早稲田大学審査学位論文

博士（人間科学）

Role of estradiol in circadian difference of body temperature and heart rate

体温、心拍リズムの時間的差異における エストラジオールの役割

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1. Introduction

1.1 Introduction of the study entitled “normal body temperature of healthy young adults at rest in a thermoneutral environment”.

In most animals, body temperature is an important determinant for metabolism, movement, and neural activity [1–3]. Homeothermic animals, in particular, maintain a constant body temperature using various autonomic and behavioral processes [4]. However, the meaning of the term body temperature is sometimes vague. In large animals, including human beings, the body temperature represents the temperatures of two separated physical compartments: core and shell [5], and reports indicate that thermal inputs from both core body and skin activate thermoregulatory responses [6,7].

I propose that the core body temperature is used as a surrogate for the body temperature in clinical medicine, and accurate monitoring involves placement of a thermometer such as a thermistor probe or thermocouple in the core body, e.g. rectum or esophagus [8,9]. More practical methods such as thermometry in the oral cavity, axilla, and ear canal are used in clinics and at home as the first step in the evaluation of infection, inflammation, and medication effects. These methods aim to assess core temperature although the temperatures measured are those of the body shell. Among them, axillary temperature measurement has been widely used to evaluate patient

temperature for years [9–11], probably due to the ease of axillary access [10]. However, the influence of the environmental temperature and incorrect placement of the thermometer lead to erroneous body temperature measurement [9]. Additionally, some more recently introduced digital thermometers, while capable of producing rapid results, utilize a predictive algorithm that could augment measurement errors and tend to show lower values [12–14].

"Normal body temperature" was defined as the axillary temperature measured using a mercury thermometer (approximately 37.0 °C) [15]. However, axillary temperature varies among people, and temperatures ranging from 36.2 to 37.5 °C are accepted as normal [15,16]. This range may compensate for various factors that influence measurement. The factors include measurement errors and environment temperature. Moreover, I speculate that the wide range of axillary temperature reflects physical and physiological characteristics affecting the shell temperature, such as fat mass, skin blood flow, or basal metabolic rate. The existence of human temperature variation indicates that a comparison of an individual's temperature with the normal range may not accurately evaluate their state of health. Instead, it is more important to compare the individual's current temperature with their personal baseline temperature. For example, I can identify a fever based on a temperature that is 0.5 °C greater than

the personal normal temperature.

In the present study, I aimed to reevaluate the meaning of the normal body temperature determined by measurements of axillary temperature. Previous studies assessed the importance of axillary temperature measurements by comparing them to core temperature measurements [11–13,17–24]. However, these studies were limited to small groups, patients, and newborns. Therefore, I first compared the axillary and tympanic temperatures of over 100 healthy subjects of a similar age in the same thermoneutral environment and during the same season. Tympanic temperature was utilized as a surrogate for core temperature [25,26]. I also compared each subject's perceived personal baseline body temperature with the axillary temperature I recorded. Finally, I tested my hypothesis that axillary temperature deviations are related to physical, physiological, and behavioral characteristics.

1.2 Introduction of the study entitled "the effect of estradiol depletion on daily changes of body temperature and heart rate in female rats".

Women entering menopause often experience a decrease in plasma female hormones (i.e., estradiol and progesterone) and changes in the rhythms of hormonal secretions [27]. In addition, some women experience physical and/or mental disorders

(e.g., hot flashes, night sweats, and mood swings). Clinical data from patients who received surgical oophorectomy or hormone replacement therapy indicate that the disorders were caused by depletion of female sex hormones [28–31]. However, such disorders are not found during the pre-pubertal period and gradually disappear after menopause. Thus, it is speculated that the effect of estradiol depletion is transient.

Hot flashes [32–34] are a common physical disorder in menopausal women and are characterized by the sudden onset of hotness and palpitation. During hot flashes, skin blood flow increases, sweating occurs, and core temperature decreases [35]. Their incidence may be related to daily rhythm, as several studies have reported that hot flashes occur at a specific time of a day [36–38]. Thus, the depletion of female hormones appears to affect thermoregulation, cardiovascular function, and circadian rhythmicity. This speculation is supported to some extent by research that indicates circulating estradiol is involved in thermoregulation in female rats. Uchida et al. (2010a, 2010b) assessed the effect of estradiol on thermoregulation by comparing ovariectomized rats that did and did not receive estradiol replacement. They suggested that estradiol lessens the reduction in core temperature during cold exposure by affecting autonomic and behavioral thermoregulatory processes [39,40]. The influence of estradiol on heart rates has also been reported in female rats: they demonstrate

tachycardia after the depletion of plasma estradiol [41–44]. Ovariectomy augments the expression of the β_1-adrenoreceptors of the cardiomyocytes in rats [45–47], which may affect heart rates by increasing pacemaker rhythm of the heart (i.e., chronotropic action) [48].

Depletion of plasma estradiol and progesterone starts within an hour after surgical removal of the ovaries in rats [49]. To assess the influence of estradiol depletion, previous studies have compared ovariectomized animals to sham-operated/estradiol-dosed animals [39,40,50] or investigated animals with genetic depletion of estradiol or estradiol receptors [51]. However, these studies could not clarify the time-effect after the estradiol depletion. In addition, I do not know if the influence includes the circadian components of the core temperature and heart rate. Thus, it remains unclear i) how the depletion of estradiol is manifested through thermoregulation and cardiovascular function, and ii) whether the influence is observed throughout the day or at a specific time of day. The aims of the present study were to clarify whether the depletion of plasma estradiol affected daily changes of core temperature and heart rates and how the effects changed after depletion. I conducted 24-h measurement of core temperature and heart rates after surgical removal of the ovaries or stopping estradiol replacement in ovariectomized rats, for 21 days. I investigated the effect of the changes

that altered plasma estradiol from high to low level, to replicate peri-menopause. In addition, I evaluated changes in plasma norepinephrine level and the expression of the β-adrenoreceptors of the cardiomyocytes.

1.3 Introduction of the study entitled “the effect of estradiol depletion on thermoregulatory response and its circadian difference during a heat exposure in female rats”.

Depletion of estradiol is involved in body temperature regulation in female animals and menopause women [33,52]. Some women suffer from hot flashes which are characterized by episodic activation of heat dissipation (i.e., skin vasodilatation) and sweating [34]. In addition, they experience drop of body core temperature after the flashes [35]. It has been reported that the symptoms of hot flashes occur at a specific of a day [36–38]. Therefore, the depletion of estradiol affects circadian rhythm of body temperature. However, it still remains unclear how estradiol affects circadian body temperature regulation.

Homeotherms have a biphasic pattern of body core temperature during a day, that is, it is low in an inactive period, and high in an active period. In rats, the inactive period is in a light phase and the active period is in a dark phase because they are nocturnal

animals. I have previously shown that the body core temperature shows the biphasic pattern during a day in female rats, although body core temperature is transiently decreased in the middle of the dark phase by the depletion of estradiol [53]. The fluctuation of body core temperature is determined by the opposing processes of heat production and heat loss. The decrease in body core temperature is associated with a decrease in heat production by reducing oxygen consumption and increase in heat loss from a tail which is a major effector of heat loss in rats [54]. The oxygen consumption in the dark phase does not change by depletion of estradiol in female rats [55,56]. It has been reported that the tail skin temperature is increased by skin vasodilation during the dark phase in the low-estradiol rats [57,58]. Therefore, the depletion of estradiol may transiently increase the heat loss in the dark phase, resulting in a decrease in body core temperature.

In a high ambient temperature, body core temperature is maintained by the promotion of heat loss and inhibition of heat production. Previous reports showed that body core temperature in the ovariectomized rats is higher than that in the estradiol-treated rats in the high ambient temperature [57,59,60]. These previous studies reported that the tail skin temperature in the ovariectomized rats are also higher than in the estradiol-treated rats [57,59]. However, there are no reports which showed

that whether the heat production was reduced or not in the ovariectomized rats exposed to heat. Thus, it is still unknown how depletion of estradiol affects heat production response in the high ambient temperature. Therefore, the mechanism for the increase of body core temperature in the ovariectomized rats in the high ambient temperature is not fully understood. Moreover, these previous reports only indicated the effect of heat exposure in the light phase on body core temperature. The regulations of heat loss and heat production may differ in the light or dark phases in the high ambient temperature in the female rats.

The purpose of the present study was to clarify whether the depletion of estradiol has an influence on body core temperature to heat exposure in the light or dark phases. I conducted 24-h measurement of core and tail temperatures and metabolic heat production in the female rats. In addition, I investigated the mechanism of the changes of body core temperature by affecting heat production and loss responses during heat exposure in the light or dark phases in the female rats.

2. Normal body temperature of healthy young adults at rest in a thermoneutral environment

2.1. Summary

The aims of this study were to 1) evaluate whether recently introduced methods of measuring axillary temperature are reliable, 2) examine if individuals know their baseline body temperature based on an actual measurement, and 3) assess the factors affecting axillary temperature. Subjects were healthy young men and women (n= 76 and 65, respectively). Three measurements were obtained: 1) axillary temperature using a digital thermometer in a predictive mode requiring 10 seconds ($T_{ax\text{-}10\ sec}$), 2) axillary temperature using a digital thermometer in a standard mode requiring 10 minutes ($T_{ax\text{-}10\ min}$), and 3) tympanic membrane temperature continuously measured by infrared thermometry (T_{ty}). Subjects answered questions about eating and exercise habits, sleep and menstrual cycles, and thermoregulation, and reported what they believed their regular body temperature to be (T_{reg}). T_{reg}, $T_{ax\text{-}10\ sec}$, $T_{ax\text{-}10\ min}$, and T_{ty} were 36.2 ± 0.4 °C, 36.4 ± 0.5 °C, 36.5 ± 0.4 °C, and 36.8 ± 0.3 °C (mean ± SD), respectively. There were correlations between T_{ty} and $T_{ax\text{-}10\ min}$, T_{ty} and $T_{ax\text{-}10\ sec}$, and $T_{ax\text{-}10\ min}$ and $T_{ax\text{-}10\ sec}$ (r = .62, r = .46, and r = .59, P < .001, respectively), but not between T_{reg} and $T_{ax\text{-}10\ sec}$ (r = .11, P

= .20). A lower $T_{ax\text{-}10\ sec}$ was associated with smaller body mass indices and irregular menstrual cycles. Modern devices for measuring axillary temperature may have changed the range of body temperature that is recognized as normal. Core body temperature variations estimated by tympanic measurements were smaller than those estimated by axillary measurements. This variation of axillary temperature may be due to changes in the measurement methods introduced by modern devices and techniques. However, axillary temperature values correlated well with those of tympanic measurements, suggesting that the technique may reliably report an individual's state of health. It is important for individuals to know their baseline axillary temperature to evaluate subsequent temperature measurements as normal or abnormal. Moreover, axillary temperature variations may, in part, reflect fat mass and changes due to the menstrual cycle.

2.2. Methods

(1) Subjects

Healthy college students were recruited for the study (76 males and 65 females, aged 20.7 ± 1.6 and 20.7 ± 1.9 years (mean ± SD), respectively). Experiments were conducted from August to October (autumn in Japan). Body temperature was measured

from 2:00 – 3:00 pm in an experimental room maintained at an ambient temperature of 25.4 ± 2.0 °C and relative humidity of 62 ± 9% (mean ± SD, respectively). All subjects were instructed to wear light clothing (such as T-shirts and long pants) and no subjects reported discomfort during the study. Written informed consent was obtained from all individual participants prior to commencing the study. The Human Research Ethics Committee of the Faculty of Human Sciences of Waseda University approved all the procedures. The study was also conducted in accordance with the Declaration of Helsinki.

Subjects were instructed to avoid exercise the day before the experiment and eschew food intake for one hour before arriving at the experimental room. In addition, I verified that subjects wore lighter clothes. While sitting in a chair for at least 30 minutes, the subjects completed a questionnaire (12 questions for males and 13 for females) about sleep and eating habits, menstrual cycle (females), exercise, and body temperature. The questionnaire included a question asking each participant to state his or her own regular body temperature (T_{reg}).

Axillary temperatures were determined with a digital thermistor probe (MC612, Omron Healthcare, Inc., Kyoto, Japan). The accuracy and resolving power of the thermistor sensor are ± 0.1 °C and 0.1 °C, respectively. The measurement was

performed twice, using different modes provided in the probe. One temperature was obtained using the standard mode: subjects were asked to place the thermometer in their axilla until the temperature displayed was stable, which usually took 10 minutes ($T_{ax\text{-}10\ min}$). The other was assessed using the predictive mode: subjects were instructed to place the thermometer in the same manner, and the value was determined by an algorithm (based on the immediate increase in temperature that occurs when the subject places the instrument) within 10 seconds ($T_{ax\text{-}10\ sec}$). Subjects conducted these measurements by themselves after instruction by a researcher.

After the measurement of $T_{ax\text{-}10\ min}$ and $T_{ax\text{-}10\ sec}$, the subject's tympanic membrane temperature (T_{ty}) was monitored with an infrared sensor probe (CE Thermo, NIPRO Corp., Osaka, Japan), as a surrogate for estimating core temperature [25,26]. The sensor probe was placed in the left ear canal with the assistance of a researcher. The probe occluded the ear canal, and the researcher adjusted the placement of the probe so as to make the sensor show the highest value (i.e., ideal direction of the probe). The data was recorded at 30-s intervals and stored on a computer, till when the value became stable (± 0.1 °C for 3min, usually took 10 – 15 min).

Body mass index was calculated as weight (kg)/height^2 (m^2) and classified as follows: underweight, 18.4 kg/m^2 or below; normal weight, 18.5 – 24.9 kg/m^2; overweight,

25.0 kg/m^2 or above [61].

(2) Statistics

I drew histograms demonstrating the grouped T_{reg}, $T_{ax\text{-}10\ sec}$, $T_{ax\text{-}10\ min}$, and T_{ty} data. The data for each temperature measurement method was divided into interval widths of 0.3 °C each, from 35.1 °C to 37.2 °C, and less than 35.1 °C and greater than 37.2 °C. The skewness and kurtosis were determined for each distribution (IBM SPSS Statistics for Windows, Version 22.0., IBM Corp., NY, USA). I hypothesized that the skewness and kurtosis were both 0 if the data showed normal distribution.

The difference of means between T_{reg}, $T_{ax\text{-}10\ min}$, $T_{ax\text{-}10\ sec}$, and T_{ty} was assessed by the one-way analysis of variance using SPSS software. A post hoc test was conducted using the Bonferroni method.

The correlations between $T_{ax\text{-}10\ min}$ and $T_{ax\text{-}10\ sec}$, $T_{ax\text{-}10\ min}$ and T_{ty}, and T_{reg} and $T_{ax\text{-}10\ sec}$ were evaluated by Pearson's test. Fisher's z-transformation test was performed to examine the difference between the correlations. Linear regression analysis was also conducted using the method of least squares.

I assumed a causal relationship between $T_{ax\text{-}10\ sec}$ results and the questionnaire answers. First, I divided the subjects into two groups based on their $T_{ax\text{-}10\ sec}$: one group

with measurements lower than the $T_{ax\text{-}10\ sec}$ median and the other with higher measurements. Each answer of the questionnaire was digitized and compared between the two groups using Student's *t*-test. The null hypothesis was rejected at $P < .05$. All values are expressed as the mean ± SD.

2.3. Results

Figures 1A-D show the frequency distributions of T_{reg}, $T_{ax\text{-}10\ sec}$, $T_{ax\text{-}10\ min}$, and T_{ty}, respectively. The mean values were 36.2 ± 0.4 °C, 36.4 ± 0.5 °C, 36.5 ± 0.4 °C, and 36.8 ± 0.3 °C, respectively. Any pair of the means was different ($P < .05$). The median values of T_{reg}, $T_{ax\text{-}10\ sec}$, $T_{ax\text{-}10\ min}$, and T_{ty} were 36.2 °C, 36.4 °C, 36.5 °C, and 36.9 °C, respectively. The skewness was -0.40, 0.04, -0.66, and -0.82 in T_{reg}, $T_{ax\text{-}10\ sec}$, $T_{ax\text{-}10\ min}$, and T_{ty}, respectively, and the kurtosis was 0.51, -0.28, 0.54, and 1.02, respectively.

Figure 2 shows scattergrams demonstrating the relationship between T_{ty} and $T_{ax\text{-}10\ min}$ (A), T_{ty} and $T_{ax\text{-}10\ sec}$ (B), $T_{ax\text{-}10\ min}$ and $T_{ax\text{-}10\ sec}$ (C), and T_{reg} and $T_{ax\text{-}10\ sec}$ (D). There were significant correlations between T_{ty} and $T_{ax\text{-}10\ min}$, T_{ty} and $T_{ax\text{-}10\ sec}$, and $T_{ax\text{-}10\ min}$ and $T_{ax\text{-}10\ sec}$ ($r = .62$, $P < .001$; $r = .46$, $P < .001$; and $r = .59$, $P < .001$, respectively). However, there was no significant correlation between T_{reg} and $T_{ax\text{-}10\ sec}$ ($r = .11$, $P = .20$). The linear regression line equations for T_{ty} and $T_{ax\text{-}10\ min}$, T_{ty} and $T_{ax\text{-}10\ sec}$, and $T_{ax\text{-}10\ min}$ and

$T_{ax\text{-}10\ sec}$ were: $y = 0.82x + 6.26$, $y = 0.64x + 12.94$, and $y = 0.62x + 13.80$, respectively. The r-value for T_{ty} and $T_{ax\text{-}10\ min}$ was greater than that for T_{ty} and $T_{ax\text{-}10\ sec}$ ($z = 2.64$, $P = .01$).

Table 1 summarizes the comparison of each answer in the questionnaire between the two groups we had defined by subject $T_{ax\text{-}10\ sec}$. The group with a $T_{ax\text{-}10\ sec}$ below the median $T_{ax\text{-}10\ sec}$ had a lower body mass index, and women in the groups had irregular menstrual cycles.

2.4. Discussion

The term “normal body temperature” is often used in clinical medicine and at home; however, the definition should be more sharply defined to avoid misunderstanding. I aimed to answer three fundamental questions regarding the normal body temperature (usually assessed by the value of axillary temperature) in the present study. First, I analyzed if variation in axillary temperature between subjects originated from a) technical errors in the measurement process, for example, incorrect placement of the measurement device, b) technological problems with the instrument itself, for instance, with the predictive algorithm, or c) individual differences in core body temperature. Second, I tested whether the body temperature subjects identified as their personal

body temperature corresponded with their measured body temperature. Third, I investigated if differences in axillary temperature reflect differences in physical, physiological, or behavioral characteristics or vice versa.

I obtained T_{ty} using continuous infrared thermometry as a surrogate for estimating core temperature. It has been reported that the value obtained by this method correlates well with the esophageal temperature at an ambient temperature 19 – 24 °C [25,26]. The ambient temperature is a factor affecting the reliability of the infrared thermometry. In addition, the sensor is needed to face to the tympanic membrane. The sensor probe used in the present study was designed to correct the influence by monitoring the ambient temperature and to fit to the ear canal, pointing to the tympanic membrane (based on the manual of the maker). I also tried to increase the reliability of the measurement by the methods as follows, besides collecting data of more than 100 subjects. Measurements were conducted in a stable thermoneutral environment to minimize deviations from the core temperature. A researcher conducted the placement of the probe, making the sensor face to the tympanic membrane. The probe occluded the ear canal, which also minimized the influence of the ambient temperature. Moreover, I continuously measure T_{ty}, till when the value became stable (usually took 10 – 15 min). I assume that, even if the sensor did not correctly point to the tympanic membrane, the

stabilizing period allowed the inner-ear temperature to become identical to the temperature of the tympanic membrane. The average was 36.8 ± 0.3 °C, and the coefficient of variation was 0.8%. This finding together with the higher value of the kurtosis for the frequency distribution of T_{ty} could suggest that there was little interindividual difference in the core temperature. Although the skewness was less than 0, the result may also suggest the accuracy of the measurement method (Figure 1D).

Reports indicate that axillary temperature, although measured at the body surface, correlates well with core temperature [11,13,17,21–24]. In the present study, I also found a significant correlation between T_{ty} and $T_{ax\text{-}10\ min}$ (Figure 2A), although the frequency distribution of $T_{ax\text{-}10\ min}$ was different from that of T_{ty} (smaller kurtosis, Figures 1C and D). Moreover, the regression slope was 0.82. These results may suggest that under the conditions present during my measurements, axillary temperature closely approximates the core temperature as previously reported. However, the value showed greater variation among subjects compared to T_{ty}. In addition, T_{ty} was higher than $T_{ax\text{-}10\ min}$ as previously reported [62]. Because the measurements were conducted in a similar environment and under the instruction and supervision of researchers, factors leading to measurement errors [9] may have been negligible.

There was a significant correlation between T_{ty} and $T_{ax\text{-}10\ min}$, and T_{ty} and $T_{ax\text{-}10\ sec}$

(Figures 2A and B). The correlation coefficient (*r*) for the correlation between T_{ty} and $T_{ax\text{-}10\ sec}$ was lower than that for the correlation between T_{ty} and $T_{ax\text{-}10\ min}$. These results confirm that $T_{ax\text{-}10\ sec}$ includes a greater error in estimated axillary temperatures as indicated by previous reports [12–14]. Moreover, the mean of $T_{ax\text{-}10\ sec}$ was lower than that of $T_{ax\text{-}10\ min}$, which suggests that it is necessary to know an individual's personal regular temperature as obtained by the standard method. This knowledge may help individuals to assess whether they have a fever correctly and, thus, evaluate their state of health more accurately.

It seems to be accepted that "normal body temperature" is around 37.0 °C on average, although a range around this value (36.2 to 37.5 °C) is considered within normal limits [15,16]. However, in the present study, the averaged values estimated by axillary temperature measurements using a digital thermometer were lower than the accepted normal value (i.e. 36.2 °C on average). The reason remains unclear. Differences in sensor material and the use of a predictive mode may have resulted in lower values.

In recent years, the axillary temperature is usually measured using a predictive mode, and all of the study participants regularly used this method of measurement. However, we did not find any correlation between T_{reg} and $T_{ax\text{-}10\ sec}$ (Figure 2D). This

result may suggest that the body temperature subjects believe to be their regular temperature is not based on the values they previously measured. However, I may have misinterpreted the data, because axillary temperature shows daily fluctuations and is influenced by the menstrual cycle [63,64].

Subjects with a $T_{ax\text{-}10\ sec}$ below the median tended to have a lower body mass index (28% underweight, 0% overweight) compared to those whose $T_{ax\text{-}10\ sec}$ was above the median (14% underweight, 78% normal weight, 8% overweight) (Table 1). A smaller subcutaneous fat mass may affect axillary temperature and be a factor involved in the interindividual difference we found. In addition, female subjects in the former group had irregular menstrual cycles. The disturbance of body temperature related to irregular menstrual cycles may influence axillary temperature.

Twenty subjects (14% of the total subjects) reported a T_{reg} below 36.0 °C. Eight of these subjects had a $T_{ax\text{-}10\ sec}$ of < 36.0 °C. Although I did not find any relationship between these two variables (Figure 2D) for all subjects, these eight subjects may have reported their regular body temperature based on the knowledge of previous actual measurements.

An individual's axillary temperature is judged as normal or abnormal based on a previously determined value (i.e., 37.0 °C), and it is well known that normal body

temperature varies [15,16]. The present results may indicate that the normal temperature range has changed due to the use of digital thermometers that utilize predictive algorithms. My experiment included young and healthy subjects in an environment controlled to minimize factors that could potentially affect axillary temperature, although I did not consider gender differences including those related to menstruation. Even in the experimental environment, I found important interindividual differences. The present study suggests that each individual needs to be aware of his or her baseline temperature. The interindividual differences in axillary temperature may, in part, reflect measurement errors. However, I found a lower $T_{ax\text{-}10sec}$ was associated with lower body mass indices in male and female subjects and with irregular menstrual cycles. Because I did not assess physiological parameters such as metabolism, skin temperature, and skin blood flow, which may influence axillary temperature, the reasons for the observed interindividual variation remain unclear. Future studies are needed to clarify the mechanism underlying this variation.

Modern axillary temperature measurement techniques may have changed the range of normal body temperature; nonetheless, they are reliable enough to estimate core body temperature adequately. However, even between young and healthy subjects at rest in a comfortable environment, normal temperature shows significant variation.

My results suggest each individual should know his or her own regular temperature. Moreover, axillary temperature may reflect individual physical differences and diverse physiological states. Axillary temperature still has importance in evaluating health status, not only for determining if a fever is present but also for defining our baseline state of health.

3. The effect of estradiol depletion on daily changes of body temperature and heart rate in female rats

3.1. Summary

We assessed the influence of estradiol depletion on daily changes of core temperature, spontaneous activity, and heart rate in female rats. In addition, we evaluated the effects of estradiol depletion on β-adrenoreceptors (AR) in the heart and plasma norepinephrine. Rats were bilaterally ovariectomized and two tubes that either contained estradiol (E_2) or were empty (C) were placed subcutaneously in each rat's abdominal cavity. The tubes were removed 10 days later. Core temperature and heart rates were continuously measured by biotelemetry before and 1, 7, and 21 days after removal of the tubes (PRE and Days 1, 7, and 21). Core temperature was higher in the E_2 group than in the C group at 2330–0130 h on PRE and on Days 1–21. Core temperature exceeded the PRE level in the E_2 group at 1430–1830 h on Days 1–21. Heart rates were lower in the E_2 group than the C group throughout the day on PRE and on Days 1–21. β_1-AR expression and plasma norepinephrine levels were lower in the E_2 group than in the C group on PRE. Heart rates in the E_2 group exceeded the PRE level on Days 1–21. We concluded that the depletion of plasma estradiol modulates daily

changes of core temperature and heart rate, an effect that occurs immediately after the estradiol depletion. In addition, plasma norepinephrine and β-AR in the heart may at least partially affect heart rate.

3.2. Methods

Adult virgin female Wistar rats (n = 65; body weight, 150–250 g; age, 7–9 w; Takasugi Experimental Animals Supply, Saitama, Japan) were used in the present study. They were individually housed in plastic cages (45 × 25 × 20 cm) at an ambient temperature of 25°C under a 12/12-h light/dark cycle (lights on at 0700 h). Food and water were available ad libitum. By obtaining vaginal smears every day for 10 days, I verified that the rats all had regular estrous cycles. All experimental procedures were approved by the Institutional Animal Care and Use Committee, Waseda University.

(1) Experiment 1: The effect of estradiol on core temperature, spontaneous activity, and heart rates in female rats

Rats (n = 14) underwent surgery under inhalation anesthesia with 2% isoflurane (Abbott Japan, Tokyo, Japan) and air. A radio transmitter device for the measurements of core temperature, spontaneous activity, and electrocardiogram (26 × 8 mm, 2.2 g; PDT-4000 HR E-Mitter, Mini Mitter Company, Bend, USA) was implanted in the

abdominal cavity of each rat. The electrocardiogram was obtained from two electrode wires of the device that were placed under the chest skin. A bilateral ovariectomy was performed from the retroperitoneum. Penicillin G (1,000 U; Meiji Pharmaceutical, Tokyo, Japan) was subcutaneously injected to prevent post-surgical infection. Then, two silicon tubes (inner diameter 1.57 mm, outer diameter 3.18 mm, length 30 mm; Kaneka, Osaka, Japan) containing 17β-estradiol (E_2; Sigma-Aldrich, St. Louis, USA) were subcutaneously placed in the rats of the treatment group (n = 7, E_2 group), and two empty tubes were subcutaneously placed in the rats of the control group (n = 7, C group). The procedure for the preparation of the estradiol-containing tubes and the effect of these tubes on plasma estradiol level after placement were previously reported [40]. Briefly, each tube was filled with 50–60 mg of 17β-estradiol powder, and both ends were sealed with glue. The placement of the E_2 tubes provided a constant plasma level of estradiol at least 9 days. In both groups, the tubes were removed 10 days after the surgery (Day 0) under anesthesia as previously described (Figure 3). Each rat's core temperature, spontaneous activity, and electrocardiogram information were recorded for 24 h before Day 0 (PRE) and on Days 1, 7, and 21. Heart rates were assessed form the R-R interval of the electrocardiogram.

(2) Experiment 2: The effect of estradiol on expression of β_1- and β_2-adrenoreceptors of the left cardiac ventricle

Rats (n = 30) were divided into two groups (n = 15 each in the E_2 and C groups), as in Experiment 1 and underwent the same surgery and removal of the tubes on the same schedule (Figure 3). In this experiment, five rats in each group were killed by i.p. injection of overdose pentobarbital Na^+ (Kyoritsu Seiyaku, Tokyo, Japan) at 1300 h on PRE and Days 7 and 21, respectively. The cardiac muscle of the left ventricle and the blood of each animal were sampled.

(3) Analyses of the cardiac muscle and blood

The sampled cardiac muscle (200 mg) was homogenized in RIPA buffer. After the supernatant was decanted, the proteins were collected in a sample buffer (4× Laemmli Sample Buffer, Bio-Rad, Hercules, USA) and denatured for 5 min at 95°C. The protein concentration was determined by the BCA method (Pierce BCA Protein Assay Kit, Thermo Scientific, Waltham, USA). A 60-μg sample of protein was separated by 8% polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate and transferred to a polyvinylidene difluoride membrane. After being incubated in blocking solution (0.3% skim milk), the membrane was allowed to react with a rabbit polyclonal

primary antibody for β_1- and β_2-adrenoreceptors (ab3442 and 36956, respectively; 1:1200; Abcam, Cambridge, England) for 1 h at room temperature. The membrane was washed three times with Tween 20 in Tris-buffered saline and then allowed to react with the secondary antibody (horseradish peroxidase-linked donkey anti-rabbit IgG; 1:5000, NA934; GE Healthcare UK, Amersham, England). The signal was developed by applying the substrate (HRP substrate; Immobilon Western; Millipore, Billerica, USA) and detected by chemiluminescence (LAS3000, FUJIFILM, Tokyo, Japan). The membrane was then blotted with mouse primary antibody of β-actin (1:1000, ab8226; Abcam, Cambridge, England) and the secondary antibody (horseradish peroxidase-linked rabbit anti-mouse IgG; 1:5000, ab6728; Abcam, Cambridge, England). The washing procedure and the signal detection were conducted in the same manner for the β_1- and β_2-adrenoreceptors. The intensity of the protein signals was determined with Multi Gauge V3.0 software (FUJIFILM, Tokyo, Japan). The expression levels of the β_1- and β_2-adrenoreceptors were shown as the relative values to that of β-actin.

The blood was centrifuged at 4°C. The estradiol and norepinephrine levels in the plasma were determined by enzyme-linked immunosorbent assay (Estradiol EIA Kit; Cayman Chemical, Ann Arbor, USA; and Noradrenaline Research ELISA; LDN,

Nordhorn, Germany, respectively). The detection limits of estradiol and norepinephrine were 20 pg/ml and 0.1 ng/ml, respectively. The coefficient of variation of estradiol and norepinephrine were <13% and <12%, respectively.

(4) Statistics

All values are shown as the means ± standard error (SE). Values for core temperature and spontaneous activity were averaged every 30 min. Heart rate was averaged over 24-h period. Differences between C and E_2 groups in each day were determined by two-way analysis of variance (ANOVA; SPSS, Chicago, USA). Differences in daily changes among four treatment days were evaluated by two-way ANOVA with repeated measures with SPSS. Post -hoc tests were conducted by the Tukey method. Differences in heart rate among four treatment days were evaluated by one-way ANOVA with repeated measures with SPSS. Difference in heart rate between C and E_2 groups was assessed by Student's *t*-test. The null hypothesis was rejected at $P < 0.05$.

3.3. Results

(1) Plasma estradiol level

Figure 4 shows the estradiol level in the plasma on PRE and Days 7 and 21 in the C and E_2 groups. On PRE, the level was higher in the E_2 group than in the C group (205.1 ± 20.6 and 36.7 ± 16.3 pg/ml, respectively). In the C group, plasma estradiol remained unchanged on PRE and on Days 7 and 21. On Days 7 and 21, the plasma estradiol level decreased from the value on PRE in the E_2 group. There were no differences in the plasma level between the two groups on Days 7 and 21.

(2) Experiment 1

Figure 5 shows the 24-h change in core temperature on PRE in the C and E_2 groups (A), and on Days 1, 7, and 21 in the C and E_2 groups (B-1 and B-2, respectively). The PRE value in each group was included in Figures 5B-1 and 2 to clarify the difference (dashed lines). On PRE, the core temperature was greater in the E_2 group than in the C group at 2330–0130 h (Fig. 5A). The difference became the greatest at 0100 h (0.8 ± 0.1°C).

There were no differences in core temperature among the values on PRE and on Days 1, 7, and 21 in the C group (Figure 5B-1). In the E_2 group, core temperature was higher than the PRE value at 1600–1800 h on Days 1, 7, and 21; however, there was no difference among the values during the three days (Figure 5B-2). Core temperature

throughout Day 7 in the E_2 group was greater than that on PRE in the C group. In addition, core temperature on Day 21 in the E_2 group was greater than that on Day 7 in the C group at 1000–1230, 2330–0130, and 0430–0630 h.

Figure 6 illustrates the 24-h change in spontaneous activity on PRE in the C and E_2 groups (A), and on Days 1, 7, and 21 in the C and E_2 groups (B-1 and B-2, respectively). The PRE value in each group was included in Figures 6B-1 and 2 to clarify the difference (dashed lines). There was no difference among the values for four days in each group (Figure 6B-1 and 2).

Figure 7 shows the average over 24-h period in heart rates on PRE in the C and E_2 groups (A), and that on PRE, Days 1, 7, and 21 in the C and E_2 groups (B-1 and B-2, respectively). The PRE value in each group was included in Figs. 7B-1 and 2 to clarify the difference (dashed lines). On PRE, heart rates in the E_2 group were smaller than those in the C group (419 ± 13 and 363 ± 13 beats/min on average, respectively; Figure 7A).

In the C group, heart rates on Days 1 and 7 remained unchanged from the values on PRE; however, those on Day 21 were lower than those of PRE and Day 1 (369 ± 16, 419 ± 13, and 409 ± 16 beats/min on average, respectively; Figure 7B-1). In the E_2 group, heart rates on Day 7 exceeded those of PRE (406 ± 13 and 363 ± 13 beats/min

on average, respectively; Figure 7B-2). Heart rates on Day 7 in the E_2 group did not differ from those on PRE in the C group. In addition, heart rates on Day 21 in the E_2 group also did not differ from those on Day 7 in the C group.

(3) Experiment 2

Figure 8 shows the protein expression of the β_1- (A) and β_2-adrenoreceptors (B) of the left ventricle. The photo images on the top of each graph denote western blotting signals from PRE and Days 7 and 21 in the C and E_2 groups. The expression of the β_1-AR was lower in the E_2 group than in the C group on PRE (Figure 8A). In the C group, the expression levels on Days 7 and 21 were lower than on PRE. However, in the E_2 group, no differences were observed on Days 7 and 21 from the value on PRE. In addition, there were no significant differences between the two groups on Days 7 and 21.

The expression of the β_2-AR was greater in the E_2 group than in the C group on PRE and on Days 7 and 21 (Figure 8B). The values on Days 7 and 21 did not differ from the value on PRE.

Figure 9 shows the norepinephrine level in the plasma. The plasma level was lower in the E_2 group than in the C group on PRE. In the C group, the level on Day 7 fell below

that of PRE, while the level on Day 21 exceeded the PRE level. In the E_2 group, the levels on Days 7 and 21 were higher than on PRE. The value on Day 21 exceeded that of Day 7 in each group. On Days 7 and 21, there were no significant differences between the C and E_2 groups.

Table 2 summarizes the data for core temperature, heart rates, spontaneous activity, expression levels of β-AR, and plasma levels of norepinephrine and estradiol during the measurement period in Experiments 1 and 2.

3.4. Discussion

In the present study, I studied whether estradiol depletion modulates daily changes of core temperature and heart rate. I evaluated the effect of the estradiol through two different comparisons: i) comparing the core temperature and heart rate rhythms in ovariectomized rats that did and did not receive estradiol replacement; and ii) comparing the core temperature and heart rate before and after estradiol withdrawal from ovariectomized rats that had been receiving estradiol replacement. The estradiol depletion affected both core temperature and heart rate in a different ways.

(1) Plasma estradiol level

On PRE, plasma estradiol levels in the E_2 group rose to 6 times those in the C group. The level in the C group remained similar to that in the diestrus phase in normal rats (i.e., the lowest level in the estrus cycle) [40]. There were no differences among the values on PRE and Days 7 and 21 (Figure 4, Table 2) in the C group. The level in the E_2 group was 2 times higher than that in the proestrus phase in normal rats (i.e., the highest level in the estrus cycle) [40]. A previous study which used the same method of estradiol replacement showed that the physiological data were similar with proestrus level [40]. The estradiol level in the E_2 group on Days 7 and 21 approached that of the C group.

(2) Comparison of daily core temperature rhythm between the C and E_2 groups

A difference in daily change of core temperature between the C and E_2 groups was observed on PRE (Figure 5A, Table 2), although it was limited to the 2-h period in the middle of the dark phase. Previous studies reported that, during the dark phase, core temperature did not differ between ovariectomized rats that did and did not receive estradiol replacement [57,58]. However, these studies did not assess the daily change. Sanchez-Alavez et al. (2011) reported a similar decrease in core temperature during the dark phase, using ovariectomized mice [50].

My data showed that spontaneous activity seemed to decrease, in association with the reduction of core temperature on PRE in the C group (Figure 6A). It has been reported that spontaneous activity decreases and paradoxical sleep during the dark phase increases after ovariectomy in rats [65–67]. As reported previously, estradiol shortened a period of the free-running activity rhythm of blind in rats [68] by changing the expression of clock-related genes [69]. Thus, the change in circadian core temperature rhythm may have partially reflected this spontaneous activity and disordered sleep rhythm.

Hosono et al. (2001) reported greater tail vasodilation in ovariectomized rats during heat exposure [70]. In women suffering hot flashes, core temperature becomes lower during a peak frequency of hot flashes than that in asymptomatic women [38]. A sudden reduction in core temperature in the C group was observed at the time when the daily core temperature rhythm peaked in the E_2 group. Nagashima et al. (2003) showed that, in male rats, the tail surface temperature exceeds that of the core temperature during dark phase [71], suggesting that tail skin blood flow is elevated. I did not assess the tail skin temperature in the present study. The results suggest that thermoregulatory skin vasodilation may have been augmented due to the circadian increase in core temperature, resulting in a decrease in core temperature.

(3) Changes in daily core temperature rhythm in the E_2 group

The comparison of the core temperature rhythms between the C and E_2 groups indicated that the depletion of plasma estradiol might be associated with the daily core temperature change in the C group. However, on Days 1, 7, and 21 in the E_2 group, the core temperature did not fall below the value on PRE was observed during the dark phase. On the contrary, core temperature increased from the PRE level during the late light phase on all three dates (Figure 5B-2, Table 2). Spontaneous activity also did not change from the PRE level on Days 1, 7, and 21 (Fig. 6B-2, Table 2). Thus, activity did not contribute to the change in daily core temperature rhythm in the E_2 group. Therefore, the estimated effect of the estradiol depletion on daily core temperature rhythm differs substantially between the two study conditions.

A comparison of the rhythms on PRE in the C group and on Day 7 in the E_2 group, (i.e., 9 days after ovariectomy and 7 days after estradiol withdrawal), revealed that core temperature in the E_2 group exceeded that in the C group throughout the day (Table 2). In addition, the same comparison between Day 7 in the C group and Day 21 in the E_2 group (17 days after the ovariectomy and 21 days after the estradiol withdrawal) indicated greater core temperature in the E_2 group during several periods in both dark

and light phases (Table 2). Previous report showed that postmenopausal women who discontinued hormone replacement therapy had vasomotor symptoms (e.g., hot flashes and night sweats) again [29]. These results suggest that hormonal state prior to estradiol depletion was reflected in a change of the core temperature rhythm. For example, greater estradiol level or presence of plasma progesterone may affect averaged level of daily core temperature change.

The effect of ovariectomy on daily core temperature rhythm remained unchanged on PRE and Days 1–21 in the C group. Moreover, on Day 7–21 in the E_2 group, the core temperature rhythm did not change from that on Day 1 in the E_2 group. Thus, the effect of estradiol depletion on the core temperature rhythm was evident immediately and lasted to Day 21.

(4) Comparison of heart rate between the C and E_2 groups

The heart rates were higher in the C group than in the E_2 group on PRE (Figure 7A, Table 2). However, the daily change of amplitude was similar between the two groups (153 ± 16 and 134 ± 9 beats/min in the C and E_2 groups, respectively). The results clearly suggest that the effect of the estradiol depletion on heart rate (but not on core temperature) was similar throughout the day. In addition, estradiol depletion appears to

affect core temperature and heart rate through different mechanisms. Previous studies reported that single injection and chronic replacement of estradiol decreased heart rates in ovariectomized rats [41–43]. The plasma norepinephrine level was higher in the C group than in the E_2 group (Figure 9). He et al. [43] reported that ovariectomized rats had greater sympathetic nerve activity, a finding that supports my results. The change in heart rates may reflect greater sympathetic nerve activity throughout the course of the day, although it remains unknown whether plasma norepinephrine was also higher throughout the day.

Another mechanism for the increased heart rates in the C group may be the expression of β-adrenoreceptors. β_1-AR is associated with the chronotropic action of the heart [48], which was greater in the C group than in the E_2 group (Figure 8A). Previous studies have also reported that protein expression [45–47] and mRNA of the β_1-AR in rats' hearts [47] increased after ovariectomy. The change of β_1-AR expression from PRE to Day 7 suggests that ongoing effects of estradiol depletion (Figure 8A). On the contrary, β_2-AR expression was lower in the C group than in the E_2 group (Figure 8B). β_2-AR is related to inotropic action of the heart. Several studies have shown that estradiol administration induces hypertension in ovariectomized rats [44,72–76]. Thus, the lower levels of β_2-AR expression may reduce the inotropic action of the heart,

inducing a compensatory increase in heart rate to maintain cardiac output.

(5) Changes in heart rate in the E_2 group

In the E_2 group, the heart rates increased from those of PRE throughout Day 7 (Figure 7B-2, Table 2). Plasma norepinephrine increased from the PRE level on Days 7 and 21. However, there was no effect of estradiol depletion on the β-adrenoreceptors. Thus, estradiol depletion may increase heart rates via sympathetic activation, but not through changes in β-adrenoreceptors expression.

I compared the heart rate between PRE in the C group and Day 7 in the E_2 group and between Day 7 in the C group and Day 21 in the E_2 group. However, there were no differences in the heart rate. Thus, estradiol depletion increases heart rates despite the previous hormonal state.

Heart rate in the C group on Days 1 and 7 remained unchanged from the PRE level; however, they were lower on Day 21 (Figure 7B-1). A previous report also showed a reduction in heart rate one month after ovariectomy in rats [74]. Thus, the effect of the estradiol depletion on heart rate may extend only for a limited period.

(6) Conclusion

In summary, the depletion of plasma estradiol in female rats immediately affects daily changes of core temperature and heart rate. The variation of core temperature after depletion of estradiol may be influenced by plasma estradiol and/or progesterone levels prior to the depletion. The depletion of estradiol affects daily heart rate rhythm in a different manner from that of the core temperature. The increase of heart rate after loss of estradiol was transient, and also was related to the β-adrenoreceptors in the heart and the plasma norepinephrine level. Among the contributing factors to heart rates, the norepinephrine level may be more important.

These results suggest that core temperature and heart rate rhythms are also modulated in peri-menopausal women, whose estradiol levels decrease rapidly. I speculate that the modulated rhythms are associated with menopause syndromes such as hot flashes and palpitations.

4. The effect of estradiol depletion on thermoregulatory response and its circadian difference during a heat exposure in female rats

4.1. Summary

I assessed the influence of estradiol on thermoregulatory response in female rats exposed to heat. Female Wistar rats were divided to four groups: ovariectomized rats with (E_2 (+)) and without (E_2 (-)) administered estradiol, and sham operated rats in proestrus (P) and diestrus (D) phases. Core temperature (T_{core}) and tail temperature (T_{tail}) were measured with thermistor probes in the abdominal cavity and the proximal part in the subcutaneous tissue of the tail. Oxygen consumption ($\dot{V}O_2$) was measured by open-circuit indirect calorimetry. Nine days after the surgery, at 2.5 h after lights-onset or lights-off, the rats were exposed to the ambient conditions of 28°C, 31°C, and 34°C subsequently for 1h each. In the light phase, there were no differences in T_{core} and $\dot{V}O_2$ between E_2 (-) and E_2 (+) group, and between D and P groups. However, in the dark phase, T_{core} was higher in the E_2 (-) than the E_2 (+) group, and was also higher in the D than P group. In addition, $\dot{V}O_2$ in the E_2 (-) and D groups were remained control level, although $\dot{V}O_2$ during 34 °C exposure in E_2 (+) and P groups were reduced from the control level. T_{tail} tended to be higher in the E_2 (-) than the E_2 (+) group both in the light

and dark phases. T_{core} may be increased in female rats, when plasma estradiol level decreases; however, such response is limited in the dark phase. The increase in T_{core} may be caused by greater metabolic rate even in heat.

4.2. Methods

Adult virgin female Wistar rats (n = 48; body wt, 249 ± 25 g (mean ± SD); age, 9–11 wks; Takasugi Experimental Animals Supply, Saitama, Japan) were used in the present study. They were individually housed in a plastic cage (45 × 25 × 20 cm) at an ambient temperature (T_a) of 25°C under a 12/12-h light/dark cycle (lights-on at 0700 h). Food and water were available ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee, Waseda University.

(1) Surgery

All rats underwent surgery under inhalation anesthesia with 2% isoflurane (Abbott Japan, Tokyo, Japan) and air. A radio transmitter device for the measurements of abdominal temperature (T_{core}), tail surface temperature (T_{tail}), and spontaneous activity (3.5 cm^3, 7.5 g; F40-TT, Data Sciences International (DSI), St Paul, MN, USA) was implanted in the abdominal cavity of each animal. Two wire-type thermistors were

connected to the device (8 cm length): one was placed in the abdominal cavity for T_{core}, and the other was passed through the abdominal cavity, and placed in the subcutaneous space of the tail (the lateral 2 cm from the tail root). Rats were further divided to two groups for two different experiments, which were to evaluate responses to heat in ovariectomized rats with and without estradiol replacement (*Experiment 1*, n = 28) and normal female rats in different phase of estrus cycle (*Experiment 2*, n = 20), respectively.

In *Experiment 1*, rats were bilaterally ovariectomized via the retroperitoneal cavity. Then, a silicon tube (inner diameter 1.57 mm, outer diameter 3.18 mm, length 30 mm; Kaneka, Osaka, Japan) was placed in the subculatenous space of the right back. The tube was filled with or without 17β-estradiol (E_2 (+) or E_2 (-) group, n = 14 each). The tube for the E_2 (+) group was prepared, filled with 50 – 60 mg 17β-estradiol powder (Sigma-Aldrich, St. Louis, MO, USA) and both ends sealed. An empty tube was used for E_2 (-) group. In our preliminary finding, the placement of the tube with E_2 provide a constant level of plasma estradiol for more than 9 days. Rats were allowed to recover from the surgery for 7 days from the surgery.

In *Experiment 2*, rats had just sham operation of the ovariectomy conducted in *Experiment 1*. The rats were sampled vaginal smears each day after the surgery. By

microscopic assessment, the estrus phase was determined [77]. The assessment was repeated until when two regular estrus cycles were observed (at least 10 days). All groups of rats had Penicillin G (1,000 U; Meiji Seika Pharma Co., Tokyo, Japan) injected to the subcutaneous tissue of the back to prevent post-surgical infection.

(2) Heat exposure protocol and measurements

In both *Experiments 1 and 2*, each rat was moved to a Plexiglas box (35 × 20 × 20 cm) in a climatic chamber (Program Incubator IN604, Yamato Scientific, Tokyo, Japan). The box was attached to an airflow system with a constant flow rate of 2.0 l/min. The difference in oxygen tension between the room air and the air that passed through the chamber was determined every 60 s with an electrochemical oxygen analyzer (model LC-700E, TORAY, Tokyo, Japan). Oxygen consumption rate ($\dot{V}O_2$) was determined as the product of the difference in oxygen tension and airflow rate (indirect calorimetry). The values were divided by the 0.75 power of body weight (Brody-Kleiber formula) and corrected to STPD condition. T_{core}, T_{tail}, and spontaneous activity were recorded every 60 s with a data collection system (Dataquest A. R. T. software, DSI), which consisted of a receiver board (model RPC-1, DSI) under the cage connected to a personal computer. T_a in the chamber was continuously monitored with a thermistor and kept at 25.0 ±

0.2 °C. Each rat stayed in the condition for more than two days. The obtained data on the day in the box were used as the time control for those on the next days when heat exposure was conducted. On the heat exposure day, at 0930 or 2130 (2.5 h after lights-onset or lights-off), rats were sequentially exposed to an environment of 28°C, 31°C, and 34°C for 1 h each. In *Experiment 2*, rats were divided to two groups so as to be in the proestrus (P group, n = 10) or diestrus phase (D group, n = 10) on the heat exposure day. Food and water were removed before the heat exposure. Body weight was measured before and after the heat exposure.

(3) Blood sampling

After the heat exposure, rats were killed by i.p. injection of overdose (50 mg/kg body wt) pentobarbital Na^+ (Kyoritsu Seiyaku, Tokyo, Japan). A 2-ml blood sample was taken from the left ventricular cavity and centrifuged at 4 °C, and the plasma was stored at –80 °C. The estradiol level in the plasma was determined by enzyme-linked immunosorbent assay (Estradiol EIA Kit; Cayman Chemical, Ann Arbor, MI, USA). The detection limit of estradiol was 20 pg/ml. The coefficient of variation of estradiol was <13%.

(4) Data Analysis

To assess overall heat loss responses from the body core to the environment, whole body thermal conductance was calculated as $\dot{V}O_2/(T_{core} - T_a)$ [78,79].

The thermistor wire under the tail skin may reflect ambient temperature and body temperature besides skin blood flow of the tail. Therefore, to evaluate the efficiency of heat loss from the tail, we calculated the heat loss index at the tail as $(T_{tail} - T_a)/(T_{core} - T_a)$ [57]. The theoretical value of the index is between 0 and 1.

All values were shown as the means ± standard error (SE). Values of T_{core}, T_{tail}, spontaneous activity, and $\dot{V}O_2$ during the heat exposure were averaged every 5 min. Differences in plasma estradiol level among three groups were determined by one-way analysis of variance (ANOVA). Differences in daily changes and mean values during the temperature exposure among three groups were evaluated by two-way ANOVA. Differences between control and heat exposure in each group were evaluated by two-way ANOVA with repeated measures. Differences in $\dot{V}O_2$, thermal conductance, and heat loss index during control and 34 °C among three groups were also evaluated. Post -hoc tests were conducted by the Tukey method. The null hypothesis was rejected at $P < 0.05$. The statistics were conducted with IBM SPSS Statistics for Windows, Version 22.0. (IBM Corp., Armonk, USA).

4.3. Results

(1) Plasma level of estradiol

Figure 10 shows plasma estradiol levels in the E_2 (-) and E_2 (+) groups (A) in *Experiment 1* and in the D and P groups (B) in *Experiment 2*. In *Experiment 1*, plasma estradiol level was lower in the E_2 (-) group than in the E_2 (+) group in the light phase (46.9 ± 5.7 and 141.9 ± 24.6 pg/ml, respectively) and in the dark phase (53.1 ± 4.4 and 158.9 ± 41.1 pg/ml, respectively). In *Experiment 2*, the level was lower in the D group than in the P group in the light phase (46.8 ± 6.0 and 124.5 ± 29.0 pg/ml, respectively) and in the dark phase (42.6 ± 11.9 and 118.7 ± 6.0 pg/ml, respectively).There were no differences in the plasma level between the E_2 (-) and D groups, and the E_2 (+) and P groups. However, the level tended to be higher in the E_2 (+) than in the P group (P = 0.056).

(2) *Experiment 1,* Responses to heat the E_2 (-) and E_2 (+) groups (influence of estradiol replacement in ovariectomized rats).

Figure 11 shows T_{core} (A and C) and T_{tail} (B and D) during heat exposure. Figures 11A and B denote the data in the light phase, and Figs. 11C and D in the dark phase.

The gray solid and dotted line in each figure is values during the same period on the previous day (i.e. time control at 25°C) in the E_2 (-) and E_2 (+) groups, respectively.

T_{core} in the E_2 (-) and E_2 (+) groups (Fig. 11A) remained at the level of each time control in the light phase. There were no differences in T_{core} between the E_2 (-) and E_2 (+) groups (37.5 ± 0.1°C and 37.3 ± 0.1°C in average, respectively). In the dark phase, T_{core} became higher than the the time control at 100 – 180 min (31°C and 34°C) in the E_2 (-) group, and at 10 – 20 (28°C) and 105 – 180 min (31°C and 34°C) in the E_2 (+) group (Fig. 11C). T_{core} was higher in the E_2 (-) group than in the E_2 (+) group at 55 – 85 (28°C and 31°C) and 120 – 180 min (34°C).

T_{tail} in the light phase were higher than the time control at 25 – 35 and 150 – 180 min (28°C and 34°C) in the E_2 (-) group, and at 0 – 15 and 160 – 180 min (28°C and 34°C) in the E_2 (+) group (Fig. 11B). In addition, T_{tail} was higher in the E_2 (-) group than in the E_2 (+) group at 10 – 30 min (28°C) and 80 – 100 min (31°C). In the dark phase, T_{tail} was higher than the time control at 75–180 min (31°C and 34°C) in the E_2 (-) group, and at 95–180 min (31°C and 34°C) in the E_2 (+) group (Fig. 11D).

Figure 12 shows $\dot{V}O_2$ (A and C) and spontaneous activity (B and D) during heat exposure. Figures 12A and B denote the data in the light phase, and Figs. 12C and D in the dark phase. The gray solid and dotted line in each figure is values during the same

period on the previous day (i.e. time control at 25°C) in the E_2 (-) and E_2 (+) groups, respectively.

$\dot{V}O_2$ in the light phase became lower than the time control at 0 – 20 and 95 – 115 min (28°C and 31°C) in the E_2 (-) group, and at 5 – 10, 95 – 105 and 125 – 135 min (28°C, 31°C, and 34°C) in the E_2 (+) group (Fig. 12A). There were no differences in $\dot{V}O_2$ between the E_2 (-) and E_2 (+) groups.

In the dark phase, $\dot{V}O_2$ was lower than the time control at 50 – 70 and 90 – 180 min (28°C, 31°C, and 34°C) in the E_2 (+) group; however, that in the E_2 (-) group remained unchanged from the time control (Fig. 12C). $\dot{V}O_2$ in the E_2 (-) group was higher than in the E_2 (+) group at 50 – 70, 120 – 125, and 150 – 160 min (28°C, 31°C, and 34°C).

Both in the light and dark phases, spontaneous activity increased from the time control level at the onset of heat exposure (at 28°C) in both E_2 (-) and E_2 (+) groups (Figs. 12B and D). However, the activity remained at the control level during the rest of the period. There were no differences between the E_2 (-) and E_2 (+) groups.

(3) *Experiment 2*, Responses to heat in the D and P groups (differences in estrus phases).

Figure 13 shows T_{core} (A and C) and T_{tail} (B and D) during heat exposure. Figures

13A and B denote the data in the light phase, and Figs. 13C and D in the dark phase. The gray solid and dotted line in each figure is values during the same period on the previous day (i.e. time control at 25°C) in the D and P groups, respectively. T_{core} in the D and P groups remained at the level of each time control in the light phase (Fig. 13A). There were no differences in T_{core} between the D and P groups (37.5 ± 0.2°C and 37.3 ± 0.3°C in average, respectively). In the dark phase, T_{core} was higher than the time control at 5 – 35, 75 – 135, and 150 – 180 min (28°C, 31°C, and 34°C) in the D group, and at 0 – 180 min (28°C, 31°C, and 34°C) in the P group (Fig. 13C). T_{core} in the D group was higher than that in the P group throughout the heat exposure period. Both in the light and dark phases, there were no differences in T_{core} between the E_2 (-) and D groups, and the E_2 (+) and P groups.

T_{tail} in the light phase were higher than the control level at 100 – 130 min (31°C and 34°C) in the D group, and at 160–180 min (34°C) in the P group (Fig. 13B). There were no differences in T_{tail} between the D and P groups. In the dark phase, T_{tail} was higher than the control level at 125 – 180 min (34°C) in the D group, and at 165 – 180 min (34°C) in the P group (Fig. 13D). There were no differences between the D and P groups. Both in the light and dark phases, there were no differences in T_{tail} between the E_2 (-) and D groups, and the E_2 (+) and P groups.

Figure 14 shows $\dot{V}O_2$ (A and C) and spontaneous activity (B and D) during heat exposure. Figures 14A and B denote the data in the light phase, and Figs. 14C and D in the dark phase. The gray solid and dotted line in each figure is values during the same period on the previous day (i.e. time control at 25°C) in the D and P groups, respectively.

$\dot{V}O_2$ in the light phase was lower than the time control at 40 – 50 min (28°C) in the D group, and at 85 – 90 and 125 – 150 min (31°C and 34°C) in the P group (Fig. 14A). In the dark phase, $\dot{V}O_2$ in the P group was lower than the time control at 115 – 135 and 170 – 180 min (31°C and 34°C) (Fig. 14C); however, that in the D group remained at the level of the time control. Both in the light and dark phases, there were no differences in $\dot{V}O_2$ between the E_2 (-) and D groups, and the E_2 (+) and P groups.

Spontaneous activity in the D and P groups were at the level of control both in the light and dark phases (Fig. 14B and D). Both in the light and dark phases, there were no differences in spontaneous activity between the E_2 (-) and D groups, and the E_2 (+) and P groups.

Figure 15 shows the averaged values of $\dot{V}O_2$ (A and C) and thermal conductance (B and D) during heat exposure in *Experiment 1*. Figures 15A and B denote the data in the light phase, and Figs. 15C and D in the dark phase.

In the light phase, the averaged values of $\dot{V}O_2$ during 31°C and 34°C exposure were lower than the value during 28°C exposure, and the value during 34°C exposure was lower than the value during 25°C exposure (Fig. 15A). In the dark phase, the averaged values of $\dot{V}O_2$ during 31°C and 34°C exposure were lower than the value during 25°C and 28°C exposure. The averaged values of $\dot{V}O_2$ during 31°C and 34°C exposure in the E_2 (-) group was higher than in the E_2 (+) group (Fig. 15C; 31°C, 16.9 ± 0.4 and 15.0 ± 0.5 mi/min·kg body $wt^{0.75}$; 34°C, 16.8 ± 0.6 and 14.9 ± 0.6 mi/min·kg body $wt^{0.75}$, respectively).

In the light phase, the averaged values of thermal conductance during 28°C, 31°C, and 34°C exposure were higher than the value during 25°C exposure, the values during 31°C and 34°C exposure were higher than the value during 28°C exposure, and the value during 34°C exposure was higher than the value during 31°C exposure (Fig. 15B). In the dark phase, the averaged values of thermal conductance during 31°C and 34°C exposure were higher than the values during 25°C and 28°C exposure, and the value during 34°C exposure was higher than the value during 31°C exposure (Fig. 15D).

Figure 16 shows the averaged values of $\dot{V}O_2$ (A and C) and thermal conductance (B and D) during heat exposure in *Experiment 2*. Figures 16A and B denote the data in the light phase, and Figs. 16C and D in the dark phase.

Both in the light and dark phases, the averaged values of $\dot{V}O_2$ during 28°C, 31°C, and 34°C in the D and P groups were remained unchanged from 25°C. There were no differences between the D and P groups (Figs. 16A and C).

In the light phase, the averaged values of thermal conductance during 31°C and 34°C exposure were higher than the value during 25°C exposure and the value during 34°C exposure was higher than the values during 28°C and 31°C exposure (Fig. 16B). In the dark phase, the averaged value of thermal conductance during 34°C exposure was higher than the values during 28°C and 31°C exposure (Fig. 16D).

Figure 17 shows the averaged values of heat loss index in the light phase (A) and in the dark phase (B) in *Experiment 1*. In the light phase, the averaged values of heat loss index during 34°C exposure was higher than the value during 28°C exposure in the E_2 (+) group (Fig. 17A; 0.8 ± 0.03 and 0.6 ± 0.02, respectively). In the dark phase, the averaged values of heat loss index during 31°C and 34°C exposure were higher than the values during 25°C and 28°C exposure (Fig. 17B).

Figure 18 shows the averaged values of heat loss index in the light phase (A) and in the dark phase (B) in *Experiment 2*. Both in the light and dark phases, the averaged values of heat loss index during 28°C, 31°C, and 34°C in the D and P groups were remained unchanged from 25°C. There were no differences between the D and P

groups (Figs. 18A and B).

4.4. Discussion

The aim of my study was to evaluate the effect of the estradiol depletion on the T_{core} during heat exposure in the light or dark phases. In addition, I investigated whether heat production or loss responses affect the changes of T_{core} during heat exposure in the female rats. My main finding was that T_{core} was not changed by heat exposure in the light phase, regardless of plasma estradiol level in the female rats; however, in the dark phase, T_{core} increased by depletion of estradiol in the female rats when exposed to heat. In addition, such increase in T_{core} may be caused by greater metabolic rate even in the heat.

(1) Plasma estradiol level

I examined whether the depletion of estradiol affects T_{core} when exposed to high T_a. The plasma estradiol level is lower in the E_2 (-) group than in the E_2 (+) group (Figs. 10A and B). In addition, I examined whether the estrous cycle affects T_{core} when exposed to high T_a in the normal female rats. During the estrous cycle in the normal female rats, the plasma estradiol level is lowest in the diestrus phase and highest in the proestrus phase

[77]. In the present study, the plasma estradiol level in the D group was also lower than that in the P group (Figs. 10C and D). In addition, the level in the E_2 (-) group was similar to that in the D group, and the level in the E_2 (+) group was similar to that in the P group.

(2) In the light phase

In the four groups, the values of T_{core} were not different between during the heat exposure and 25°C exposure (Fig. 11A and Fig. 13A). In the high environmental temperature, homeothermic animals maintain T_{core} by increasing heat loss and decreasing heat production. T_{tail} in a part of the light phase increased from the control in the four groups (Fig. 11B and Fig. 13B), although the values of HLI were not different between 34°C exposure and 25°C exposure (Fig. 17A and Fig. 18A). In addition, $\dot{V}O_2$ in the light phase decreased from the control in the four groups (Fig. 12A and Fig. 14A). These results suggest that the female rats may maintain T_{core} during heat exposure in the light phase by increasing heat loss from the tail and decreasing heat production.

It has been reported that T_{core} in the ovariectomized rats in the light phase increase after heat exposure: 2–3 h exposure at 31°C [59], 3 h exposure at 33°C [57], 2–3 h exposure at 32.5–34°C [80], and 1.5 h exposure at 38°C [60]. However, in the present study, T_{core} was not different between the E_2 (-) and E_2 (+) groups (Fig. 11A), and

between the D and P groups (Fig. 13A). Therefore, the condition of heat exposure (i.e., exposure time and temperature) may affect the change in T_{core} in the light phase in the female rats.

(3) In the dark phase

In the four groups, the values of T_{core} were higher during the heat exposure than during 25°C exposure (Fig. 11C and Fig. 13C), although the T_{core} in the light phase was not different from the control level. It has been reported that the rats exposed to 42°C, which is a high T_a greater than their normal body core temperature, markedly decreased their spontaneous activity not to increase the heat production [81]. However, my data showed that the spontaneous activity during 28–34°C exposure in the dark phase was unchanged from the control level (Fig. 12D and Fig. 14D). My data also showed that there were no differences in the spontaneous activity in the light phase between 28–34°C exposure and the control level (Fig. 12B and Fig. 14B); however, the values of spontaneous activity showed almost zero, because the light phase is the inactive phase in the rats. The previous report showed that the Wistar rats' thermoneutral zone, which is a range of temperature requiring little energy, is approximately range of 28–32°C [82]. Thus, exposure to 28–34°C for the Wistar female rats in the present study may not have

been strong enough to decrease the spontaneous activity in the dark phase. Therefore, the female rats at the hot environmental temperature not in excess of their normal body temperature may increase T_{core} in the dark phase by the effect of this spontaneous activity compared to the light phase.

In the dark phase at T_a of 28–34°C, T_{core} was higher in the female rats which had low plasma estradiol than those which had high plasma estradiol (Fig. 11C and Fig. 13C). In the present study, there were no remarkable differences in T_{tail} and HLI between E_2 (-) and E_2 (+) groups and between D and P groups (Fig. 11D and Fig. 13D, and Fig. 17B and Fig. 18B). Therefore, the heat loss response from the tail during the heat exposure may not be affected by the depletion of estradiol. Previous studies show that the depletion of estradiol increased T_{tail} and HLI during the dark phase in female rats at a T_a of 24–25°C [57,58]. One possible factor is that, by the depletion of estradiol, the heat loss from the tail may become insufficient to reduce T_{core} in the high T_a because the tail skin vasodilation already maximal occurred.

I also assessed the effect of heat production by estimating $\dot{V}O_2$ in the high T_a. In a part of the dark phase at T_a of 28–34°C, $\dot{V}O_2$ was higher in the E_2 (-) group than in the E_2 (+) group (Fig. 12C). In addition, in the E_2 (+) and P groups, $\dot{V}O_2$ decreased from the control level; however, $\dot{V}O_2$ was unchanged from the control condition both in the E_2 (-)

and D groups (Fig. 12C and Fig. 14C). Therefore, the hyperthermia in the E_2 (-) and D groups may be induced by greater heat production. Previous studies show that the depletion of estradiol has no effect in $\dot{V}O_2$ during the dark phase in female rats at a T_a of 20–24°C [55,56]. However, it remains unknown whether the heat exposure affects the mechanism of heat production during the dark phase in the female rats. One possible factor for the great heat production in the heat by depletion of estradiol is greater sympathetic nerve activity. The sympathetic nerve activity induces an increase in metabolic rate. I previously showed that ovariectomized rats, from 9 days after the surgery, had greater heart rates in the dark phase at T_a of 25°C [53] by the greater sympathetic nerve activity.

(4) Behavioral thermoregulatory response

In a high environmental temperature, behavioral thermoregulatory responses (i.e., a grooming with saliva, an extended posture, and an escape behavior) are seen in rats [83]. The escape behavior occurs early in exposure to a high temperature [83]. In the present study, the spontaneous activity seemed to increase in the early phase of heat exposure both in the light and dark phase in the four groups (Figs. 12B and D, and Figs. 14B and D). Therefore, the heat escape behavior may occur equally, regardless of the

plasma estradiol level and light/dark phases. I did not assess the behavioral thermoregulatory response in the present study. However, Baker et al. reported that an evaporative cooling by saliva decrease in ovariectomized rats exposed to 38°C [60]. Therefore, the behavioral response may be associated with the change of T_{core} in the female rats in the heat.

(5) Conclusion

In summary, the depletion of plasma estradiol in female rats increases T_{core} in the dark phase in the heat. The increase of T_{core} in the dark phase may be influenced by not the heat loss but the heat production response even in the heat. In addition, such phenomenon was confirmed in the estrous cycle in the normal female rats.

These results suggest that menopausal women have a risk of the increase in T_{core} in the active phase even if they are exposed to the low T_a than their normal body temperature.

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6. References

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7. Figure Legends

Figure 1. Histograms demonstrating the grouped T_{reg}, $T_{ax\text{-}10\ sec}$, $T_{ax\text{-}10\ min}$, and T_{ty} data. Histograms of (**A**) T_{reg} (median = 36.2 °C, skewness = -0.40, kurtosis = 0.51), (**B**) $T_{ax\text{-}10\ sec}$ (median = 36.4 °C, skewness = 0.04, kurtosis = -0.28), (**C**) $T_{ax\text{-}10\ min}$ (median = 36.5 °C, skewness = -0.66, kurtosis = 0.54), and (**D**) T_{ty} (median = 36.9 °C, skewness = -0.82, kurtosis = 1.02) in healthy young men and women (n = 141). The data for each temperature measurement method was divided into interval widths of 0.3 °C, from 35.1 °C to 37.2 °C, and less than 35.1 °C and greater than 37.2 °C. T_{reg}, the regular body temperature each subject reported in the questionnaire, $T_{ax\text{-}10\ sec}$, axillary temperature measured with a digital thermometer in a predictive mode (10-second measurement), $T_{ax\text{-}10\ min}$, axillary temperature obtained using a standard method (10-minute measurement), T_{ty}, tympanic membrane temperature by infrared thermometry.

Figure 2. Scattergrams for T_{ty} and $T_{ax\text{-}10\ min}$, T_{ty} and $T_{ax\text{-}10\ sec}$, $T_{ax\text{-}10\ min}$ and $T_{ax\text{-}10\ sec}$, and T_{reg} and $T_{ax\text{-}10\ sec}$. Scattergrams for T_{ty} and $T_{ax\text{-}10\ min}$ (**A**), T_{ty} and $T_{ax\text{-}10\ sec}$ (**B**), $T_{ax\text{-}10\ min}$ and $T_{ax\text{-}10\ sec}$ (**C**), T_{reg} and $T_{ax\text{-}10\ sec}$ (**D**) from healthy young men and women (n = 141).

Figure 3. Experimental protocol. C group, ovariectomy without estradiol replacement; E_2 group, ovariectomy with estradiol replacement, and the replacement was stopped one day before Day 1 (Day 0). PRE, one day before Day0; Days 1, 7, and 21, 1, 7, and 21 days after the tubes' removal. E_2 depletion, the plasma estradiol level is similar to that in the diestrus phase in normal rats (shaded area).

Figure 4. Plasma estradiol levels on PRE and Days 7 and 21 in the C and E_2 groups. The values are the means ± SE (n = 5 on each day in the C and E_2 groups, respectively). * Significant difference between the C and E_2 groups, $P < 0.05$. # Significant difference from the value on PRE, $P < 0.05$. E_2, 17β-estradiol.

Figure 5. Daily change of core temperature on PRE and Days 1, 7, and 21 in the C and E_2 groups. **A**) 24-h core temperature on PRE in the C and E_2 groups. **B-1** and **B-2**) the core temperature on Days 1, 7, and 21 in the C and E_2 groups, respectively. Each value of PRE is superimposed as a dashed line. Each datum denotes a 30-min average. The values are the means ± SE (**A**, n = 7 in each group; **B-1**, n = 5; and **B-2**, n = 5) *

Significant difference between the C and E_2 groups, $P < 0.05$. # Significant difference from the value on PRE, $P < 0.05$.

Figure 6. Daily change of spontaneous activity on PRE and Days 1, 7, and 21 in the C and E_2 groups. **A**) 24-h spontaneous activity on PRE in the C and E_2 groups. **B-1** and **B-2**) the spontaneous activity on Days 1, 7, and 21 in the C and E_2 groups, respectively. Each value of PRE is superimposed as a dashed line. Each datum denotes a 30-min average. The values are the means ± SE (**A**, n = 7 in each group; **B-1**, n = 5; and **B-2**, n = 5).

Figure 7. Average heart rate on PRE and Days 1, 7, and 21 in the C and E_2 groups. **A**) average heart rate during 24-h on PRE in the C and E_2 groups. **B-1** and **B-2**) average heart rate during 24-h on PRE, Days 1, 7, and 21 in the C and E_2 groups, respectively. The values are the means ± SE (**A**, n = 7 in each group; **B-1**, n = 5; and **B-2**, n = 5). * Significant difference between the C and E_2 groups, $P < 0.05$. # Significant difference from the value on PRE, $P < 0.05$. † Significant difference between Day 1and Day 21, $P < 0.05$.

Figure 8. Western blotting analysis of the β_1- and β_2-adrenoreceptors (AR) of the left ventricle of the heart. The bands denote the majority of the results obtained from the blotting. The graphs show the relative values of the signal strength of the β_1- and β_2-ARs to β-actin expression. The values are the means ± SE (n = 5 in the C and E_2 groups, respectively). * Significant difference between the C and E_2 groups, $P < 0.05$. # Significant difference from the value on PRE, $P < 0.05$.

Figure 9. Plasma norepinephrine levels on PRE and Days 7 and 21 in the C and E_2 groups. The values are the means ± SE (n = 5 on PRE, Day 7, and Day 21 in the C and E_2 groups, respectively). * Significant difference between the C and E_2 groups, $P < 0.05$. # Significant difference from the value on PRE, $P < 0.05$. + Significant difference between Day7 and Day 21, $P < 0.05$.

Figure 10. Plasma estradiol levels in the E_2 (-) and E_2 (+) groups in the light phase (**A**) and in the dark phase (**B**), and in the D and P groups in the light phase (**C**) and in the dark phase (**D**). The values are the means ± SE (**A, B** n = 7 on each group, **C, D** n = 5 on each group). * Significant difference between the E_2 (-) and E_2 (+) groups or D and P groups, $P < 0.05$. *E_2* 17β-estradiol, *D* diestrus, *P* proestrus.

Figure 11. Changes in body core temperature (T_{core}) and tail skin temperature (T_{tail}) in the light phase (**A, B**) and in the dark phase (**C, D**) in *Experiment 1*, respectively. T_{core} and T_{tail} are shown as the 5 min average. Each value of the control level is superimposed as a gray line. The values are the means ± SE (n = 7 on each group). * Significant difference between the E_2 (-) and E_2 (+) groups, $P < 0.05$. § Significant difference from the baseline value in the E_2 (-) group, $P < 0.05$. # Significant difference from the baseline value in the E_2 (+) group, $P < 0.05$.

Figure 12. Changes in oxygen consumption ($\dot{V}O_2$) and spontaneous activity in the light phase (**A, B**) and in the dark phase (**C, D**) in *Experiment 1*, respectively. $\dot{V}O_2$ and spontaneous activity are shown as the 5 min average. Each value of the control level is superimposed as a dashed line. The values are the means ± SE (n = 7 on each group). * Significant difference between the E_2 (-) and E_2 (+) groups, $P < 0.05$. § Significant difference from the baseline value in the E_2 (-) group, $P < 0.05$. # Significant difference from the baseline value in the E_2 (+) group, $P < 0.05$.

Figure 13. Changes in body core temperature (T_{core}) and tail skin temperature (T_{tail}) in the light phase (**A, B**) and in the dark phase (**C, D**) in *Experiment 2*, respectively. T_{core} and T_{tail} are shown as the 5 min average. Each value of the control level is superimposed as a gray line. The values are the means ± SE (n = 5 on each group). * Significant difference between the D and P groups, $P < 0.05$. § Significant difference from the baseline value in the D group, $P < 0.05$. # Significant difference from the baseline value in the P group, $P < 0.05$.

Figure 14. Changes in oxygen consumption ($\dot{V}O_2$) and spontaneous activity in the light phase (**A, B**) and in the dark phase (**C, D**) in *Experiment 2*, respectively. $\dot{V}O_2$ and spontaneous activity are shown as the 5 min average. Each value of the control level is superimposed as a dashed line. The values are the means ± SE (n = 5 on each group). § Significant difference from the baseline value in the D group, $P < 0.05$. # Significant difference from the baseline value in the P group, $P < 0.05$.

Figure 15. Average $\dot{V}O_2$ and thermal conductance at 25°C, 28°C, 31°C and 34°C in the light phase (**A, B**), and in the dark phase (**C, D**) in *Experiment 1*, respectively. The values are the means ± SE (n = 7 on each group). * Significant difference between the

E_2 (-) and E_2 (+) groups, $P < 0.05$. † Significant difference from the value at 25°C, $P < 0.05$. ‡ Significant difference from the value at 28°C, $P < 0.05$. ¶ Significant difference from the value at 31°C, $P < 0.05$.

Figure 16. Average $\dot{V}O_2$ and thermal conductance at 25°C, 28°C, 31°C and 34°C in the light phase (**A, B**), and in the dark phase (**C, D**) in *Experiment 2*, respectively. The values are the means ± SE (n = 5 on each group). † Significant difference from the value at 25°C, $P < 0.05$. ‡ Significant difference from the value at 28°C, $P < 0.05$. ¶ Significant difference from the value at 31°C, $P < 0.05$.

Figure 17. Average heat loss index 25°C, 28°C, 31°C and 34°C in the light phase (**A**), and in the dark phase (**B**) in *Experiment 1*. The values are the means ± SE (n = 5 on each group). † Significant difference from the value at 25°C, $P < 0.05$. ‡ Significant difference from the value at 28°C, $P < 0.05$.

Figure 18. Average heat loss index 25°C, 28°C, 31°C and 34°C in the light phase (**A**), and in the dark phase (**B**) in *Experiment 2*. The values are the means ± SE (n = 5 on each group).

8. Figures

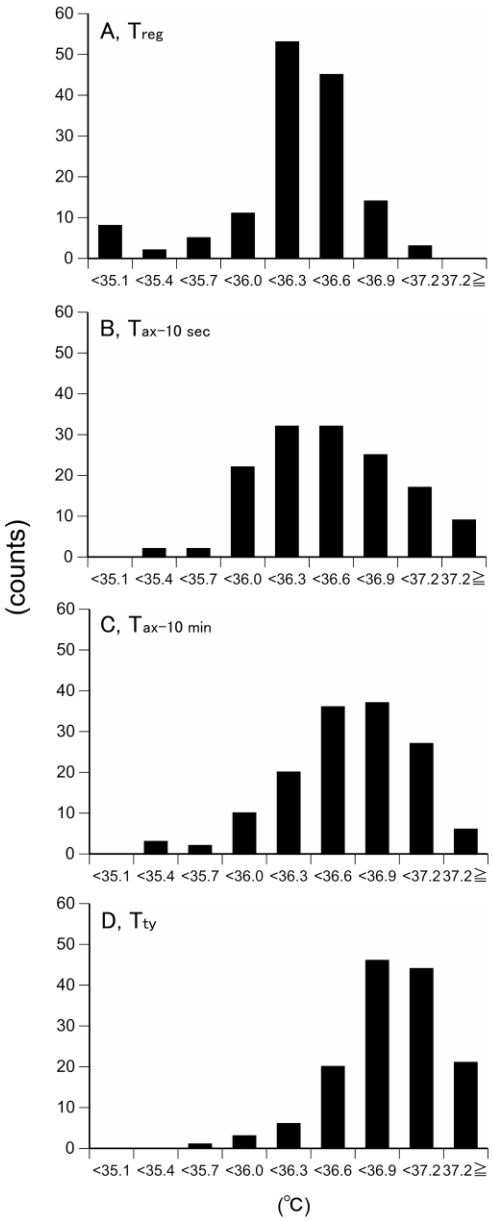

Figure 1. Histograms demonstrating the grouped T_{reg}, $T_{ax\text{-}10\ sec}$, $T_{ax\text{-}10\ min}$, and T_{ty} data

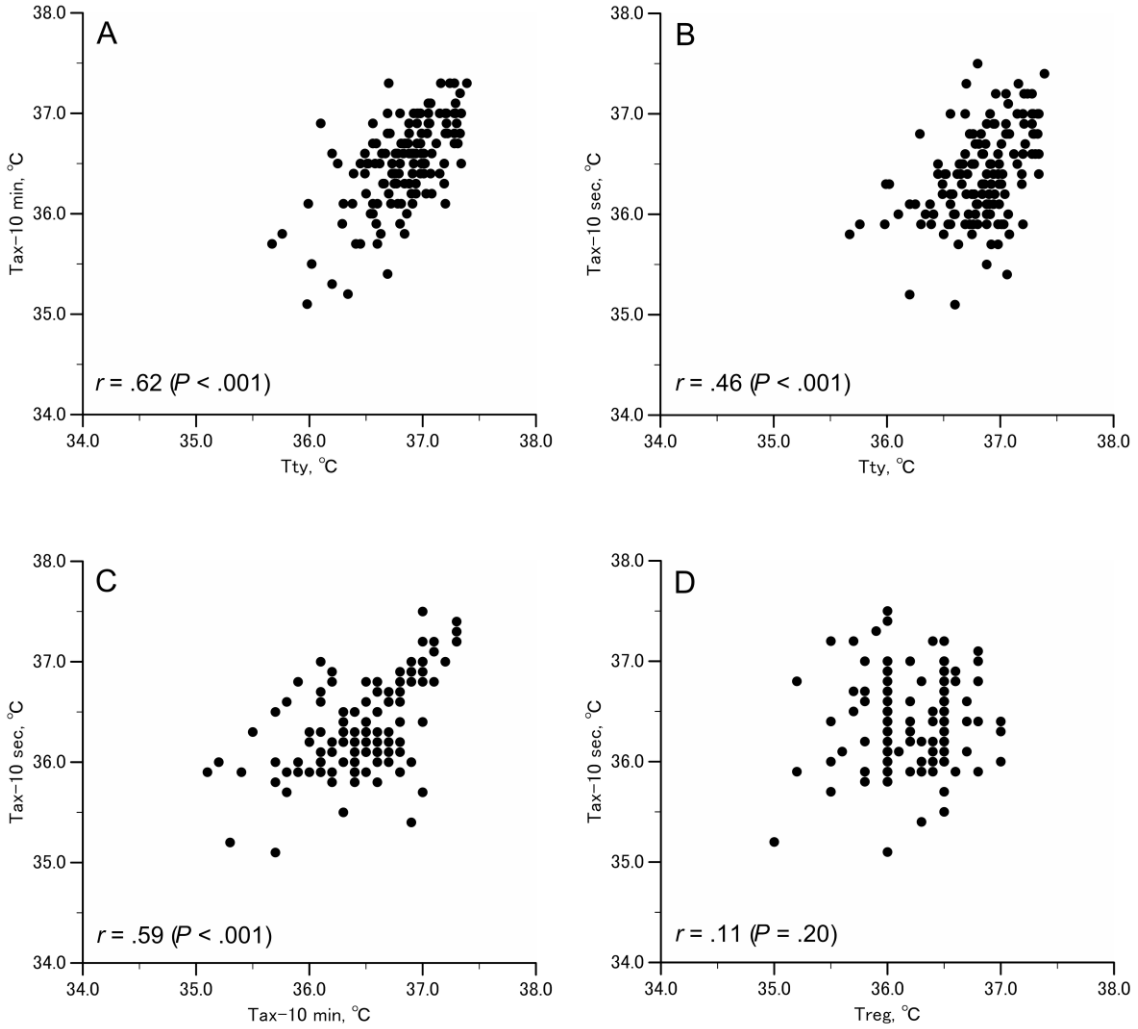

Figure 2. Scattergrams for T_{ty} and $T_{ax\text{-}10\ min}$, T_{ty} and $T_{ax\text{-}10\ sec}$, $T_{ax\text{-}10\ min}$ and $T_{ax\text{-}10\ sec}$, and T_{reg} and $T_{ax\text{-}10\ sec}$

Table 1. The comparison of each answer in the questionnaire between the two groups defined by $T_{ax\text{-}10\ sec}$

	mean ± SD	Tax-10sec< 36.4 ℃	Tax-10sec≥ 36.4 ℃	*P* value (*, if < 0.05)
1. Body mass index (height and body weight)?	20.5 ± 2.2	20.0 ± 1.9	21.1 ± 2.4	.01 *
2. How many times do you have meal a day?	2.8 ± 0.5	2.8 ± 0.4	2.9 ± 0.6	.38
3. Do you eat something between meals? Answer (1: many times 2: sometimes 3: seldom)	1.9 ± 0.8	1.9 ± 0.8	2.0 ± 0.7	.56
4. Do you eat something in midnight? Answer (1: many times 2: sometimes 3: seldom)	2.3 ± 0.8	2.3 ± 0.8	2.4 ± 0.8	.81
5. How many days do you take alcohol a week?	1.0 ± 1.5	1.0 ± 1.4	1.0 ± 1.5	.75
6. Do you think your meal is well-balanced diet? Answer (1: Yes 2: No)	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 0.5	.82
7. Do you try reducing caloric intake to control body weight? Answer (1: Yes 2: No)	1.9 ± 0.3	1.9 ± 0.3	2.0 ± 0.2	.16
8. How many hours do you sleep?	6.7 ± 1.2	6.7 ± 1.3	6.8 ± 1.2	.82
9. How many hours do you exercise a week?	3.9 ± 6.1	3.4 ± 5.3	4.8 ± 6.9	.19
10. Do you think that you are very sensitive to cold environment? Answer (1: Yes 2: No)	1.6 ± 0.5	1.5 ± 0.5	1.7 ± 0.5	.12
11. How often do you use an air conditioner in summer? Answer (1: many times 2: sometimes 3: seldom)	1.9 ± 0.8	1.9 ± 0.8	2.0 ± 0.8	.51
12. How high is your normal body temperature?	36.2 ± 0.4	36.2 ± 0.4	36.2 ± 0.4	.20
13. For only female: Do you have a regular menstruation cycle? Answer (1: Yes 2: No)	1.3 ± 0.5	1.5 ± 0.5	1.2 ± 0.4	.01 *

* *P* values were calculated using Student's *t*-test.

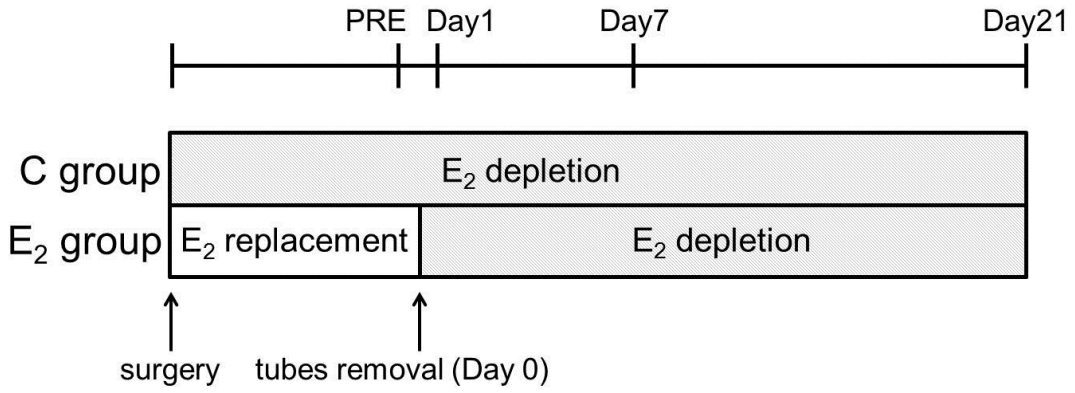

Figure 3. Experimental protocol

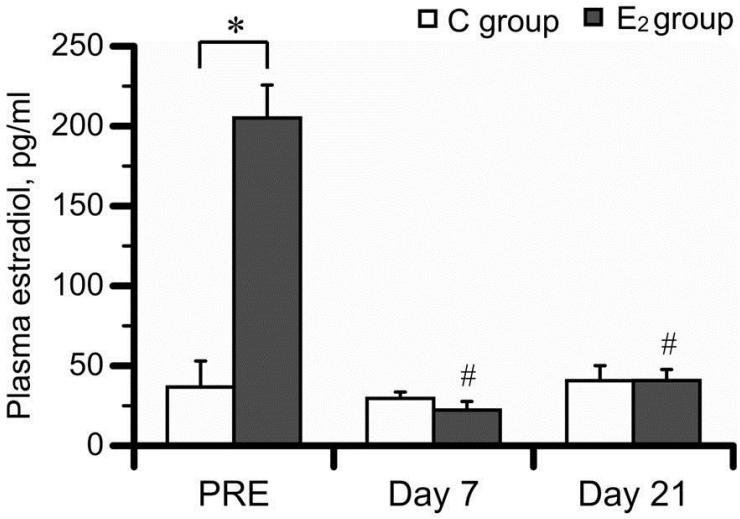

Figure 4. Plasma estradiol levels on PRE and Days7 and 21 in the C and E_2 groups

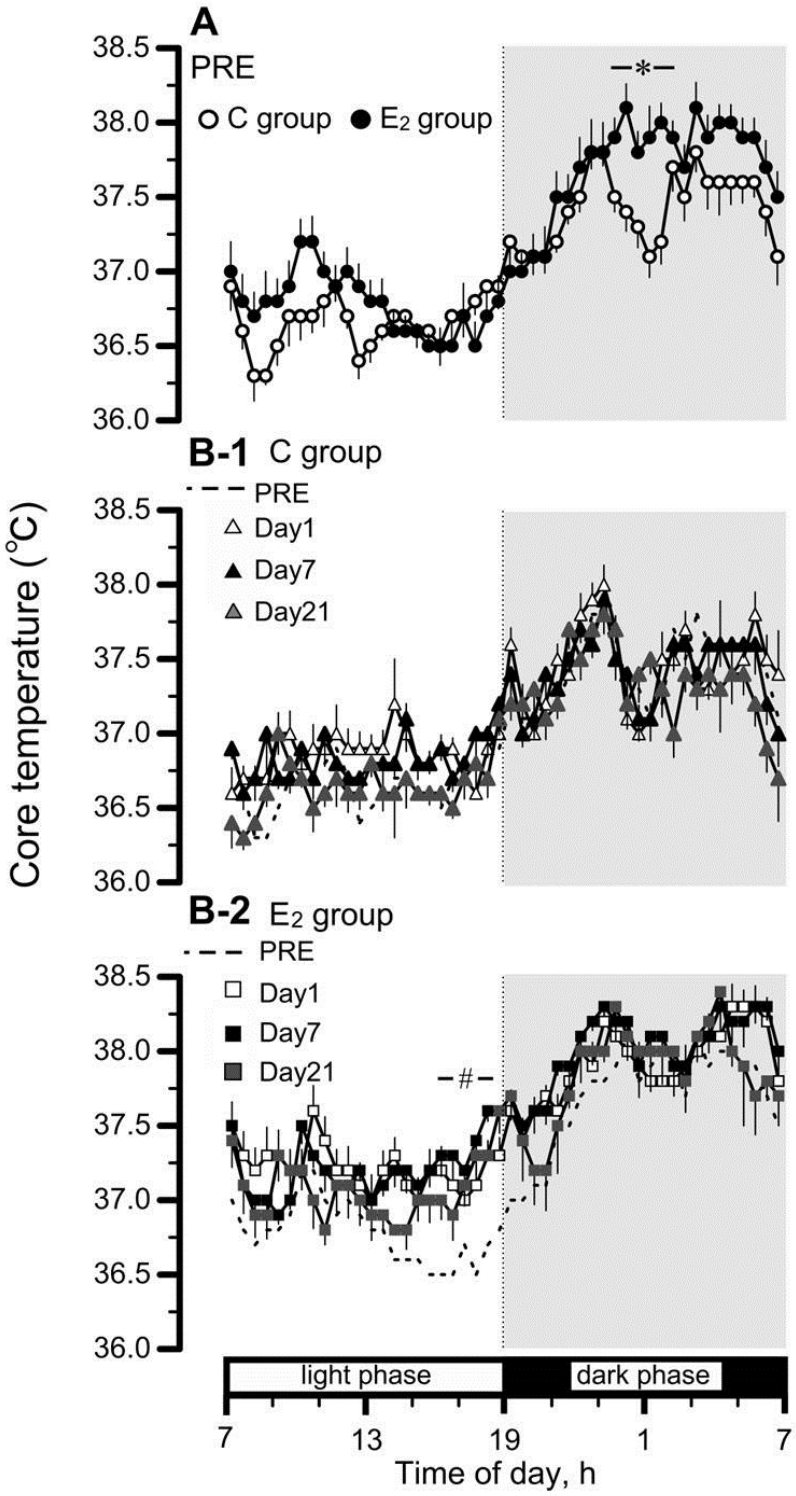

Figure 5. Daily change of core temperature on PRE and Days 1, 7, and 21 in the C and E_2 groups

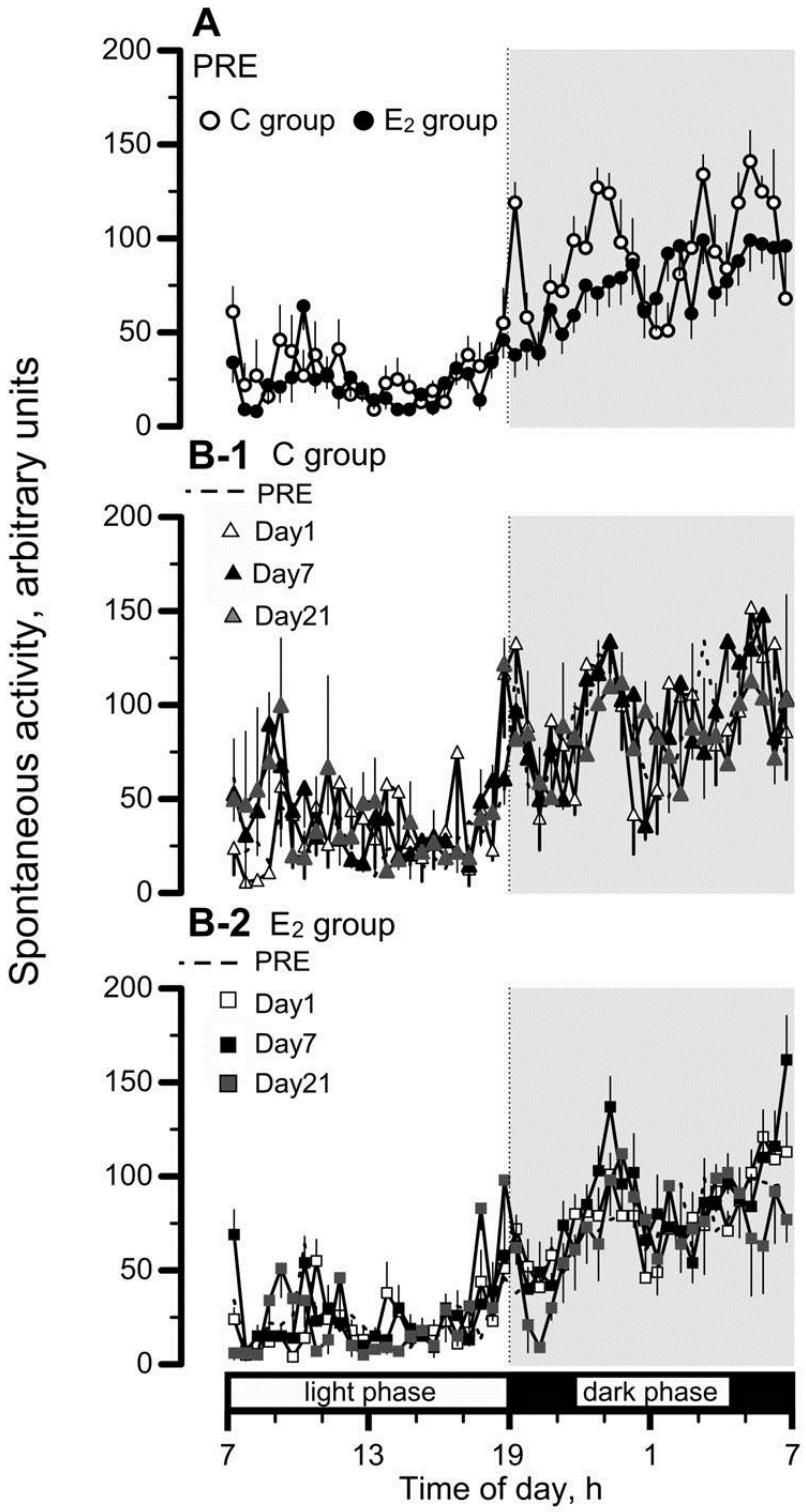

Figure 6. Daily change of spontaneous activity on PRE and Days 1, 7, and 21 in the C and E_2 groups

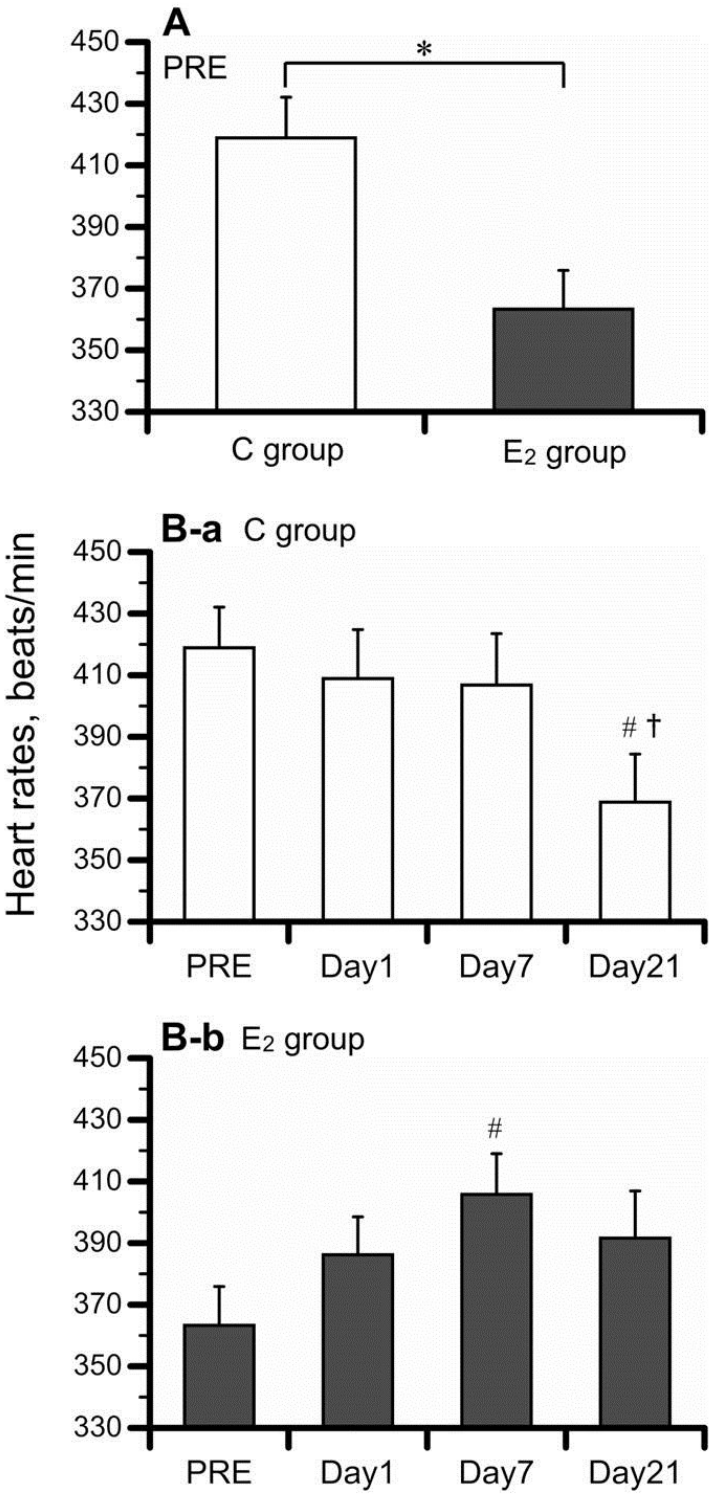

Figure 7. Average heart rate on PRE and Days 1, 7, and 21 in the C and E_2 groups

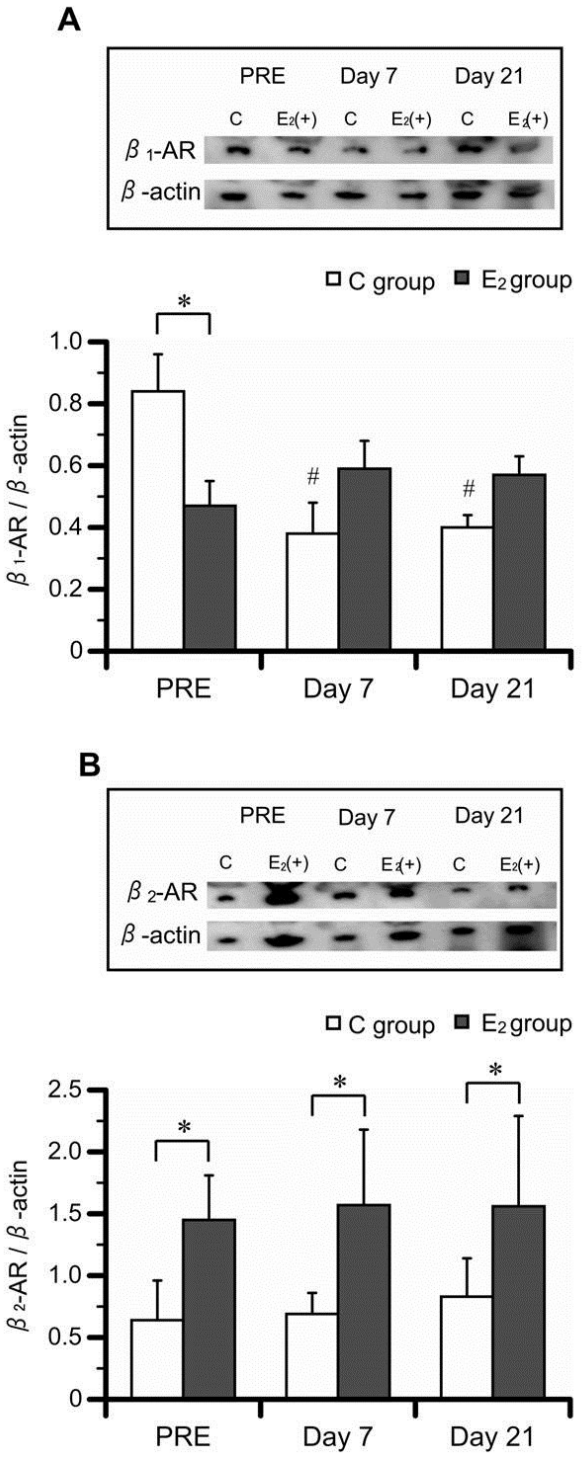

Figure 8. Western blotting analysis of the β_1- and β_2-adrenoreceptors of the left ventricle of the heart

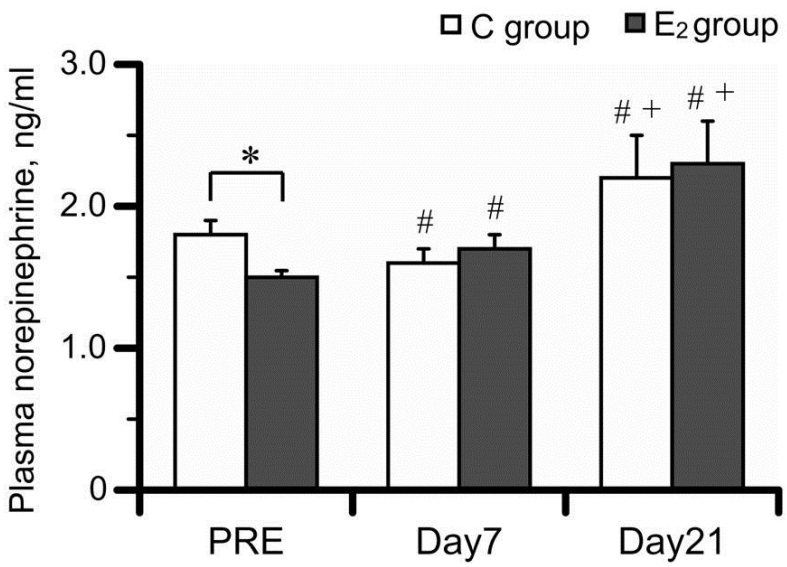

Figure 9. Plasma norepinephrine levels on PRE and Days7 and 21 in the C and E_2 groups

Table 2. Summary of the data of core temperature, heart rates, spontaneous activity, expressions of the β_1 and $_2$-adrenoreceptors (AR), and plasma levels of norepinephrine and estradiol in Experiments 1 and 2. OVX, bilateral ovariectomy; C group, ovariectomy without estradiol replacement; E_2 group, ovariectomy with estradiol replacement, and the replacement was stopped the day before Day 1.

		PRE (9 days after OVX)	Day 1 (11 days after OVX)	Day 7 (17 days after OVX)	Day 21 (31 days after OVX)
C group	core temperature		no change from PRE	no change from PRE	no change from PRE
	heart rates		no change from PRE	no change from PRE	lower than PRE and Day 1
	spontaneous activity		no change from PRE	no change from PRE	no change from PRE
	β1-AR expression			smaller than PRE	smaller than PRE
	β2-AR expression			no change from PRE	no change from PRE
	noradrenaline			lower than PRE	higher than PRE
	estradiol			no change from PRE	no change from PRE
E_2 group	core temperature	greater than the C group at 2330-0130 h	greater than PRE at 1600-1800 h	greater than PRE at 16-1800 greater than PRE of the C group	greater than PRE at 1600-1800 h greater than PRE of the C group at 1000-1230, 2330-0130, and 0430-0630 h
	heart rates	smaller than the C group	no change from PRE	greater than PRE no change from PRE of the C group	no change from PRE no change from Day 7 of the C group
	spontaneous activity		no change from PRE	no change from PRE	no change from PRE
	β1-AR expression	smaller than the C group		no change from PRE	no change from PRE
	β2-AR expression	greater than the C group		greater than the C group	greater than the C group
	noradrenaline	lower than the C group		higher than PRE	higher than PRE
	estradiol	higher than the C group		lower than PRE	lower than PRE

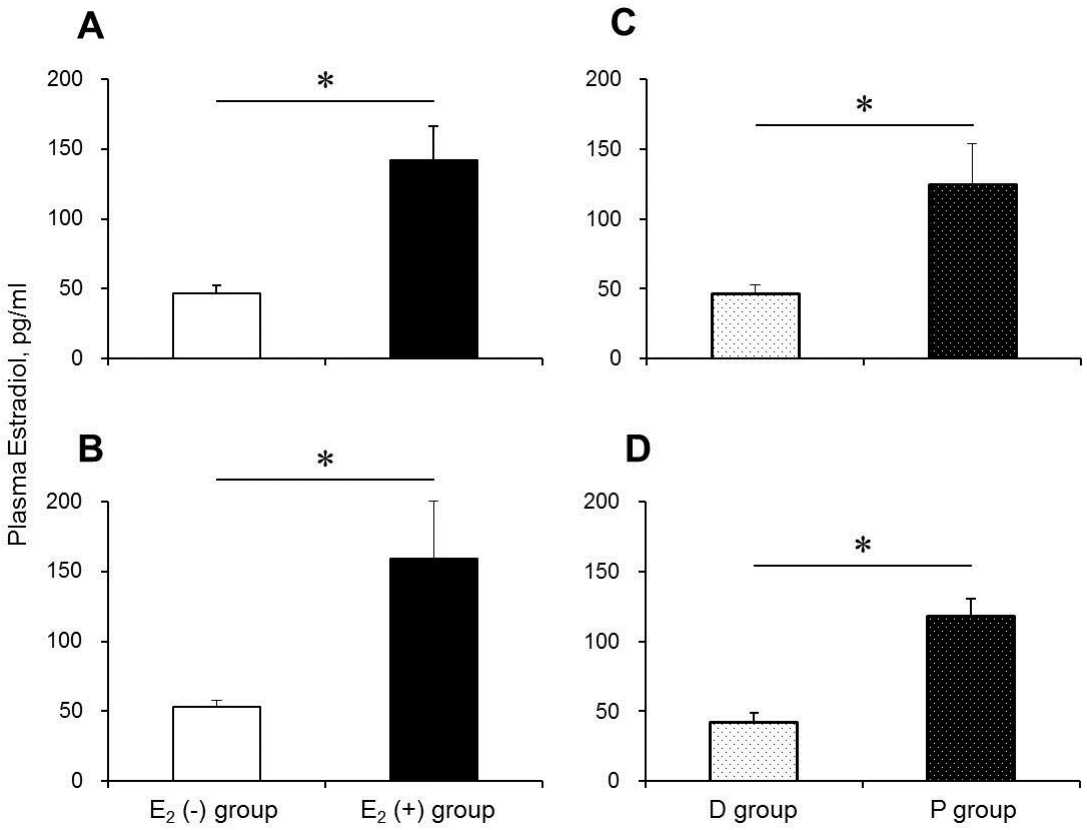

Figure 10. Plasma estradiol levels in the E_2 (-) and E_2 (+) groups in the light phase (**A**) and in the dark phase (**B**), and in the D and P groups in the light phase (**C**) and in the dark phase (**D**)

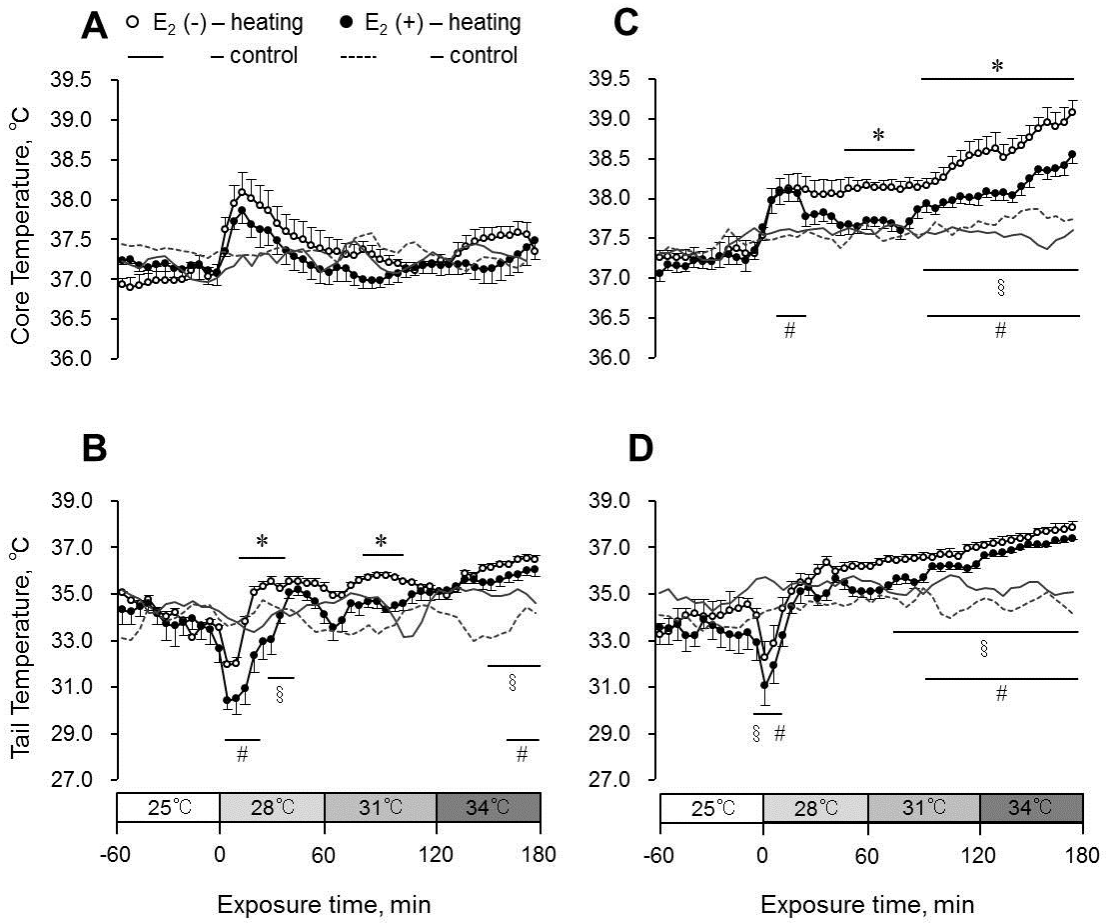

Figure 11. Changes in body core temperature (T_{core}) and tail skin temperature (T_{tail}) in the light phase (**A, B**) and in the dark phase (**C, D**) in *Experiment 1*

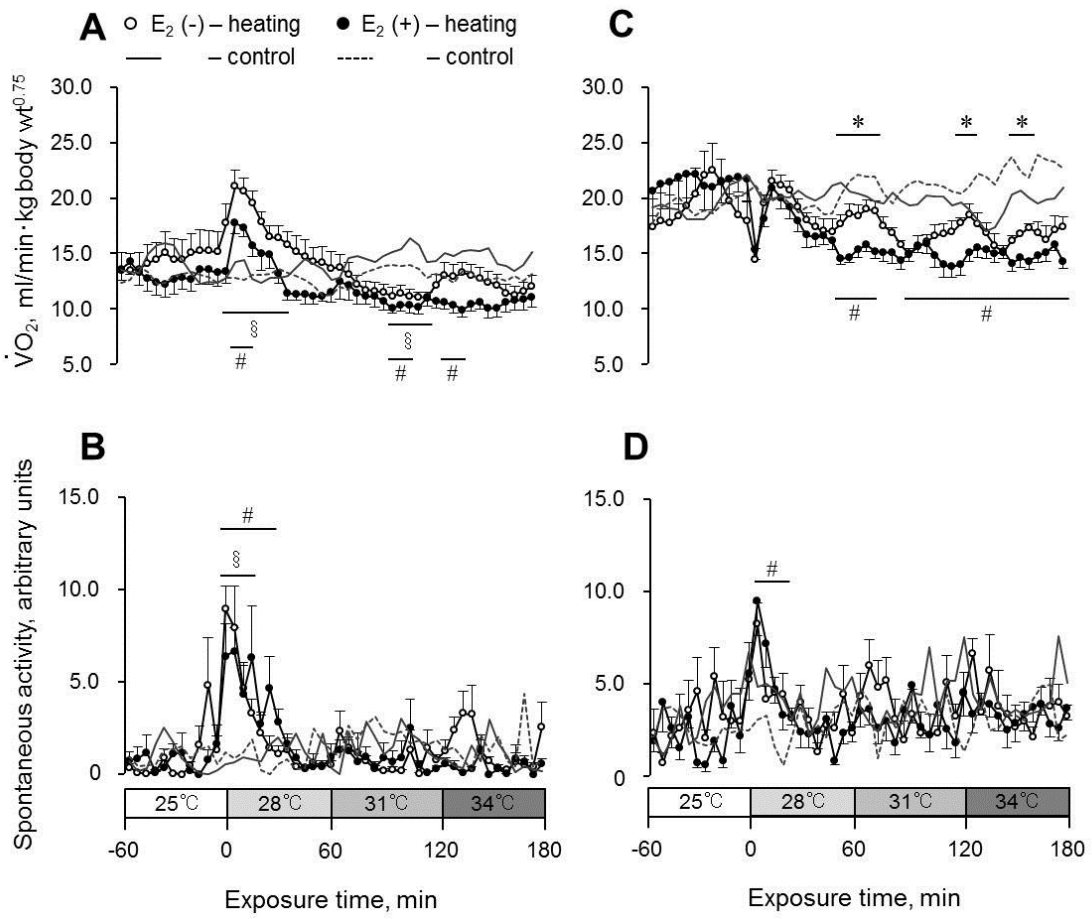

Figure 12. Changes in oxygen consumption ($\dot{V}O_2$) and spontaneous activity in the light phase (**A, B**) and in the dark phase (**C, D**) in *Experiment 1*

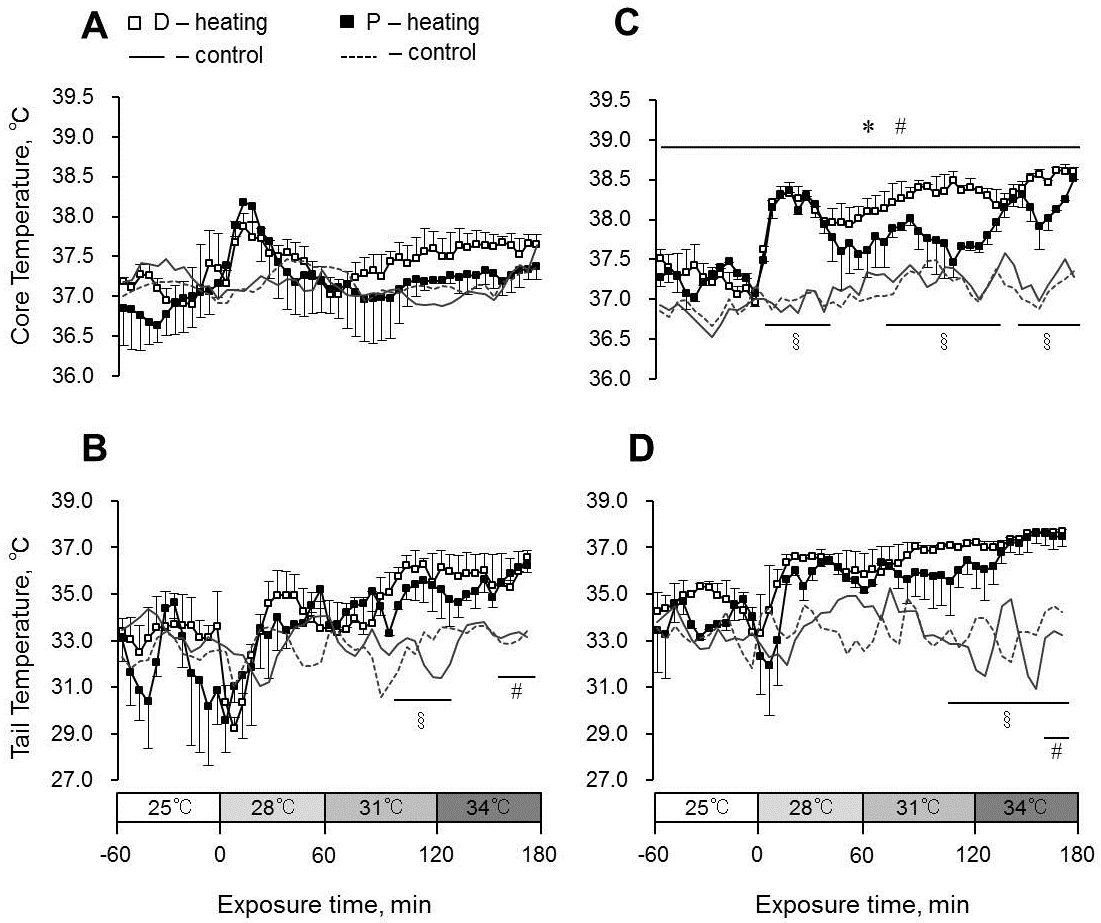

Figure 13. Changes in body core temperature (T_{core}) and tail skin temperature (T_{tail}) in the light phase (**A, B**) and in the dark phase (**C, D**) in *Experiment 2*

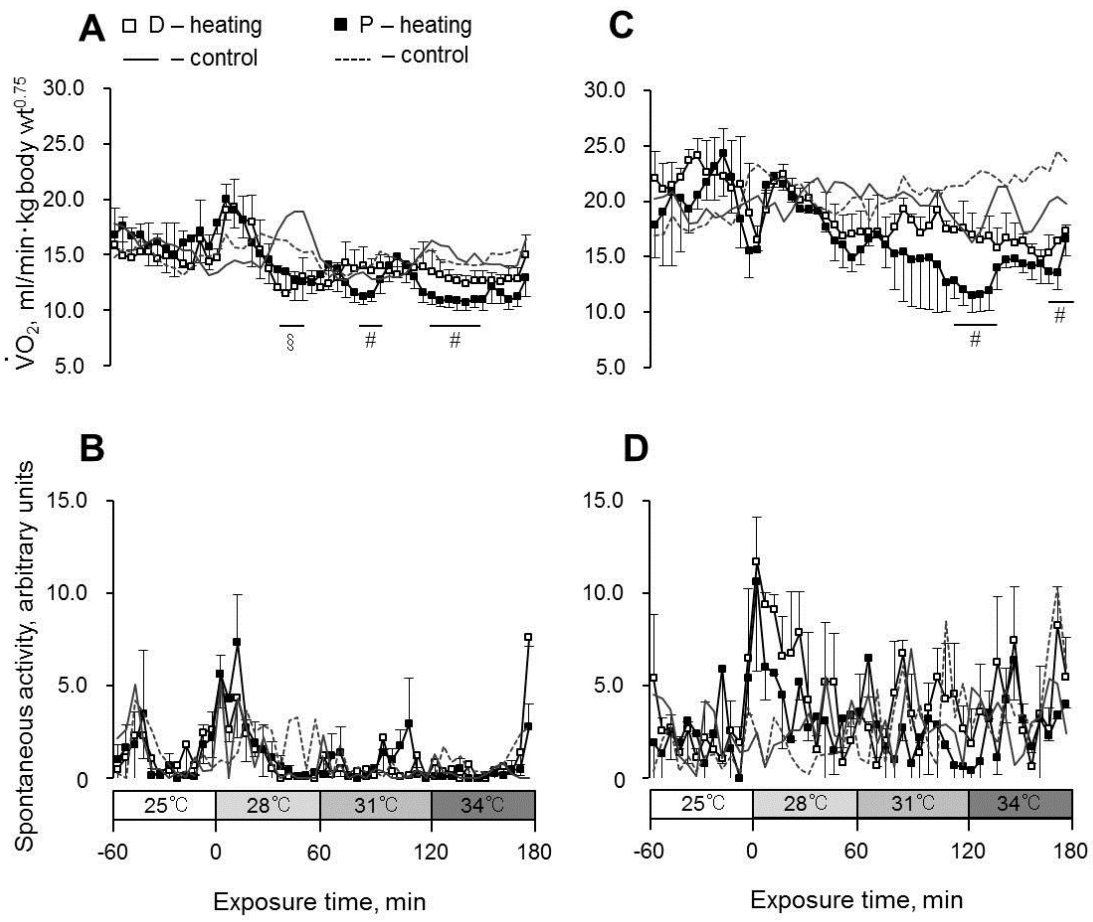

Figure 14. Changes in oxygen consumption ($\dot{V}O_2$) and spontaneous activity in the light phase (**A, B**) and in the dark phase (**C, D**) in *Experiment 2*

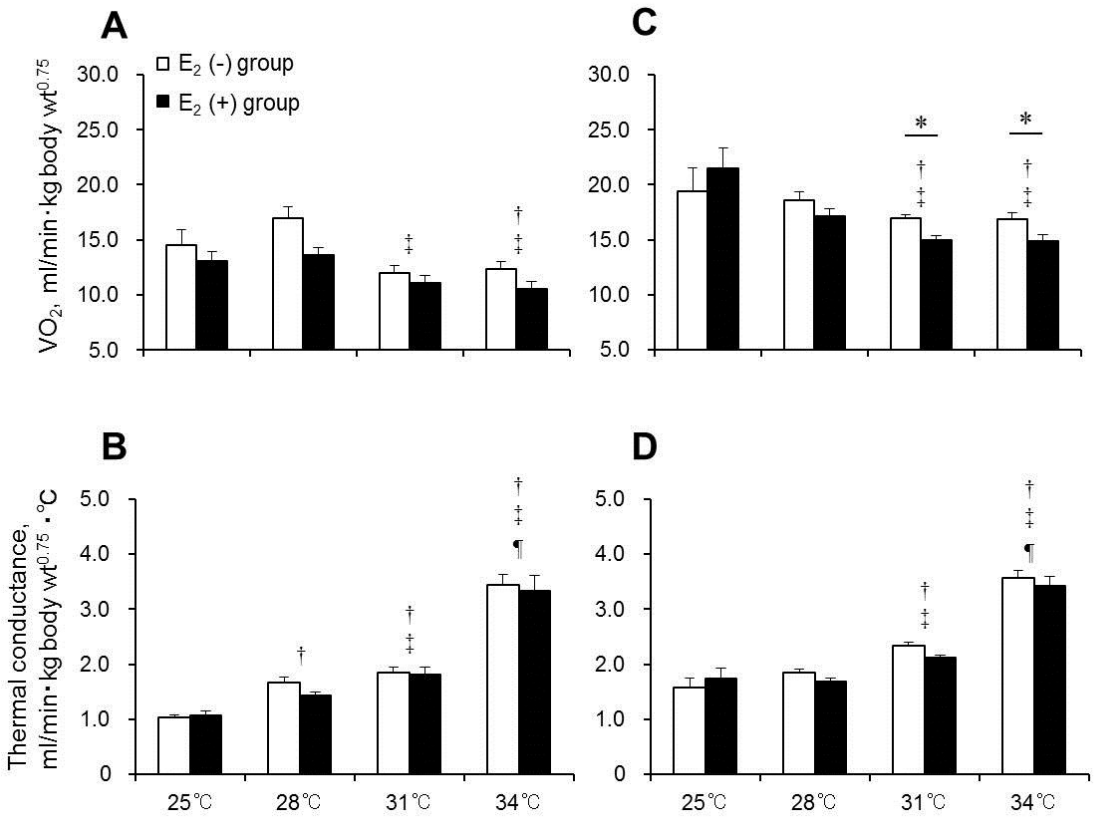

Figure 15. Average $\dot{V}O_2$ and thermal conductance at 25°C, 28°C, 31°C and 34°C in the light phase (**A, B**), and in the dark phase (**C, D**) in *Experiment 1*

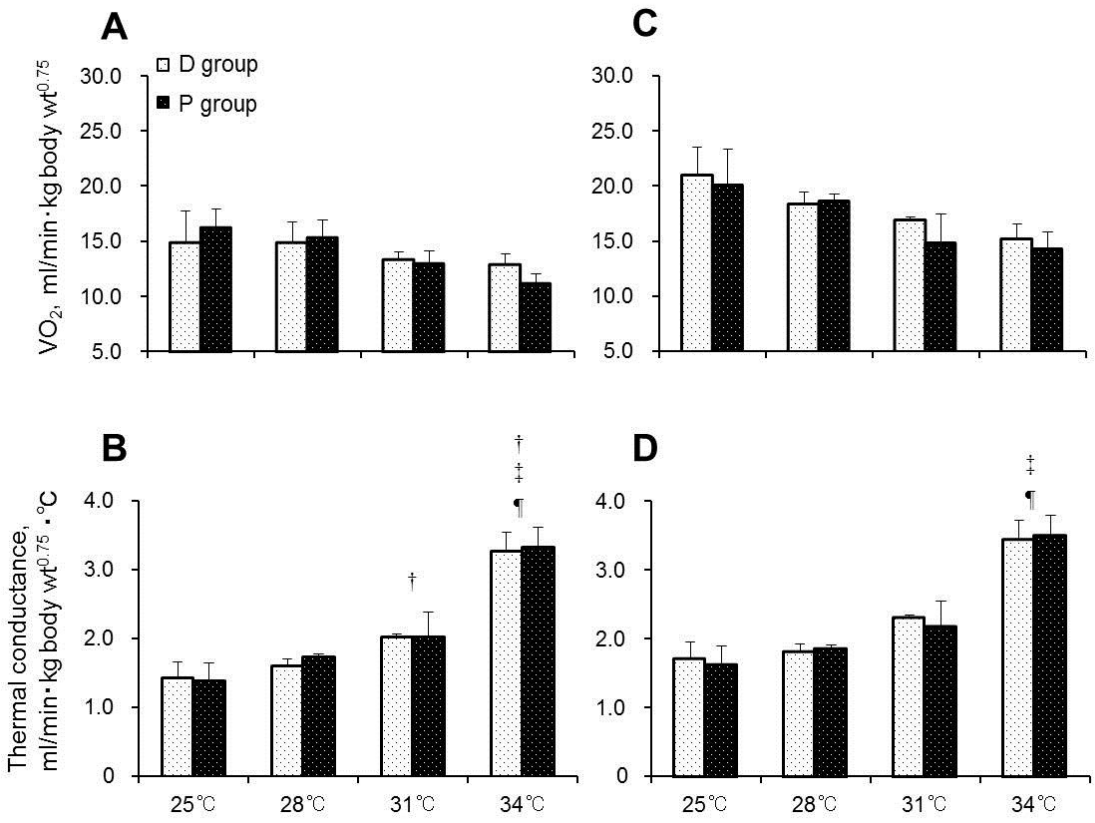

Figure 16. Average $\dot{V}O_2$ and thermal conductance at 25°C, 28°C, 31°C and 34°C in the light phase (**A, B**), and in the dark phase (**C, D**) in *Experiment 2*

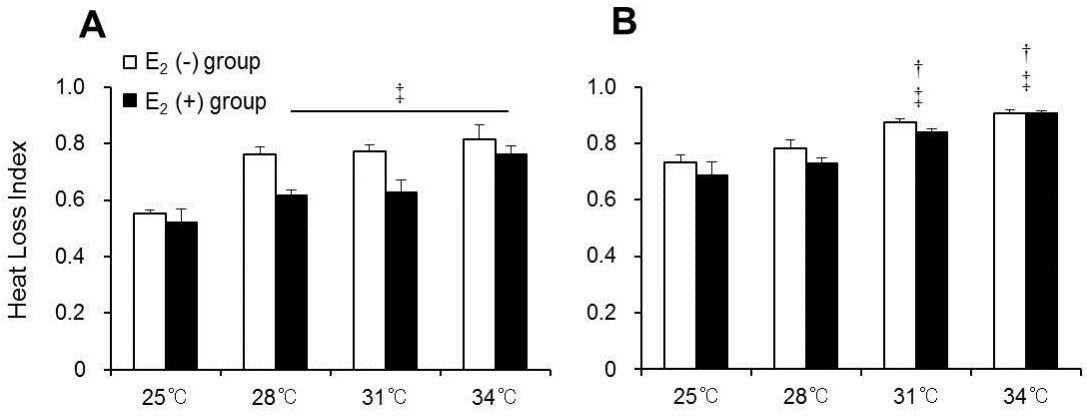

Figure 17. Average heat loss index 25°C, 28°C, 31°C and 34°C in the light phase (**A**), and in the dark phase (**B**) in *Experiment 1*

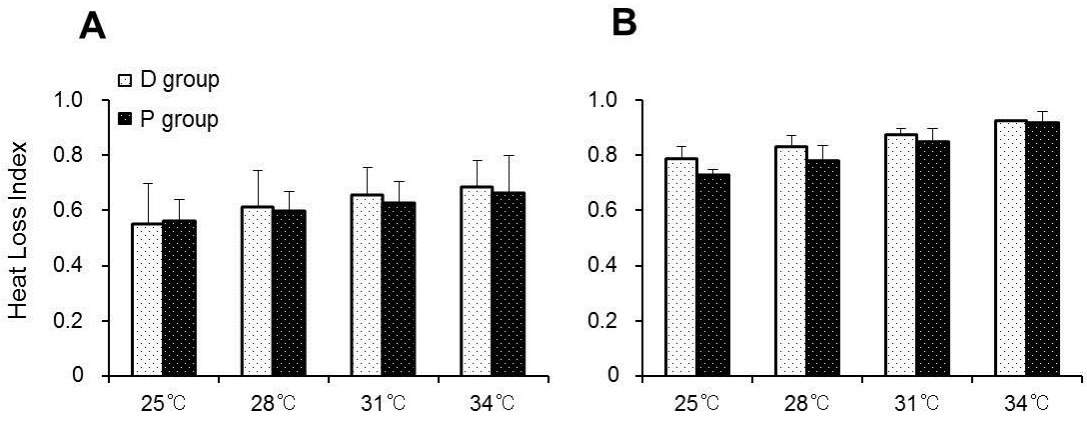

Figure 18. Average heat loss index 25°C, 28°C, 31°C and 34°C in the light phase (**A**), and in the dark phase (**B**) in *Experiment 2*