in the pulsatile secretion of LH causes the suppression of the reproductive ability. Thus, pulsatile LH secretion is one of the fundamental indicators of the reproductive ability of animals. In addition, the pulsatile secretion of LH varies during the oestrous cycle and is affected by many endogenous factors, such as energy status or stress (Nagatani *et al.*, 1996; Tsukahara *et al.*, 1999; Maeda *et al.*, 2011). These known factors also modulate the amplitude of the CRT (Maloney *et al.*, 2019). It seems that the T_c and the LH secretion are affected in parallel by common factors, such as energy balance, ambient conditions, the oestrous cycle, stress, and probably other factors.

The pulsatile secretion of LH from the pituitary is induced by the pulsatile secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus. The pulsatile secretion of GnRH is indispensable for the maintenance of reproductive function as the pulsatility is required to maintain the secretion of LH. It should be noted that, for the development of follicles, the level of LH secretion is more important than the frequency of LH pulses (Campbell *et al.*, 2007). The normal development of ovulatory follicles responds, in a dose-dependent manner, to the basal level of secretion of LH (Campbell *et al.*, 2007), and the pulsatile pattern of LH is not necessary for the acceleration of follicular development or subsequent luteal function in sheep (Campbell *et al.*, 2007). In addition, in the superovulation technique, that is frequently used in farm animals to induce excess ovulation, the administration of a single dose of FSH is enough to promote the development of multiple follicles (Kimura, 2016). Thus, it is not necessary to give gonadotrophins in a pulsatile manner to drive follicular development.

In this study, I hypothesized that there was a relationship between the CRT and LH secretion, as an indicator of the activity of HPG axis that regulates reproductive function in mammals. To test this hypothesis, I measured LH in blood samples from goats taken frequently and over a relatively long-term while T_c was recorded. I recorded the T_c of goats and analysed the correlation between the parameters of CRT and LH secretion.

4.2 Materials and Methods

4.2.1 Animals

Adult (3 – 4 years old) female Shiba goats (n = 7; 25 - 35 kg) were used for T_c recording and blood sampling. All of the goats were ovariectomized >2 months prior to the start of the experiment to exclude the effect of estrogen on the control of core body temperature through the oestrous cycle. The goats were housed loosely tied to an individual stanchion in a condition-controlled room (12:12 h light:dark cycle; 25ºC; 50% relative humidity) and maintained on dry hay (85 % of maintenance energy intake) supplemented with standard pelleted diet (15 % of maintenance energy intake). The formula is described in Chapter 3. Water and supplemental minerals were available *ad libitum*.

4.2.2 Core body temperature recording

The protocol for T_c recording is described in Chapter 3.

4.2.3 Surgery for temperature logger implantation

The protocol for surgery is described in Chapter 3.

4.2.4 Experimental procedure and blood sampling

The characteristics of the CRT were analysed for five days leading up to a day of frequent blood sampling on which the activity of the reproductive axis was assessed by measuring LH. Blood samples were collected every six minutes from 9:00 to 15:00. The plasma was separated by centrifugation (1,500 *g*, 30 min, 4 °C) and stored at −20 °C until the LH assay.

4.2.5 LH assay

Plasma LH concentration was determined using a double-antibody radioimmunoassay, as previously described (Sasaki *et al.*, 2019). The lowest detectable LH concentration was 0.1 ng ml^{-1} for 50-µl plasma samples, and the intra- and inter-assay coefficients of variation were 6.2% at 3.14 ng ml^{-1} and 7.3% at 3.11 ng ml^{-1}, respectively.

4.2.6 Data analysis

The characteristics of the circadian rhythm of ambient temperature and T_c (mesor, amplitude, cosinor minimum, cosinor maximum and acrophase) were calculated using a cosinor analysis on 24 hours of data starting at 0:00 each day (Maloney *et al.*, 2019).

The profile of LH secretion was analyzed using the PULSAR computer program (Merriam and Wachter, 1982) to detect the pulse of LH, and calculate the mean LH secretion, number of pulses, amplitude of the pulses and inter-pulse interval.

Linear modelling (R Core Team, 2020) was used to fit a model to examine the relationship between the characteristics (amplitude, mesor, minimum, and maximum) of the circadian rhythm and the LH secretion (mean LH, baseline LH, LH pulse amplitude, mumber of LH pulse, and inter pulse interval).

4.3 Results

4.3.1 The profile of core body temperature

The core body temperature (T_c) followed a circadian rhythm in every individual before the day of sampling (Figure 4.1). An increase of T_c was observed on the day of blood sampling (Day 6).

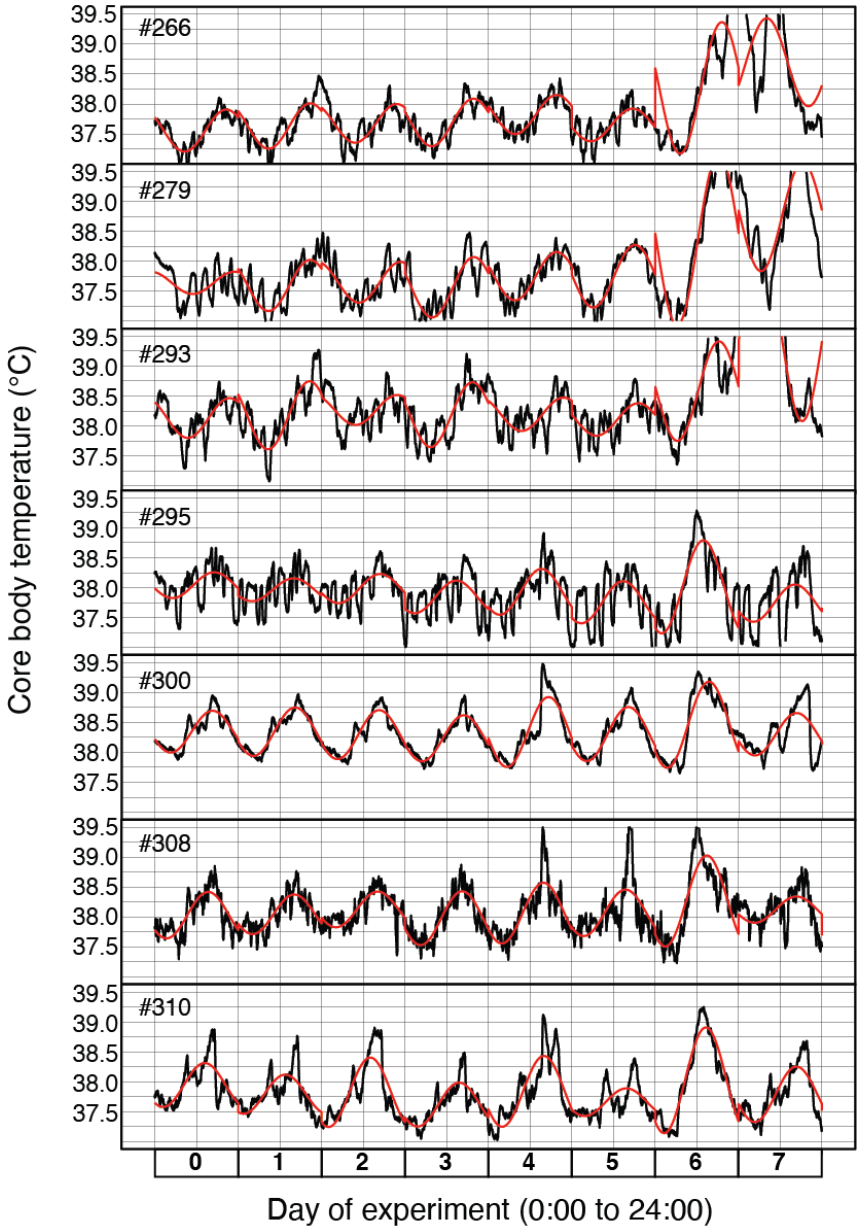


Figure 4.1: Profiles of core body temperature (black line) and fitting curve of circadian rhythm of core body temperature (red line) in seven individual (#266, #279, #293, #295, #300, #308, and #310). Day 1 to day 5 were used of for the analyses of the CRT, and blood sampling was conducted in day 6.

4.3.2 The profile of LH secretion

LH secretion was pulsatile for every individual animal (Figure 4.2).

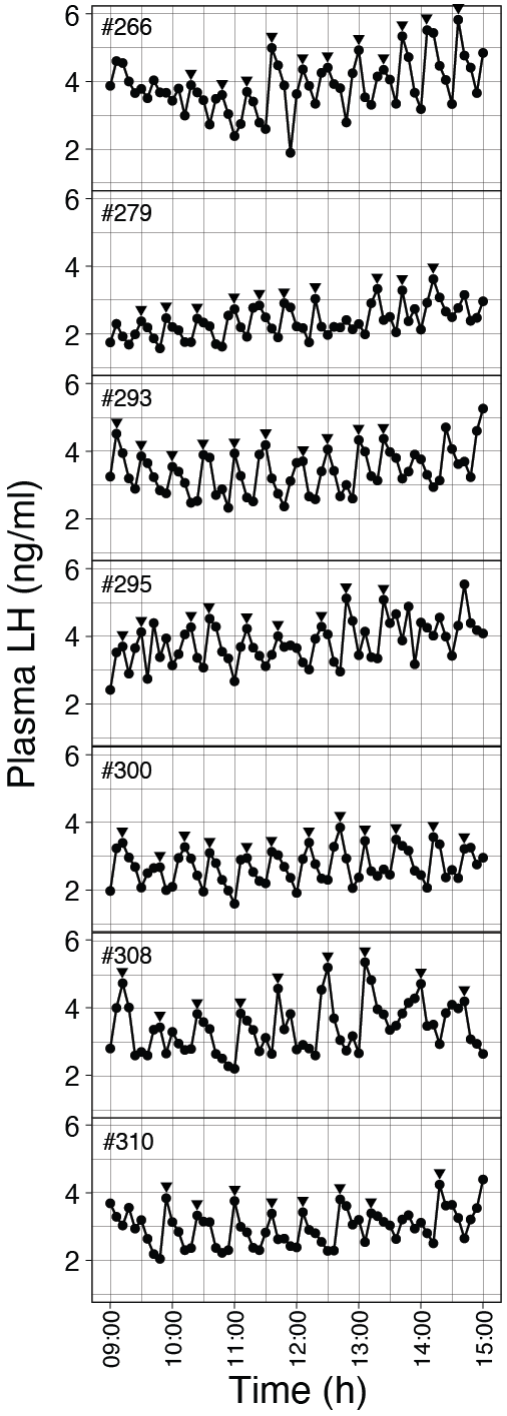


Figure 4.2: Profiles of plasma LH concentrations in seven individual goats (#266, #279, #293, #295, #300, #308, and #310). Arrowheads indicate LH pulses identified by the PULSAR computer program.

4.3.3 Relationship between the parameters of CRT and LH secretion

When the parameters of the CRT were correlated to the descriptors of the LH profile, the mean LH concentration during the 6 hours on the day of blood sampling (day 6) was correlated negatively with the amplitude of CRT (y = 6.65 – 8.86x, p< 0.01, r = 0.80) (Figure 4.3 a). The baseline LH concentration was correlated negatively with the amplitude of the CRT (y = 6.17 – 8.65x, p< 0.01, r = 0.87) (Figure 4.3 e). There were no other significant relationships between the other parameters of LH secretion and characteristics of the CRT.

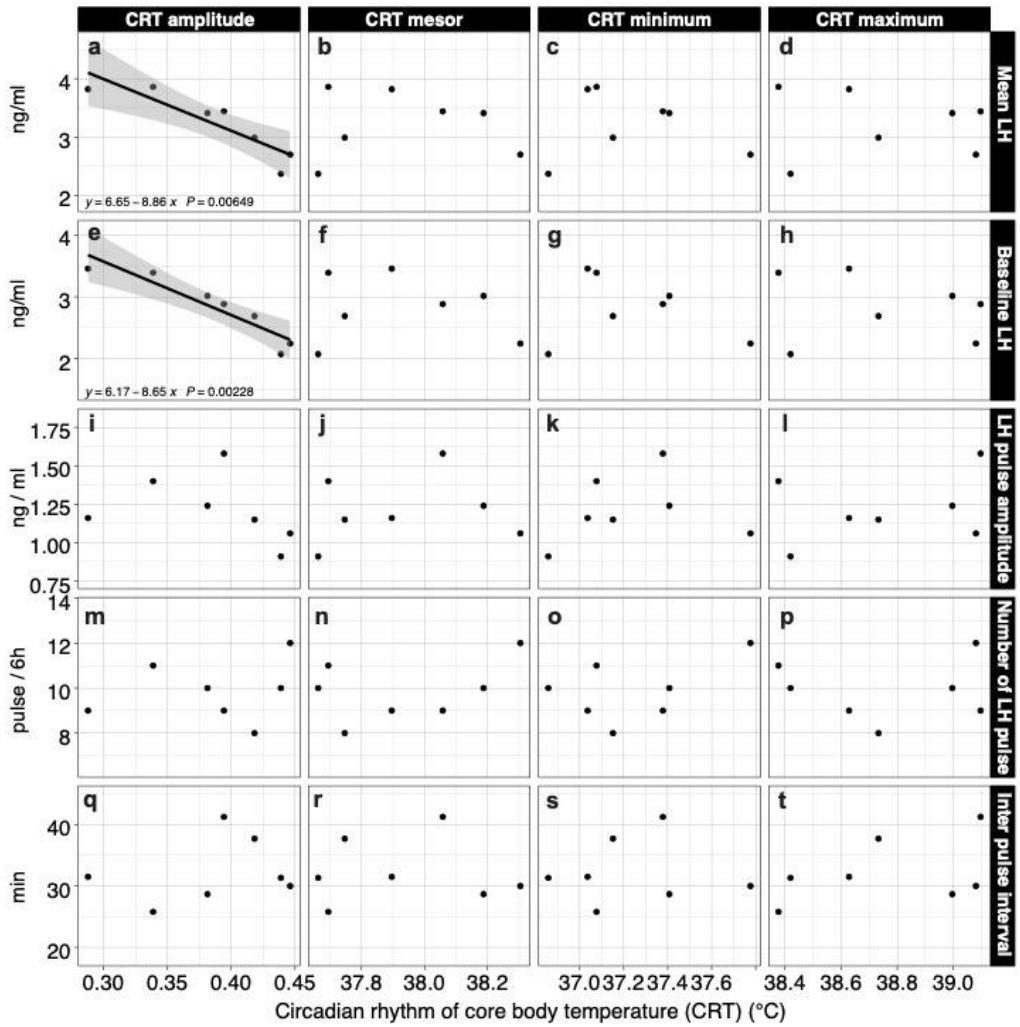


Figure 4.3: Relationship between the characteristics of CRT (amplitude, mesor, cosinor minimum, and cosinor maximum) and LH secretion (mean LH, baseline LH, LH pulse amplitude, number of pulses, and inter-pulse interval) for 6 hours. Each dot represents one goat used in the experiment.

4.3.4 Relationship between live body weight versus the CRT amplitude and LH

The amplitude of the CRT was not correlated with live body weight (p = 0.80) (Figure 4.4 A). The mean and baseline LH also were not correlated with live body weight (p = 0.8, p = 0.74, respectively) (Figure 4.4 B, C).

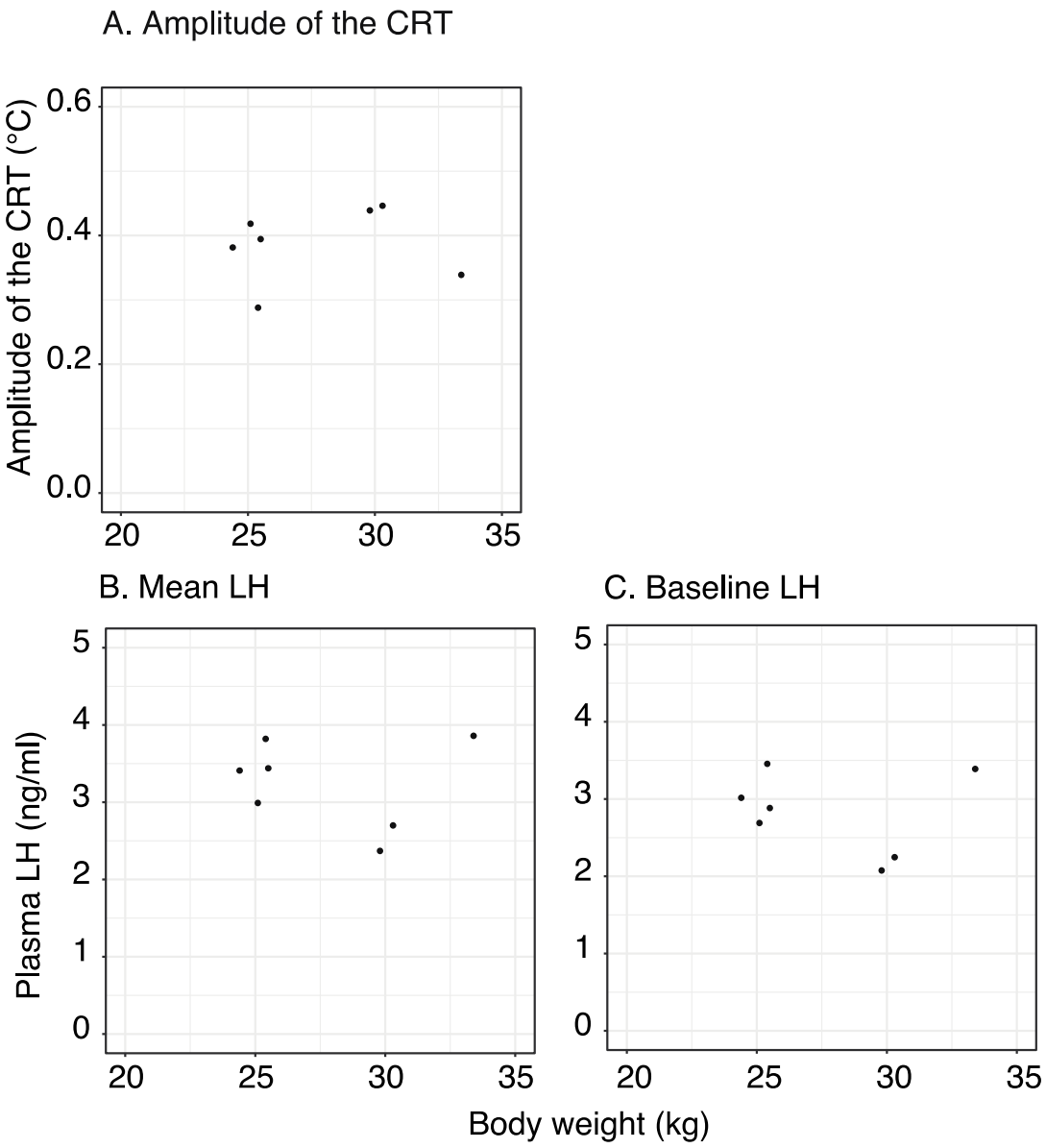


Figure 4.4: The relationship between live body weight and the amplitude of the CRT for five days before the blood sampling day (A), mean LH (B), and baseline LH (C) for six hours during blood sampling.

4.4 Discussion

The present study demonstrated a negative correlation between the mean and baseline concentrations of plasma LH and the amplitude of the CRT, suggesting that the amplitude of the CRT can be used to predict the LH concentration that drives reproductive function in females. An increase of the amplitude of the CRT may indicate attenuation of the activity of the HPG axis, as measured by the plasma concentration of LH. Interestingly, the amplitude of the CRT varied over the course of the short experiment while the goats used in this study were all apparently in good condition (they ate all offered feed with maintenance energy intake) and in the environmental conditions remained constant. Our data suggest that the amplitude of the CRT could reflect the overall state of the animal and that that state is associated with reproductive ability, a state that is not apparently linked to either the environment or to the energy status of the animal as measured in the present study.

The relationship between the amplitude of the CRT and the basal secretion of LH has not previously been reported. The studies that have measured the amplitude of the CRT in relation to reproductive status in humans and rats (Marrone *et al.*, 1976; Lee, 1988; Parry *et al.*, 1997; Coyne *et al.*, 2000; Crew *et al.*, 2018; Webster and Smarr, 2020) did not report the concentration of LH. The basal level of LH is not different between the luteal phase and the follicular phase in goats (Kanai and Ishikawa, 1988) or humans (Häggström, 2014), while the LH pulse frequency is dramatically higher in the follicular phase than in the luteal phase (Schillo, 2009). Therefore, it seems that the basal secretion of LH is not responsive to physiological variations in the concentration of sex steroids that occurs during the ovarian cycle. The present study suggests that the relationship between amplitude of CRT and basal LH secretion is also steroid independent since I used the ovariectomized goats.

4.4.1 Possible pathway linking the CRT and LH secretion

The absence of a correlation between LH pulse frequency and any characteristic of the CRT and the presence of the relationship between mean LH concentration and amplitude of CRT raised the question of the nature of pathways linking the two parameters. The relationship between the amplitude of the CRT and the mean LH would not be mediated via the GnRH pulse generator as there are no correlation between LH pulse frequency and the CRT. An

alternative possibility could be that factors that affect the CRT also act on the downstream of the GnRH pulse generator, such as at the pituitary level. Testing an action on the basal secretion of GnRH is not easy since it is technically difficult to distinguish whether the effect of the exogenous stimuli on the basal LH secretion is mediated by the GnRH pulse generator because it is difficult to investigate the activity of the GnRH pulse generator for long period of time under natural conditions (Clarke and Cummins, 1982; Caraty *et al.*, 1984). The understanding of the direct effect of exogenous stimuli on the pituitary functions is limited because, so far, investigations have focused on the interactions of GnRH and sex steroids on the pituitary functions or the effect of environmental toxicants on reproduction (Fraites *et al.*, 2010; Melmed, 2017). Interestingly, the mean LH secretion can be supressed without the change of the frequency of the LH pulses. For example, in cattle experiencing heat stress, the basal secretion of the LH was supressed without changes in the frequency of the LH pulse(Gilad *et al.*, 1993). In addition, it is reported that several neuropeptides have direct effect on pituitary gonadotrophs *in vitro*. Neuropeptide Y (Morel *et al.*, 1989), prolactin (Cheung, 1983) and kisspeptin (Gutiérrez-pascual *et al.*, 2007) stimulate and ghrelin (Fernández-Fernández *et al.*, 2007) suppress the LH secretion into culture medium. These neuropeptides would be good candidate for further analysis as the possible mechanism link the CRT and LH. While it seems more probable that the variation of the mean LH secretion is not mediated via the activity of the GnRH pulse generator but via the deterioration of the pituitary functions.

In addition, there is another possibility that the GnRH neuron located in the POA is affected by the synchronous input of circadian rhythms from thermoregulatory centres within the POA. Since the whole mechanisms regulating the CRT is not elucidated enough, it is difficult to discuss the interaction of GnRH neuron and the neural circuit regulating the CRT. However, recently some reports suggest that the GnRH neuron receives glutamatergic regulation (Iremonger *et al.*, 2010). Considering that the central mechanism regulating the core body temperature is located in POA and it is consist with glutamatergic neurons (Nakamura and Morrison, 2008, 2010a; Morrison and Nakamura, 2019), it might be possible that there is synchronous input on the GnRH secretion and thermoregulation.

Whether the GnRH pulse generator is indeed involved in the relationship between the CRT amplitude and mean LH could be ruled out by measuring multiple unit activity (MUA). The

recording of MUA, that is used in goats, enable to measure the activity of the GnRH pulse generator directly and not through LH secretion (Mori *et al.*, 1991b; Ohkura *et al.*, 2009). Using this technique, I could confirm that the relationship that the activity of the GnRH pulse generator has no relationship with the amplitude of the CRT. However, the MUA technique would not help to test if the amplitude if the CRT change the basal level of GnRH secretion because this technique solely reflects the activity of the pulse generator (Mori *et al.*, 1991a). The measure of the basal secretion of GnRH is only possible with the frequent sampling of the hypophyseal portal blood(Clarke and Cummins, 1982; Caraty *et al.*, 1994).

4.4.2 What induce the relationship of the CRT and LH secretion?

It is possible that the variation of the amplitude of the CRT might have occurred because of differences in the energy status among individual goats. These variation in energy could have then affected the values of mean LH. In the previous report, increased amplitude of the CRT was associated with the restricted energy intake, while decreased amplitude of the CRT was associated with the abundant energy intake (Maloney *et al.*, 2013; Goh *et al.*, 2016). Similarly, in ovariectomized goats, short-term energy restrictions that reduced pulsatile LH secretion is associated with the energy status as measured in body mass index (Tanaka *et al.*, 2002).

It is difficult to conclude if a variation of energy status in my goats could explain the relationship the variability in the amplitude of CRT since the goats were fed at maintenance energy requirement. In addition, body weight and the amplitude of the CRT, and body weight and the mean LH were not correlated, suggesting that change in energy status was not responsible for neither the variation of amplitude of CRT or the relationship between amplitude of CRT and basal level of LH. However, the energy homeostasis cannot be measured using body weight only. Energy status can be assessed by measuring the level of fatness or measuring the levels of endocrine indicators of energy metabolism such as leptin and insulin (Zhang *et al.*, 2004). Unfortunately, the body condition score of goats was not recorded in the present experiment. Including an assessment of the metabolic state, by measuring body condition score or metabolic makers in plasma, in future experiments, would help to elucidate the relationship between energy homeostatic condition, the amplitude of the CRT, and the mean LH.

4.5 Conclusion

In summary, the present study demonstrated that the amplitude of CRT negatively correlates with the mean and basal LH concentration. The existence of a causal relationship needs to be confirmed with the identification of the pathway(s) linking these two parameters is still unclear and could involve brain pathways controlling the amplitude of T_c (thermoregulatory system) and LH secretion (reproductive system). Further studies required to investigate the mechanism parallelly controlling both CRT amplitude and LH secretion. Regardless of our lack of understanding of the mechanism linking both CRT and basal LH secretion, the measurement of T_c and the amplitude of CRT could be used to predict the level of LH secretion of individual animals and their ability of reproduction.

Chapter 5: Central administration of amylin has both facilitatory and inhibitory actions on the gonadotropin-releasing hormone pulse generator in goats

5.1 Introduction

This chapter of the present dissertation was aimed to explore the possible pathway that links the variation of core body temperature (T_c) and reproductive function. This work has been published.

The frequency of pulsatility of gonadotropin-releasing hormone (GnRH) secretion with physiological frequency is important in maintaining normal reproduction in mammals, as evidenced by the suppression of gonadotropin release in rhesus monkeys following chronic GnRH administration (Belchetz *et al.*, 1978b). Alterations in the frequency of pulsatile GnRH secretion are dependent on reproductive status: a higher frequency of pulsatile GnRH secretion is observed in the follicular phase, which includes the accelerated development of ovarian follicles, compared with the luteal phase in ewes (Moenter *et al.*, 1991). Furthermore, pulsatile GnRH administration at a higher frequency results in a higher baseline of pulsatile luteinizing hormone (LH) secretion in ovariectomized (OVX) ewes with hypothalamic-pituitary disconnection (Clarke *et al.*, 1984). Taken together, these studies show that the frequency of pulsatile GnRH secretion is a key determinant for gonadotropin release, which in turn stimulates folliculogenesis.

Pulsatile GnRH secretion is governed by a neural mechanism called the GnRH pulse generator. Accumulating evidence has indicated that kisspeptin neurons in the hypothalamic arcuate nucleus (ARC) are the most probable candidates for the GnRH pulse generator. The kisspeptin neurons in the ARC co-express neurokinin B and dynorphin A; therefore, they are referred to as KNDy neurons in rodents and ruminants (Goodman *et al.*, 2007; Navarro *et al.*, 2009b; Wakabayashi *et al.*, 2010; Hassaneen *et al.*, 2016). Previously, I demonstrated that periodic increases in the multiple unit activity (MUA) volley, an electrophysiological manifestation of the GnRH pulse generator activity (Knobil, 1981; Kawakami *et al.*, 1982; Mori *et al.*, 1991a), are recorded when an electrode is placed near

the ARC KNDy neurons in goats, whereas the MUA volleys are not recorded in the lateral to median eminence, where GnRH neuronal terminals are densely located (Ohkura *et al.*, 2009). This suggests that ARC KNDy neurons are the main component of the GnRH pulse generator.

Recently, I reported that a certain population of kisspeptin neurons express the calcitonin receptor (CTR) gene, *CALCR*, in the ARC and anteroventral periventricular nucleus of female rats (Assadullah *et al.*, 2018), implying that CTR signalling may regulate the GnRH pulse generator activity by affecting ARC KNDy neurons. CTR is a multi-ligand receptor that is widely distributed in the central nervous system of mice (Nakamoto *et al.*, 2000) and rats (Sheward *et al.*, 1994; Becskei *et al.*, 2004). The most characterized physiological action mediated by central CTR-positive neurons is inducing anorexia *via* CTR expressed in the area postrema. Previous studies have shown that infusion of amylin, an endogenous ligand for CTR (Hay *et al.*, 2015), into the area postrema reduces food intake in rats. In line with this, administration of the amylin antagonist, AC187, increased food intake (Mollet *et al.*, 2004). Therefore, I argue that the role of CTR signalling on the activity of KNDy neurons, a putative GnRH pulse generator, should be investigated to understand the mechanisms modulating the GnRH pulse generator activity by using amylin as a CTR agonist.

Amylin-CTR signalling is one of the candidate neural pathways that is involved in the both of thermoregulation and reproduction by mediating the energy-homeostatic cues. Amylin is known to be involved in the regulation of food intake by controlling glucose homeostasis by co-secreted with insulin from pancreatic β-cells (Hay *et al.*, 2015). Amylin is also produced in the brain, and it is suggested to have a role in metabolism (Young, 2005). In addition, amylin is involved in the regulation of core body temperature. Central amylin administration induces an increase in sympathetic nerve activity and increased core body temperature (Fernandes-Santos *et al.*, 2013). Taken together, the amylin-CTR signaling is possible to be the neural circuit involved in the parallel control of the core body temperature and HPG axis.

In the present study, I investigated the role of CTR signalling in the modulation of GnRH pulse generator activity in goats. First, I confirmed *CALCR* expression in the hypothalamus of female goats by RT-PCR. Second, I investigated the effect of central administration of

amylin, an endogenous ligand of CTR, on GnRH pulse generator activity using the MUA recording technique and on LH release in OVX goats to assess the possibility that central amylin-CTR signalling is involved in the regulation of gonadotropin release and GnRH pulse generator activity. Third, I examined the distribution of *CALCR*-expressing cells in the hypothalamus of OVX goats by *in situ* hybridization. Further, I performed double *in situ* hybridization for *KISS1* (kisspeptin gene) and *CALCR* in the ARC of OVX goats to investigate whether KNDy neurons are possible action sites of CTR ligand.

5.2 Materials and Methods

5.2.1 Animals and MUA recording

Adult (2–6 years old) female Shiba goats (n = 6; 20–35 kg) were used for all MUA recordings. All goats were ovariectomized >1 month prior to the start of the MUA experiment. The goats were housed loosely tied to an individual stanchion in a condition-controlled room (12:12 h light: dark cycle; 23 ºC; 50% relative humidity) and maintained with a standard pelleted diet and dry hay. Water and supplemental minerals were available *ad libitum*.

Goats were stereotaxically implanted with an array of bilateral recording electrodes targeting the caudal ARC under halothane anaesthesia, as described previously (Mori *et al.*, 1991a). An 18-gauge stainless steel guide cannula was implanted in the lateral ventricle (LV) for intracerebroventricular administration of amylin, as described previously (Ichimaru *et al.*, 2001). The MUA was recorded in conscious animals and a characteristic increase in the MUA (MUA volley) was determined as the electrophysiological manifestation of the GnRH pulse generator activity, as reported previously (Mori *et al.*, 1991a). In addition, the goats were fitted with an 18-gauge jugular catheter (Medicut; Covidien Japan, Ltd, Tokyo, Japan) for blood sampling at least 1 day before the sampling occurred. All experimental procedures were approved by the Committee on the Care and Use of Experimental Animals in the Graduate School of Bioagricultural Sciences, Nagoya University.

5.2.2 Analysis for *CALCR* mRNA expression in the goat hypothalamus by RT-PCR

To investigate *CALCR* mRNA expression, the mediobasal hypothalamus (MBH), including the ARC or preoptic area (POA) was collected from adult female Shiba goats (n = 3). Tissue was homogenized and total RNA was extracted with Tripure Isolation Reagent (Roche

Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 µg total RNA from each sample using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Goat *CALCR* gene expression was analysed *via* RT-PCR using TaKaRa Gflex polymerase (Takara Bio Inc., Shiga, Japan). The forward and reverse gene-specific primers used for RT-PCR were 5'-CACACTTACACGCTGGTGCT-3' and 5'-GCGGTTGCAGTACAGACCTT-3', respectively. Primers were designed from a predicted goat *CALCR* mRNA sequence (GenBank accession number XM_018047421.1). PCR products were sequenced to determine the partial mRNA sequence of goat *CALCR*. The sequence was compared with the predicted sequence of goat using ClustalW (http:// clustalw.ddbj.nig.ac.jp/).

5.2.3 Central administration of amylin and blood sampling

Central administration of amylin was used to investigate the role of CTR signalling in regulating GnRH pulse generator activity. Amylin was chosen as an endogenous ligand for CTR, because amylin specifically binds to CTR or CTR and other protein complex, namely amylin receptors, to exert its physiological effect (Hay *et al.*, 2015). Further, amylin was expressed in the rat brain, suggesting that amylin is a suitable endogenous CTR ligand to investigate a central role for CTR signalling (Dobolyi, 2009). The mean interval of MUA volleys (T min) was determined by recording the MUA volleys for 6 h (09:00–15:00 h) one day before the experiment. The following day, amylin solution (1.5 or 15 nmol/head in 400 µl of saline) or 400 µl of saline was intracerebroventricularly infused for 1 min into the LV at 0.5T min after the last MUA volley. Blood samples were collected every 6 min for >4 h before and after amylin administration. The plasma was separated from the blood by centrifugation (1,500 rpm, 30 min, 4 °C) and stored at −20 °C prior to use in the LH assay.

5.2.4 LH assay

Plasma LH concentrations were determined using a double-antibody radioimmunoassay, as previously described (Sasaki *et al.*, 2019). The lowest detectable LH concentration was 0.098 ng/ml for 50 µl plasma samples, and the intra- and inter-assay coefficients of variation were 6.2% at 3.14 ng/ml and 7.3% at 3.11 ng/ml, respectively.

5.2.5 *In situ* hybridization for *CALCR* and KISS1

Three OVX goats were sacrificed with an overdose of sodium pentobarbital (19.4 mg/kg body weight). The heads were perfused bilaterally through the carotid arteries with 10 mM PBS containing 13,000U heparin/L and 0.7% sodium nitrite, followed by fixative consisting of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. A brain tissue containing the MBH was immersed in the same fixative overnight at 4°C, then in 30% sucrose in 0.1 M phosphate buffer at 4°C until it sank. Coronal sections of the brain (50-µm thickness) obtained by freezing microtome were rinsed in 0.05 M PBS and stored in the cryoprotectant solution (Watson *et al.*, 1986) at -20°C until used for *in situ* hybridization.

For single *in situ* hybridization for *CALCR*, the *CALCR*-specific digoxigenin isothiocyanate (DIG)-labeled cRNA probe (position 951-1854, GenBank Accession No. XM_018047421) had been synthesized from the goat hypothalamic cDNA by using a DIG-labelling kit (Merck, New Jersey, US). The single *in situ* hybridization was performed as previously described(Assadullah *et al.*, 2018), except for the amount of the probe: 2 µg/mL of anti-sense cRNA probe were used. The *CALCR* signals were examined by an optical microscope (BX53; Olympus, Tokyo, Japan).

Double *in situ* hybridization for *CALCR* and *KISS1* mRNA was performed as previously described [12] using the *CALCR*-specific fluorescein isothiocyanate (FITC)-labeled cRNA probe and the *KISS1*-specific DIG-labeled cRNA probe (position 279-624; GenBank Accession No. MN_001285710). Probes were synthesized from the goat hypothalamic cDNA by using a DIG-labelling kit and FITC-labelling kit (Merck). Brain sections were mounted to gelatin-coated slides and fluorescence images were obtained on a fluorescence microscope (ApoTome; Carl Zeiss, Oberkochen, Germany). The number of *KISS1*-expressing cells or *KISS1*- and *CALCR*-co-expressing cells was counted twice bilaterally in the ARC of one sections of each animal and the number was averaged. No positive signal for *CALCR* or *KISS1* was detected in the brain sections that had been hybridized with the corresponding sense probes.

5.2.6 Statistical analysis

The mean and standard deviation (SD) were calculated for all MUA data (spikes per 20 s) during the experimental period in each treatment for each individual. The start of a MUA

volley was designated as when the count at any time point exceeded 2× SD of the mean MUA. To analyse the MUA data, I determined the interval between the start of two successive MUA volleys (intervolley interval). The intervolley interval between the MUA volleys before and after amylin administration was designated as the first posttreatment interval. The second to fourth posttreatment intervals were also analysed. Statistical differences between amylin-treated and control groups were analysed using two-way ANOVA with repeated measures (between group factor = treatment, within group factor = time), followed by contrast test for multiple comparisons.

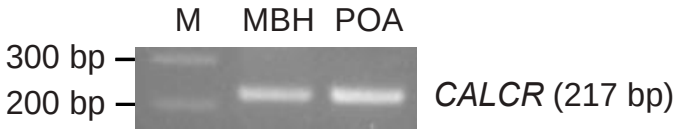
To evaluate the effect of central amylin administration on LH secretion, the percentage change in mean LH concentration during treatment period from the administration to the third volley against same time window during pre-treatment period was calculated within each animal, because basal LH secretion markedly varied among goats within the same group. Statistical differences between amylin-treated and control groups were determined using one-way ANOVA, followed by Dunnett's test. $P < 0.05$ was considered statistically significant for all analyses.

5.3 Results

5.3.1 Examination of *CALCR* mRNA expression in the MBH and POA in OVX goats by RT-PCR

CALCR mRNA was detected in the MBH and POA at the expected size (217 bp) in goats (Figure 5.1A). The mRNA sequence of amplicons (n = 3) was found to be a 100% match to the predicted goat sequence (GenBank Accession No. XM_018047421.1 Figure 5.1B).

A



B

```
goat_#16    CACACTTACACGCTGGTGCTTGACCCTCTTCATCTTCCTGAATCGCCCACTTCCGGTCCT 60
goat        CACACTTACACGCTGGTGCTTGACCCTCTTCATCTTCCTGAATCGCCCACTTCCGGTCCT 60
cattle      CACACTTACACGCTGGTGCTTGACCCTCTTCATCT TCCTGAATCGCCCGCTTCCGGTTCT 60
            ********************************************** ******** **

goat_#16    TCCAGCCAGTTCAGATTCCACCTACTCTCCAACACTGGAGCCGGAGCCATTTCTCTTCCT 120
goat        TCCAGCCAGTTCAGATTCCACCTACTCTCCAACACTGGAGCCGGAGCCA TTTCTCTTCCT 120
cattle      TCCTACCAGTTCAGATTCCACCTACTCTCCAACACTGGAGCCGGAGCCATTTCTCTTCCT 120
            ***  ****************************************************

goat_#16    TTTAGGAAAACAGAGACTGTTGCAGGCACAGCACAAATGCTTTGACCGAATGCAGAAGTT 18 0
goat        TTTAGGAAAACAGAGACTGTTGCAGGCACAGCACAAATGCTTTGACCGAATGCAGAAGTT 180
cattle      TGTAGGAAAACAGAAGCTGTTGCAGGCACAGCACAAATGCTTTGACCGAATGCAGAAGTT 180
            * ************  ******************************************

goat_#16    ACCCCCCTACCAAGGAGAAGGTCTGTACTGCAACCGC 217
goat        ACCCCCCTACCAAGGAGAAGGTCTGTACTGCAACCGC 217
cattle      ACCCCCCTACCAAGGAGAAGGTCTGTACTGCAACCGC 217
            *************************************
```

Figure 5.1: *CALCR* mRNA expression in the goat hypothalamus

RT-PCR analysis of *CALCR* gene expression in the mediobasal hypothalamus (MBH) and preoptic area (POA) of a representative goat (No. 16). The gene-specific primers were designed from a predicted goat *CALCR* mRNA sequence (GenBank Accession No. XM_018047421.1). M, 100-bp ladder. (B) Alignment of *CALCR* mRNA sequences of goat No. 16 and predicted sequence of goat. *, Identical.

5.3.2 Effects of central administration of amylin on MUA volleys and LH secretion as an indicator for GnRH pulse generator activity in OVX goats

Profiles of MUA, plasma LH concentration, and MUA volley intervals in a representative animal receiving an intracerebroventricular infusion of amylin at 1.5 or 15 nmol head^{-1} or vehicle are shown in Figure 5.2. The central administration of amylin immediately evoked an MUA volley in 4 out of 6 goats. Following this evocation, the second and third MUA volley intervals were prolonged. In contrast, MUA volley intervals were constant in the vehicle-treated control group throughout the sampling period. Central amylin administration at 1.5 and 15 nmol head^{-1} significantly decreased the percentage change values of the mean first MUA volley interval compared with vehicle-treated controls, indicating that amylin administration shortened the interval to the first MUA volley (Figure 5.3, $p < 0.05$, contrast

test). Conversely, 1.5 nmol amylin administration significantly increased the percentages change values at the third MUA volley interval after amylin treatment compared with vehicle-treated controls, indicating that the amylin treatment significantly prolonged MUA volley intervals after the second volley (Figure 5.3, $p < 0.05$, contrast test). The percentage change in the concentration of mean plasma LH during the treatment period is shown in Figure 5.4. Both 1.5 nmol and 15 nmol of amylin significantly lowered the mean plasma LH concentration when compared with the vehicle-treated control group (Figure 5.4, $p < 0.05$, Dunnett's test).

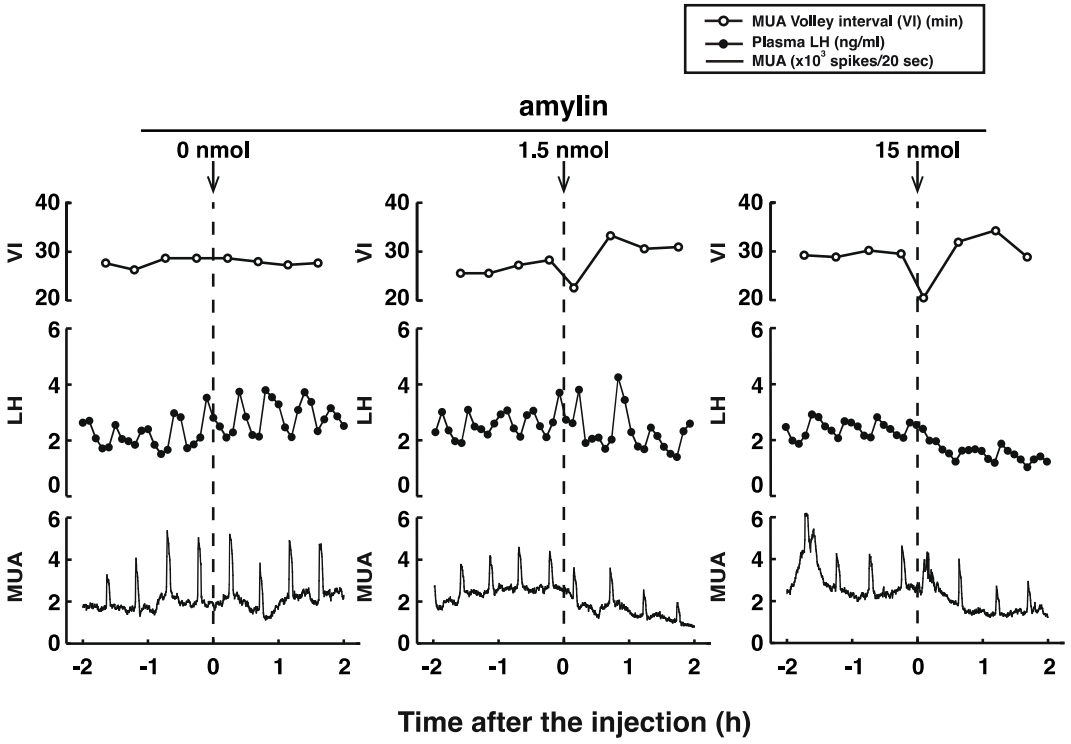


Figure 5.2: Effects of intracerebroventricular injection of amylin on the MUA and pulsatile LH secretion. (Profiles of MUA (solid line), MUA volley intervals (VI, open circles), and plasma LH concentrations (closed circles) in a representative OVX goat treated with 1.5 or 15 nmol amylin or saline. Arrows represent the time of injection (0 h).

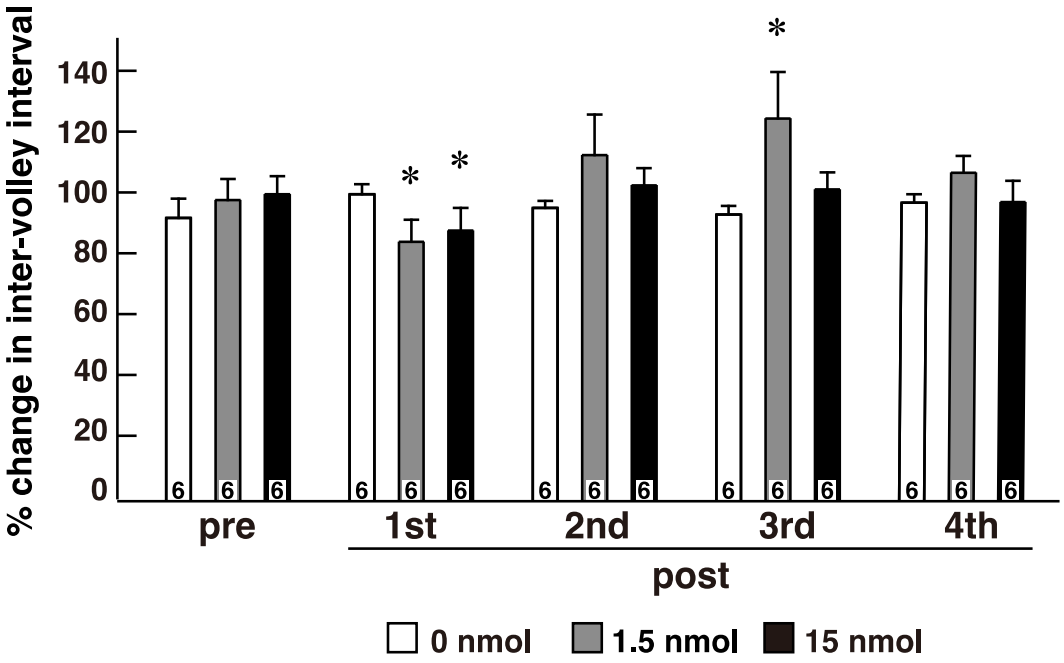


Figure 5.3: Effect of amylin administration on MUA volley intervals. Percentage changes (means ± S.E.M.) in pre- and post- (1st to 4th) MUA volley intervals after intracerebroventricular amylin injection in OVX goats (n = 6). *, $p < 0.05$ vs vehicle-treated group (contrast test).

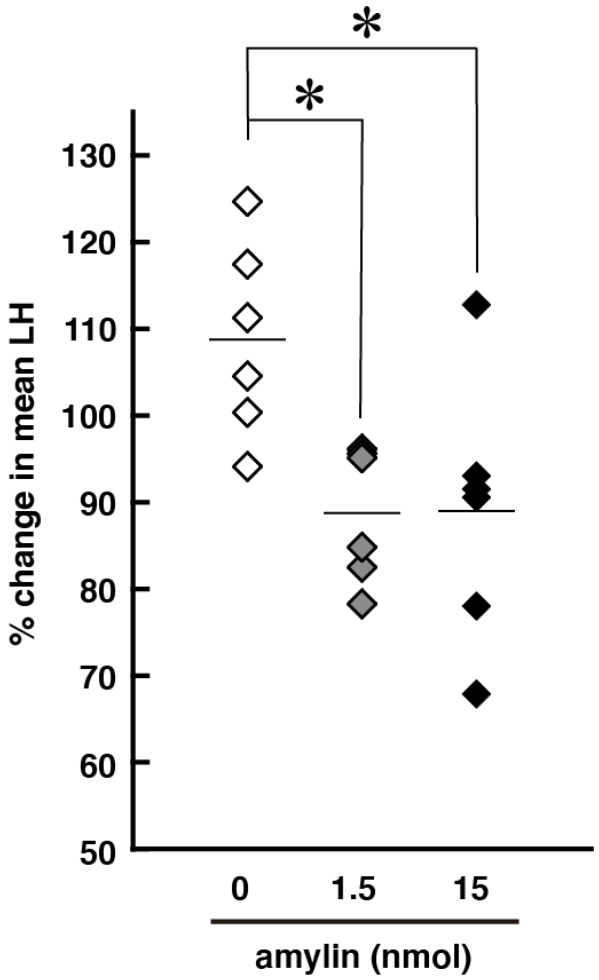


Figure 5.4: Effect of amylin administration on plasma LH concentrations

Percentage changes in mean plasma LH concentration during the treatment period from the time of amylin injection to the third MUA volley against same time window during pretreatment period after 1.5 or 15 nmol amylin or saline. Each square represents individual animals and horizontal bars show mean values of each group. *, $p < 0.05$ *vs* vehicle-treated group (Dunnett's test).

5.3.3 Distribution of *CALCR*-expressing cells in the goat hypothalamus

CALCR-expressing cells were found in the medial POA (MPOA), bed nucleus stria terminalis (BST), suprachiasmatic nucleus (SCN), paraventricular hypothalamic nucleus (PVN), dorsomedial (DMH) and ventromedial (VMH) hypothalamic nucleus, and ARC in OVX goats (Figure 5.5).

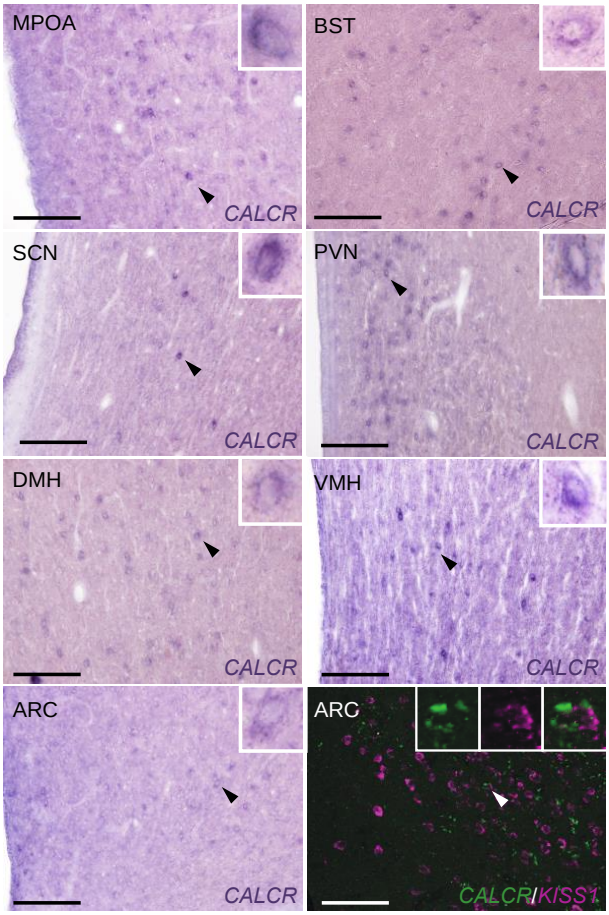


Figure 5.5: Distribution of *CALCR*-expressing cells in the hypothalamus and localization of KISS1- and *CALCR*-coexpressing cells in the ARC in OVX goats

CALCR (purple) expression was visualized by single in situ hybridization in the MPOA, BST, SCN, PVN, DMH, VMH and ARC. The insets indicate *CALCR*-expressing cells (black arrowheads) at higher magnification. *KISS1* (magenta)- and *CALCR* (green)-expression was visualized by double in situ hybridization in the ARC. The inset indicates *CALCR*- and *KISS1*-coexpressing cell (white arrowhead) at a higher magnification. Scale bars, 100 µm.

5.3.4 Examination of *CALCR* expression in the ARC *KISS1*-expressing cells in OVX goats

Little *CALCR* expression was found in *KISS1*-expressing cells in the ARC of goats (Figure 5.5). The number of *KISS1*-expressing cells and *KISS1*- and *CALCR*-co-expressing cells in the goat ARC were 342.0 ± 57.8 and 4.3 ± 1.1 (mean ± SEM, n = 3), respectively. Thus, 1.32 ± 0.27 % (n = 3) of *KISS1*-expressing cells in the ARC showed *CALCR* mRNA expression.

5.4 Discussion

The present study demonstrated that amylin-CTR signalling exerted a facilitatory followed by an inhibitory effect on GnRH pulse generator activity in OVX female goats. This biphasic effect indicates that central CTR signalling has a role in the fine-tuning of reproductive functions by affecting GnRH pulse generation activity in mammals. Indeed, the current study showed that *CALCR*-expressing cells were found in the several brain regions, such as the MPOA, BST, SCN, PVN, DMH and VMH in goats. Further, amylin was infused into the LV, suggesting that it may spread over a wide area in the brain; therefore, amylin-CTR signalling may affect some CTR-expressing cells to exert an facilitatory and inhibitory action on GnRH pulse generation. To the best of our knowledge, this is the first study to raise the possibility that CTR signalling may have a role in the modulation of the activity of the GnRH pulse generator and consequent gonadotropin release.

5.4.1 Facilitatory effect

Central amylin administration immediately induced an MUA volley in OVX goats and *CALCR* mRNA expression was evident in the goat MBH. CTR is a subfamily member of the Gq protein-coupled GPCR superfamily with seven-transmembrane domains (Morfis *et al.*, 2008), indicating that it is a stimulatory receptor. Unexpectedly, *CALCR* mRNA was co-expressed in few ARC KNDy neurons in goats, suggesting that amylin stimulates GnRH pulse generator activity by activating on CTR-expressing cells other than ARC KNDy neurons. It was previously reported a population of ARC KNDy neurons co-expressed *Calcr* mRNA in female rats (Assadullah *et al.*, 2018). Further studies are needed to elucidate the species difference in a role of CTR signalling in regulating GnRH pulse generator activity.

5.4.2 Inhibitory effect

Interestingly, central amylin administration prolonged the MUA volley intervals and suppressed LH secretion after the second MUA volley. The distribution of CTR has reported in various brain regions, such as the POA and DMH, and in the cells surrounding the cerebroventricular in rats (Nakamoto *et al.*, 2000) and monkeys (Paxinos *et al.*, 2004). The current inhibitory effect of amylin-CTR signalling on LH release is consistent with previous studies showing that intravenous injection of calcitonin, another endogenous ligand of CTR,

decreases plasma LH concentration in OVX rats (Tsai *et al.*, 1999). Since calcitonin does not cross the blood-brain barrier, calcitonin administered peripherally might affect GnRH secretion through the circumventricular organs. In addition, a central injection of amylin suppresses sexual behaviour in male rats (Clementi *et al.*, 1999) and amylin mRNA expression increases in the rat MPOA during lactation (Dobolyi, 2009) when LH secretion suppressed (Tsukamura *et al.*, 1990; Maeda *et al.*, 2011). It should be noted that the current intracerebroventricular amylin administration caused only 10 % reduction of LH concentrations, suggesting that amylin-CTR signalling has a subsidiary role in suppression of LH secretion. In this context, immediate stimulatory effect of amylin on MUA volley was also limited (~20 % reduction of inter volley intervals). In the present study, the facilitation of LH release via a rapid stimulatory effect of amylin on the GnRH pulse generator may be negated by its corresponding inhibitory effect on GnRH/LH release. These results suggest that the activation of CTR signalling has a fine-tuning role in regulation of GnRH pulse generator activity and then LH release. Further studies are required to clarify which neurons mediate the stimulatory and suppressive effects of amylin-CTR signalling on GnRH pulse generator activity and then LH release, and biological importance of this signalling. Amylin is reported to specifically bind to CTR or CTR and other protein complex, namely amylin receptors (AMY1, AMY2 and AMY3) (Hay *et al.*, 2015). Determination of amylin expression in the brain and usage of specific CTR antagonist would be a next approach to elucidate the physiological role of central amylin-CTR signalling to orchestrate the GnRH pulse generator activity.

5.5 Conclusion

The present study is the first to raise the possibility that amylin-CTR signalling has a biphasic regulatory role in the modulation of GnRH pulse generator activity and gonadotropin release in female goats. Further study using the antagonistic agents are required to elucidate the physiological role of amylin-CTR signalling on the activity of the GnRH pulse generator. Likewise, it is likely that GnRH pulse generator activity is fine-tuned in males by activation of amylin-CTR signalling, since pulsatility of GnRH/gonadotropin secretion is the fundamental endocrine feature regulating reproduction in both sexes. I hypothesize that the rapid stimulatory and slow inhibitory effects of amylin-CTR signalling on GnRH pulse

generator activity may be mediated by some non-KNDy cells in the goat brain, because few KNDy neurons co-expressed CTR and CTR widely distributed in the goat hypothalamus.

Chapter 6: General discussion

6.1 Summary

In this dissertation, I assess the hypothesis that variation in the core body temperature (T_c) is potential biomarker of fitness and reproductive performance in ruminants. In addition to that, I tried to find a possible neural pathway involved in the dual control of the T_c and the reproductive axis. The topics discussed in the thesis were, 1) the thermal responses of sheep with different temperaments in response to psychological stress, 2) the thermal response of goats to the potential stressors that livestock can experience in farming settings and that they need to adapt to, 3) the relationship between the profile of the circadian rhythm of the core body temperature (CRT) and the LH secretion; an endocrine signal that represents the central control of reproduction, and 4) the role of amylin-calcitonin (CTR) signalling in the control of the GnRH pulse generator as the possible neural pathway that could parallelly control the T_c and reproductive axis. For the first two topics, I examined the effect of possible stressors on the control of the T_c. From the results of these two chapters, I suggest that variation of the T_c could be a good indicator of an animals' fitness. In Chapter 3, I demonstrated a correlation between the amplitude of the CRT and LH secretion, suggesting that the amplitude of the CRT can be used as an index of the endocrine axis. In Chapter 4, I showed that amylin-CTR signalling that is involved in the regulation of both energy homeostasis and the core body temperature may have a role in the modulation of the activity of the GnRH pulse generator. Output from this dissertation adds further evidence to the significance of the measurement of the core body temperature as a biomarker of the fitness of ruminants and indicates the possible neural circuit that affects the central regulator of reproduction and core body temperature.

Validation of core body temperature as a biomarker of animal fitness

My investigations presented on changes in the parameters of the profile of T_c in sheep and goats. My results suggest that the profile of core body temperature is sensitive to several

environmental and physiological changes and therefore could be reliable biomarker of animal fitness to challenging situations.

First, the amplitude of the CRT increased when animals were exposed to energetically challenging situations. In my thesis, it is newly suggested that a strong psychological stress can induce an increase in the amplitude of the CRT (Chapter 2). In addition, my results show that, in hot and humid conditions, the amplitude of the core body temperature is strongly correlated to the ambient temperature (Chapter 3). The amplitude of the CRT would be an indicator of the degree of stress that animals receive from the environment. Furthermore, I report that increased amplitudes of the CRT are associated with a lower level of the LH secretion. My results support that the amplitude of the core body temperature increases when the animals face a range of stressors (Maloney *et al.*, 2019), not only by the psychological stress but also environmental stress such as an increase in ambient temperature since most of these stressors can affect reproductive function, the amplitude of the CRT could be a useful biomarker of animal fitness and could be used to manage animals and maintain their reproduction and productivity.

Aside from the amplitude of the CRT, stress-induced hyperthermia (SIH) could be a useful biomarker, especially to assess the impact of psychological stress on farm animals. Regarding acute psychological stress, sheep displayed stress-induced hyperthermia during the isolation box test and also during exposure to a dog. The stress-induced hyperthermia seemed to be discriminative because it was observed during exposure to psychological stressors but not when the sheep walked without dog guidance even though the motor activity of the sheep significantly increased. Similarly, the amplitude of the SIH response was greater during the exposure to the dog phenotypic in the high responders that reacted more in the isolation box test than the lower responders. However, the SNP in TPH2 had no effect on the size of SIH, indicating the different thermal response of the individuals with contrasting temperament could not be explained by only one SNPs. So SIH seems to be discriminative of the stressors but also is affected by the temperament of a sheep.

The variation in T_c suggests the changes in CRT amplitude could be a strategy to limit the energy expenditure of an animal facing challenges. Thermodynamically, an increase of core body temperature could result in a wastage of energy by livestock, and therefore a loss in

productivity. It has been suggested that a lower core body temperature results in a lower basal metabolic rate BMR (du Bois, 1921). An increase of one degree in core body temperature is associated with a 10 – 13 % increase in oxygen consumption (du Bois, 1921), suggesting an increase of energy expenditure. As the variation of the T_c is energy consuming for animals, the increased amplitude of the CRT and hyperthermia such as SIH might result in a wastage of the energy for livestock. Further investigation into the true energy cost of the variation of the core body temperature in response to psychological or physiological challenges, and the interactions between these two types of challenges, would help to demonstrate that variation in the amplitude of CRT is part of a mechanism that potentially saves energy.

My results indicate that tethering induced a chronic increase of core body temperature under energy restriction when energy restriction is known to induce a decrease in core body temperature. It seems that both psychological stress, such as tethering, and physiological stress such as energy restriction, can act independently to modulate the shape of the CRT. The mechanisms by with these two different stressors can converge in the brain centres that control the CRT are unknown, but my thesis has opened the possibility to explore further these mechanisms.

6.2 Application of the detection of the variation of the temperature

Technically, the measurement of the core body temperature can be both instantaneous and less invasive than most of the classical biomarkers of stress, such as the concentrations of cortisol in saliva or blood. The measurement of hormones in blood or plasma is always post-hoc in that there is a lag between the sampling time and the obtaining of the results from the hormone assay. In my experiments, I used temperature loggers that need to be implanted into the abdominal cavity of sheep and goats, and the implantation of the logger (and their recovery) is invasive as it required abdominal surgery under general anaesthesia. Recently, real-time and non-invasive techniques to measure core body temperature have been developed. One possible method is infrared thermal imaging. A number of studies have assessed the practicability of infrared thermal imaging (IR) for monitoring body

temperature in farm animals (LokeshBabu *et al.*, 2018; Giro *et al.*, 2019a, b; Giannetto *et al.*, 2020) and in wild animals for research purpose (Cilulko *et al.*, 2013). Even though the technology and equipment used for IR detection systems have been improved and algorithm to estimate the core body temperature from the surface temperature have been developed, the current resolution is about ± 0.5 °C which is still too large to accurately measure changes in the amplitude of CRT (Kimata, 2020). In addition, the location of the measurement on the body also affects the result (Blache and Maloney, 2017b)

Recently, another non-invasive T_c monitoring system has been developed. Skin-attachable sensors for measuring core body temperature based on the heat-flux principle enable to monitoring of the core body temperature with an accuracy of 0.1 °C, even when the airflow rate is 5 m s^{-1} (Kitamura *et al.*, 2010; Feng *et al.*, 2017; Tanaka *et al.*, 2021). In near future, these new technologies might be applicable as an instantaneous device to monitor variation in the core body temperature.

6.3 A possible neural circuit involved in the parallel control of T_c and reproduction

Reproductive function is one of the most important components to consider when strategies are designed to improve animal production, as it directly affects the number of animals in the production system. Reproduction is sensitive to an animal's energetic, stress, and health status. Therefore, to predict the capacity of an individual to reproduce, it is important to have a means to assess the animals overall status which could encompass the energetic, stress, and health status. As I describe in my thesis, the factors that affect the T_c are common to the factors that affect reproductive ability. From the results of Chapter 4, it is possible that some mechanisms could parallelly control core body temperature and reproduction. I focused on amylin-CTR signalling because it is one of the candidate neural pathways that is involved in the control of T_c, energy balance, and the activity of the GnRH pulse generator that regulates reproductive function.

The mechanism that generates the CRT is not fully understood. There are strong connections with the master clock in suprachiasmatic nucleus (SCN) that controls the central circadian rhythm. Lesion of the SCN abolishes the CRT in rodents (Krieger *et al.*, 1977; Eastman *et al.*, 1984). The CRT is not only influenced by the SCN, but also by other nuclei.

For example, the amplitude of the CRT is increased in the rat with lesions of the medial preoptic are (MPOA) lesion (Szymusiak *et al.*, 1985). Lesion of the subparaventricular zone (SPZ) that receives a large number of projection from SCN abolishes the CRT while the daily rhythm of activity is maintained (Lu *et al.*, 2001). Recently, it was reported that the arcuate nucleus (ARC) is involved in the control of the CRT in cooperation with SCN. Retrochiasmatic knife cuts that disconnect the SCN and the ARC attenuate the autonomous CRT in animals exposed to continuous dark (Buijs *et al.*, 2017). Thus, the origins of the CRT cannot be explained by a single neural circuit, and several possible brain regions and neural connections that are involved in the generation of the CRT have been reported. Although several possible neural circuits have been proposed to be involved in the generation of the CRT, there are few reports on circuit involved in the investigating the modulation of the amplitude of the CRT. Further investigations are required to elucidate the drivers and neural mechanisms that control the amplitude of the CRT.

Amylin-CTR signalling is a possible mechanism in the control of both the core body temperature and reproductive function. I showed that amylin-CTR signalling has a role in the modulation of the activity of the GnRH pulse generator. I showed that only around 1% of kisspeptin neurons express *CALCR* (CTR gene), therefore, it seems that the modulative effect on the activity of the GnRH pulse generator is mediated via other neurons in the hypothalamus that are responsive to calcitonin and then relay to kisspeptin neurons. Amylin is a peptide hormone that has an anorexigenic and body weight loss effect, suggesting that it has an important role in the control of energy homeostasis. In addition, it has been reported that the administration of amylin induces hyperthermia in rats (Bouali *et al.*, 1995b), and both i.v. and i.c.v. administration of amylin increases energy expenditure (Osaka *et al.*, 2008). Since the effect of amylin is attenuated by the pre-administration of propranolol, the effect of the amylin seems to occur via the sympathetic nervous system (Osaka *et al.*, 2008). Thus, amylin signalling could be an integrator of energy homeostasis and T_c. In addition, it has been reported that amylin-CTR signalling activates proopiomelanocortin (POMC) neurons (Lutz *et al.*, 2018) and a deficiency of the CTR in POMC neurons attenuates the thermogenic response and energy expenditure in response to the administration of amylin (Coester *et al.*, 2020). Thus, the thermogenic effect of amylin could be mediated by the POMC neurons. Furthermore, the administration of MT II, an

agonist of α-MSH that is produced by POMC neurons, increases the activity of the GnRH pulse generator (Matsuyama *et al.*, 2005). It has also been revealed that the suppressive input on the kisspeptin neurons during malnutrition and lactation neuron is mediated by β-endorphin- μ-opioid receptor signalling (Tsuchida *et al.*, 2021) and Dyn- kappa-opioid receptor signalling (Tsuchida *et al.*, 2020). These pathways mediated by opioid receptors are another candidate to mediate the neural input on the GnRH pulse generator. Taken together, amylin-CTR signalling may have an integrative role in T_c, energy homeostasis, and reproduction. Further, as I describe in Chapter 1, the calcitonin receptor is suggested to be an ancient modulator of the CRT (Goda *et al.*, 2018b). Given that *CALCR* mRNA is expressed quite widely in the goat hypothalamus, including the nuclei suggested to be involved in the CRT such as the SCN and the POA, CTR-mediated signalling also has the possibility to control the CRT.

Several studies suggest the parallel control of the central regulator of reproduction and T_c. For example, the activity of the GnRH pulse generator is affected by glucose availability. Disturbance to glucose metabolism by administration of 2-Deoxy-d-glucose (2DG) supresses the activity of the GnRH pulse generator in goats (Ohkura *et al.*, 2004b). The administration of 2DG also has a hypothermic effect (Osaka, 2015). Ghrelin is also related to energy homeostasis and is secreted during food deprivation. The administration of ghrelin suppressed the LH secretion in monkeys, suggesting the suppression of the GnRH pulse generator (Vulliémoz *et al.*, 2004). It has also been reported that the administration of ghrelin induces hypothermia (Sato *et al.*, 2021). Taken together, it seems that the mechanisms activated by high energy availability promote the activity of the GnRH pulse generator and increase T_c, in other hand, mechanisms that are activated during the low energy availability suppress the activity of the GnRH pulse generator and decrease T_c. Unfortunately, few studies have investigated the reproductive status and T_c simultaneously.

In the present study, I didn't measure core body temperature during the administration of amylin. T_c needs to be investigated in concert with activity of the reproductive axis to clarify the relationship between energy homeostasis, the regulation of the T_c, and reproduction. The stimulatory effect of amylin-CTR signalling on the activity of the GnRH pulse generator was transient, after which, the activity of the GnRH pulse generator was suppressed. The activation seemed to be negated by an inhibitory effect afterward. As the administration

was a single injection into cerebral ventricle, the effect of chronic administration should be investigated.

6.4 Merit of application of the lmer method in large animal experiments

In my study, I used linier mixed effect modelling that enabled me to conduct covariate analysis. lmer is a statistical model that enables the inclusion of both of fixed effects and random effects and it is useful to apply to the repeated measurements experimental designs. When we conduct experiments with large animals, it is not realistic to make the experimental situation uniform, as usually the experiments are conducted outside, and various factor affect the result. In addition, large animals such as farm animals have more inter-individual genetic variability than do inbred experimental animals, such as rodents. In addition, we often use the same animals repeatedly in a Latin square design as it is sometimes more difficult to obtain and house a large number of large animals than when working with small laboratory animals. Therefore, I suggest that the lmer is very good way to analyse the results obtained from the experiments conducted with large animals in outside condition, although it has not often been used in the field of physiology.

6.5 Future research direction

I would suggest a few directions for future research based on the results that I obtained during my thesis.

1) Investigation of the relationship between variation in T_c and productivity of livestock

   While there are a lot of studies that have investigated the relationship between stressors and T_c variation, only a few studies have reported the relationship between T_c and productivity itself. To understand the physiological significance of the variation of T_c, it is necessary to obtain the evidence that core body temperature variation is associated with not only physiological status but also with productivity. Such studies will support the paradigm that the profile of T_c is a reliable biomarker that also gauges the productivity of an animal as well as its fitness to the environment. In addition, such

comprehensive analysis would enable creation of new guidelines to monitor the condition of animals and potentially forecast any decline in productivity.

2) Explore of the neural circuitry involved in the integrative control of T_c, energy status, and reproduction

   The mechanisms that generate the CRT and modulate the amplitude of the CRT are still unclear. These mechanisms should be investigated to understand the integration of both energy homeostasis and core body temperature. Especially, the central mechanisms that are involved in the control of energy homeostasis should be investigated to understand its role in the control of T_c and the CRT. Traditional agonist/antagonist approaches are difficult to apply continuously, and so are probably not an appropriate way to investigate these control mechanisms. To analyse any effect on the CRT, chronic modulation of the neural circuitry would be required. The new technologies of gene modification or the use of methods that enable the manipulation of specific neural pathways, such as chemogenetics and optogenetics, could be used to investigate these central mechanisms.

3) Investigate the usefulness of T_c variation as a selection tool to develop better strains of animals with high resilience to environmental stress

   While the selection of animals based on their productivity or emotional reactivity has been conducted, strategies to select animals based on the T_c response to stressors has never been done. In Chapter 2, I found that selection based on phenotype is associated with the size of SIH, as more reactive sheep exhibited more SIH in response to psychological stimuli. It would be interesting to select animals based on their profile of T_c and investigate the association with emotional reactivity.

The selection of the animals based on their resilience to environmental stressors may become more important in future farming as it is predicted that the environment of the livestock industry will become more severe. In my thesis, I found a large variability in the response of T_c to changes in ambient conditions. Therefore, it would be worth investigating the power of selection based on their ability to maintain T_c homeostasis and its effect on their productivity.

6.6 Concluding remarks

The results from this dissertation have added evidence to support the concept that the profile of T_c is a useful biomarker of animals' fitness. The variation in the T_c indicates the fitness of an animal and how it copes with exogenous challenges. In addition, it is also suggested that there is an underlying mechanism that integrates energy balance, core body temperature, and reproductive function. The next challenge is to develop a new method to measure changes in T_c to predict an animals' fitness to both artificial and natural environments.

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