EFFECT OF ESTROGEN STATUS ON CIRCADIAN CORE AND TAIL SKIN TEMPERATURE RHYTHMS IN FEMALE MICE

By

JESSI RAE MCMINN

_____

A Thesis Submitted to The Honors College

In Partial Fulfillment of the Bachelors degree
With Honors in

Physiology

THE UNIVERSITY OF ARIZONA

M A Y 2 0 1 7

Approved by:

_____

Dr. Naomi Rance
Department of Pathology

Abstract

Body temperature in women is altered by changes in circulating gonadal steroids during the menstrual cycle and estrogen withdrawal after menopause. In contrast to numerous studies in the rat, there is limited information on the effects of estrogen on body temperature regulation in the mouse. The present studies were designed to measure circadian rhythms of core temperature (T_{CORE}) and tail skin temperature (T_{SKIN}) in the mouse. In experiment 1, female mice were implanted with an intraperitoneal temperature probe to measure T_{CORE} and a temperature probe was attached to the tail to measure T_{SKIN}. Daily vaginal smears were taken to assess estrous cycle stage and T_{CORE} and T_{SKIN} were measured continuously for 14 days. In experiment 2, temperature measurements were recorded for ovariectomized (OVX), and low and high estrogen (E_2) replaced animals. We report circadian rhythms of T_{CORE} and T_{SKIN} were not affected by day of the estrous cycle. There was also no significant effect of estrogen replacement on T_{SKIN} during the dark phase, regardless of the E_2 dose. However, low and high E_2 significantly decreased T_{CORE} in OVX mice during the light phase. These studies reveal a significant species difference in estrogen modulation of thermoregulation in the rat and mouse.

Introduction

Menopausal hot flushes are a central nervous system disorder and occur due to estrogen withdrawal. Flushes represent an episodic activation of heat dissipation effectors in which cutaneous blood vessels dilate to raise skin temperature and release heat from the body. The inappropriate activation of the heat dissipation pathway can also include sweating and behavioral changes which, along with flushes, may be effective enough to cause a reduction in core body temperature (Freedman 2001). Several environmental factors can induce flushes in menopausal women, such as higher ambient temperature, spicy food (chemical stimuli), or stressed psychological states (Archer, Sturdee et al. 2011).

It has been shown that flushes are caused by a rapid decline in circulating gonadal hormones (Kronenberg 2010). In addition to the loss of circulating estrogens in postmenopausal women, flushes also occur in oophorectomized (removal of ovaries) premenopausal women, men undergoing androgen ablation therapy for prostate cancer, or those prescribed tamoxifen (estrogen receptor competitor) for breast cancer (Santoro 2008). Estrogen replacement therapy effectively treats flushes in postmenopausal women. However, a Women's Health Initiative study demonstrated an increased risk of coronary heart disease and breast cancer in postmenopausal women undergoing estrogen plus progestin replacement (Rossouw, Anderson et al. 2002). In order to determine alternative treatments, it will be useful to understand the mechanism involved in menopausal hot flushes.

Body temperature is regulated by the hypothalamus, which receives input from both external (ambient temperature) and internal (visceral and brain temperature) cues (Morrison and Nakamura 2011). Based on these signals, the central nervous system can activate heat- or cold-defense pathways to maintain body temperature within a homeostatic range. Endothermic, homeothermic organisms, like humans, maintain body temperature within a set range and can use heat produced internally or externally (Hammel 1965). These organisms have a thermoneutral zone of body temperature, a range of ambient temperatures within which metabolic rate does not need to change in order to maintain body temperature (Commission for Thermal Physiology 2001). Outside of this range, metabolism increases and heat- and cold-defense pathways are upregulated. The heat defense pathway includes vasodilation of cutaneous blood vessels, downregulation of brown adipose tissue (BAT) thermogenesis, evaporative cooling (sweating in humans and panting in furry animals), and cold-seeking behaviors. The cold-defense pathway is characterized by vasoconstriction of cutaneous blood vessels, thermogenesis via BAT, shivering, and heat-seeking behaviors.

In addition to understanding thermoregulatory controls in the body, it is important to understand that there are a few biological processes set to internal "clocks," or measurable rhythms. The patterns used to describe these processes are labeled as infradian, circadian, and ultradian rhythms. Infradian rhythms characterize a periodicity of greater than 24 hours, such as the menstrual cycle. Circadian rhythms maintain a periodicity of 24 hours, such as the sleep/wake cycle. An ultradian rhythm is one with a periodicity of less than 24 hours but greater than 1 hour, such as the phases of sleep or appetite. In humans, the body temperature set-point experiences fluctuations matching all three rhythm types: small fluctuations over the course of the day

(ultradian), daily increases and decreases over the sleep/wake cycle (circadian), and changes over the course of the menstrual cycle (infradian) (Refinetti and Menaker 1992).

Estrogen is an integral component of the neuroendocrine reproductive axis and has also been shown to affect body temperature regulation. Where the reproductive axis ties into the thermoregulatory circuit is still indeterminate. Neurons in the postmenopausal human hypothalamus expressing neurokinin B (NKB) and estrogen receptor-α demonstrate hypertrophy and increased gene expression, which has led a hypothesis that these neurons participate in the generation of hot flushes (Rance, McMullen et al. 1990, Rance and Young 1991). In the rat, these neurons project from the arcuate nucleus (homologous to the infundibular nucleus in the human) to the preoptic area of the hypothalamus, the efferent control center for temperature regulation pathways (Krajewski, Burke et al. 2010). Our lab is interested in core and skin temperature rhythms and the effect of estrogen modulation on these patterns in the rodent model. In human circadian rhythms, core body temperature is increased during the light phase (or daytime) and decreased during the dark phase (or nighttime). However, rodents are nocturnal and therefore exhibit an inversed pattern.

Animal models have been invaluable for exploring the central nervous system pathways involved in temperature regulation and their tie-in with the reproductive axis. In contrast to the numerous studies in the rat, there is limited information on the effects of estrogen on body temperature regulation in the mouse. In Experiment 1, we characterize core and tail skin temperature of intact cycling mice throughout the estrous cycle. Furthermore, in Experiment 2, we will determine the effects of different doses of estrogen on core and tail skin temperature rhythms in ovariectomized mice. These data will provide useful baseline information on temperature rhythms in the mouse model and help with design and interpretation of future experiments.

Materials and Methods

Animals

Female Hsd:ICR (CD-1) mice (2.5-3 months old, 25-37g; Harlan Sprague Dawley, Inc., Houston, TX) received *ad libitum* access to water and a low-phytoestrogen diet (Harlan Teklad 2019 Global 19% Protein Extruded Rodent Diet). They were individually housed in the University of Arizona Animal Care Facility with a 12:12 hour light/dark cycle (lights on at 7:00 am). The ambient temperature ($T_{AMBIENT}$) of the animal room ranged from 22.4-22.6°C with 50% humidity. The animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Arizona and followed National Institutes of Health guidelines.

Monitoring T_{SKIN} and T_{CORE} in the mouse

T_{SKIN} was monitored using a Star-Oddi DST Nano-T temperature probe (Emka Technologies, Falls Church, VA). The temperature probes were confirmed to be accurate against a National Institute of Standards and Technology certified device (TC101A thermocouple recorder). A thermocouple inside the telemetry device was visible through the clear end cap, which allowed for orientation against the ventral surface of the tail. The housing was manufactured (by WB Enterprises, Tucson, Arizona) from Delrin plastic with an assortment of channel widths (5.0, 5.5, and 6.0mm) to accommodate different tail sizes (Fig. 1). The housing was attached to the tail using Loctite 454 prism instant adhesive gel glue (Fisher Scientific, Pittsburgh, VA) on the lateral surfaces of the tail. The tails were held flush against the temperature probe while the glue cured, and it was ensured that no glue was applied between the surface of the probe and tail. The housing was glued under brief isoflurane anesthesia 2.5cm from the base of the tail (Fig. 2). Tails were inspected daily to ensure that there was no swelling, erythema, or other signs of irritation. In a few instances in which the tail probes fell off, they were reattached and the data from that day was discarded. T_{CORE} and activity were measured concurrently with a DSI telemetry probe (TA-F10, Data Sciences International, St. Paul, MN) implanted in the peritoneal cavity. $T_{AMBIENT}$ was recorded with a DSI ambient temperature probe.

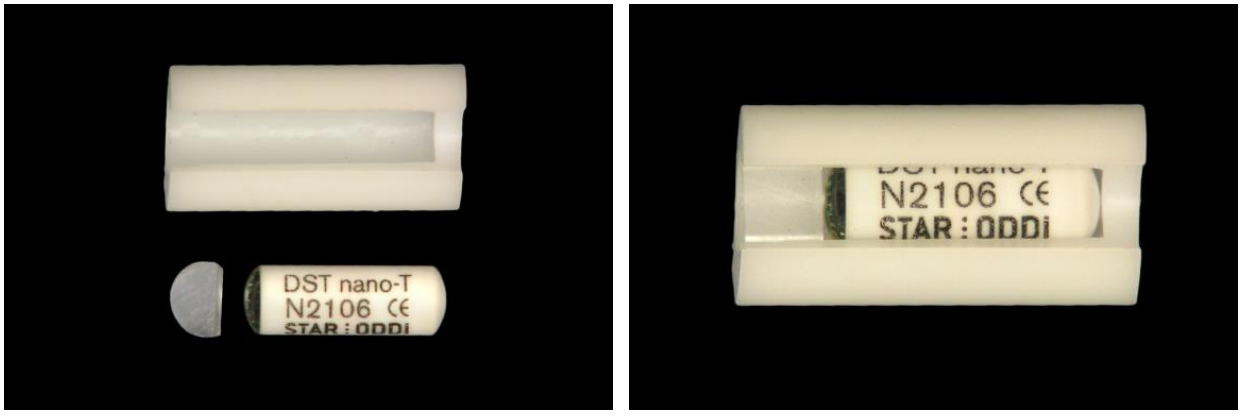


Figure 1: T_{SKIN} was recorded using a Star-Oddi DST Nano-T sensitive temperature probe (Emka Technologies, Falls Church, VA) encased in a Delrin housing.

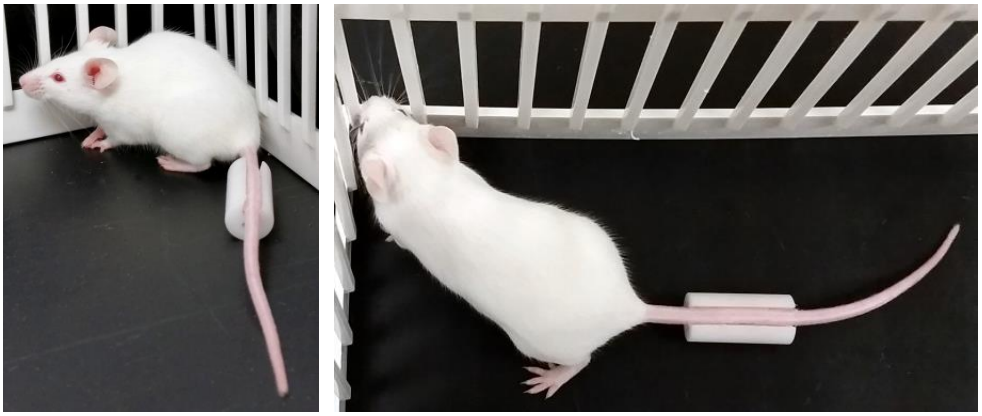


Figure 2: The probe was placed in direct contact with the ventral surface of the tail ~2.5cm from the base and secured using gel glue.

Experiment 1: Measurements of circadian rhythms of T_{SKIN} and T_{CORE} throughout the estrous cycle.

Ten mice were implanted intraperitoneally with the DSI telemetry transmitter under isoflurane 25-26 days before the start of vaginal smear collection to allow for healing and reduced influence on circadian temperature collection. The Nano-T probe was attached to the tail as described above two days before the start of data collection. For two weeks (experimental days 0-13, Fig. 3), daily vaginal smears were collected at approximately 7:45-8:15 am in the animal facility and analyzed for estrous cycle stage. T_{CORE}, T_{SKIN}, and activity were recorded every five minutes continuously over the two weeks. The mice were housed individually in microisolator cages and placed on PhysioTel receiver boards in a dedicated room in the animal facility that was relatively free from noise and had restricted access. The mice were weighed weekly during the time of vaginal smearing.

Experiment 2: Circadian rhythms of T_{CORE} and T_{SKIN} in OVX mice treated with low and high concentrations of 17β-estradiol.

The ten mice from Experiment 1 were ovariectomized (OVX) on experimental days 14-15 (Fig. 3) and housed in the animal facility as described above. A one-week wash-out period of circulating estrogens was allowed before circadian T_{CORE}, T_{SKIN}, and activity were continuously collected every five minutes for one week. Then, the low dose of estrogen was administered via subcutaneous implantation of a SILASTIC capsule (1.57mm inner diameter, 3.18mm outer diameter, effective length 20mm; Dow Corning Copr., Midland, MI) containing 360µg/mL 17β-estradiol in sesame oil (low E_2) under isoflurane anesthesia (experimental day 30). Temperature and activity recordings were collected for seven days, then the capsules were removed and a blood sample was collected for serum E_2 analysis. After another wash-out week, a 720µg/mL E_2 capsule (high E_2) was implanted (experimental day 44) and measurements were collected as before. A blood sample was collected seven days after capsule implantation for serum E_2 analysis.

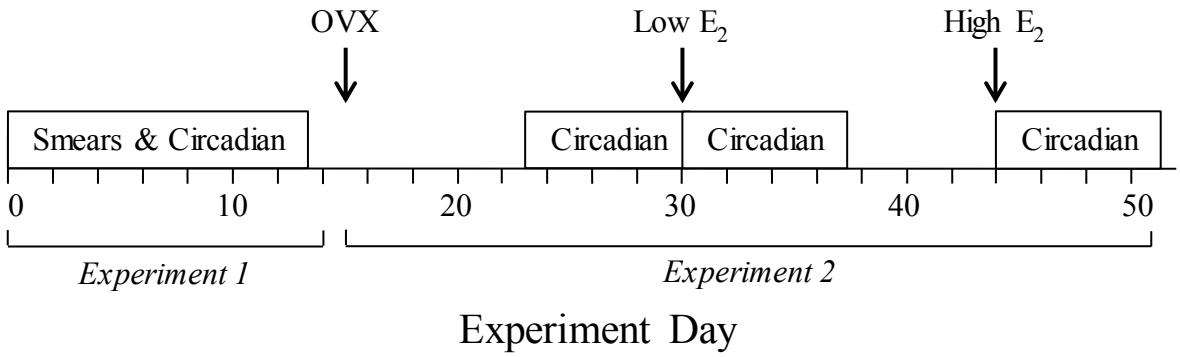


Figure 3: Protocol for Experiments 1 and 2. Mice were implanted with telemetry probe 25-26 days before day 0. Smears and circadian recordings were collected days 0-13. Animals were OVX days 14-15 and circadian recordings were collected days 23-29. Animals were implanted with high and low E_2 on days 30 and 44, respectively, and circadian recordings were collected for the following seven days.

Data Analysis

Circadian Recordings: Data were calculated for six-hour blocks of time in the light (9:00-14:55h) and dark (21:00-2:55h) phases over days 3-5 of recordings. Values were averaged for each mouse and their values were used to calculate group averages. The heat loss index (HLI), which is a measurement of active tail skin vasomotion (Romanovsky 2002) was calculated using the formula: $HLI = (T_{SKIN}-T_{AMBIENT})/(T_{CORE}-T_{AMBIENT})$. Two-way analysis of variance (ANOVA, cycle day vs light/dark phase or estrogen status vs light/dark phase) was performed for T_{CORE}, T_{SKIN}, HLI, and activity using Tukey's *post hoc* test with $\alpha<0.05$.

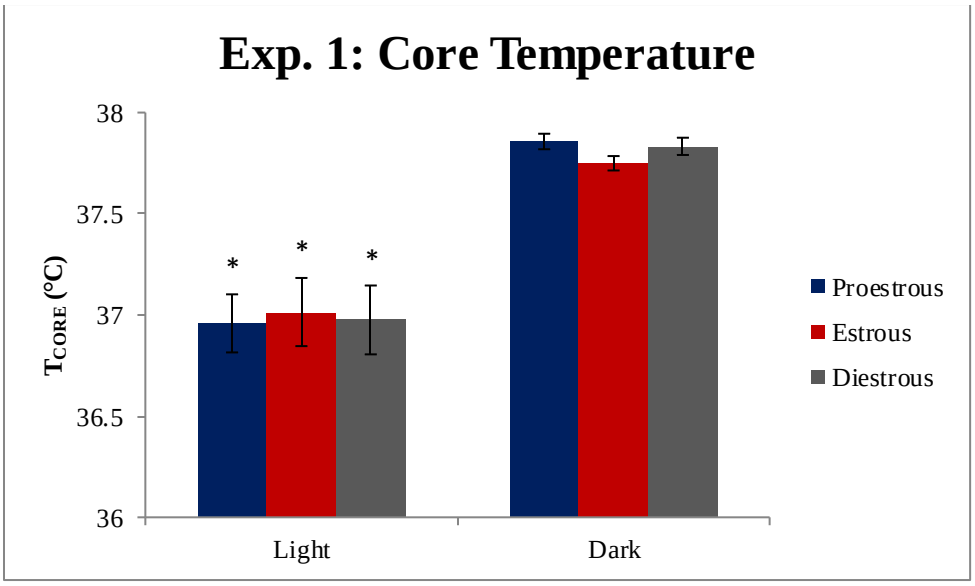
Serum assays: Serum samples were sent to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core Facility to measure estradiol (Calbiotech Estradiol Elisa). The sensitivity of the Estradiol Elisa was 3pg/mL with an intraassay coefficient of variation of 6.1%.

Results

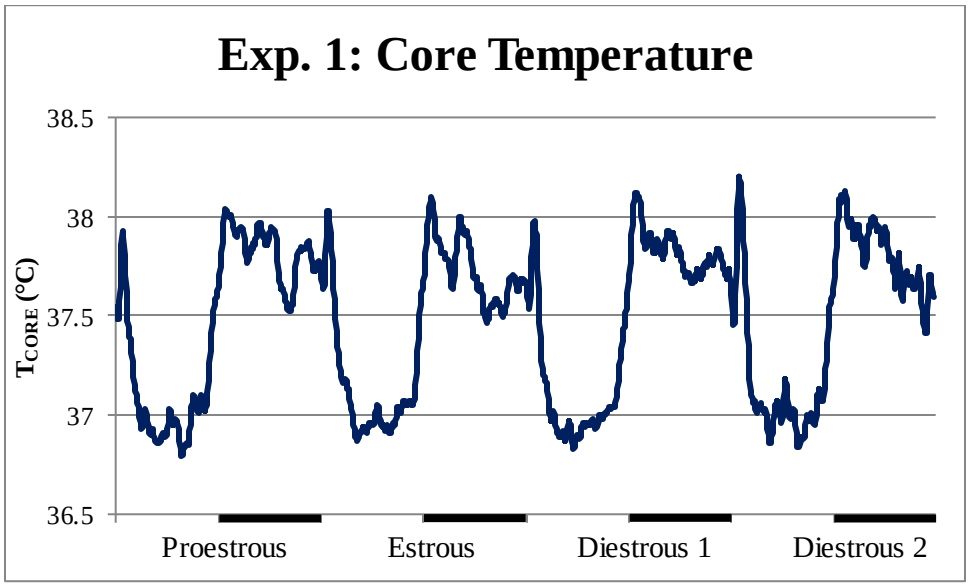
Experiment 1: Effect of Estrous Cycle Phase

Intact cycling animals demonstrated prominent circadian rhythms of T_{CORE} and T_{SKIN}. There were significant differences between light and dark phase during each cycle stage (Proestrous, Estrous, Diestrous) for T_{CORE}, HLI, and Activity but not for T_{SKIN} (Fig. 4). There was no significant difference across cycle stage within light or dark phase for any experimental parameter. Refer to Appendix A for mean ± SEM and p values.

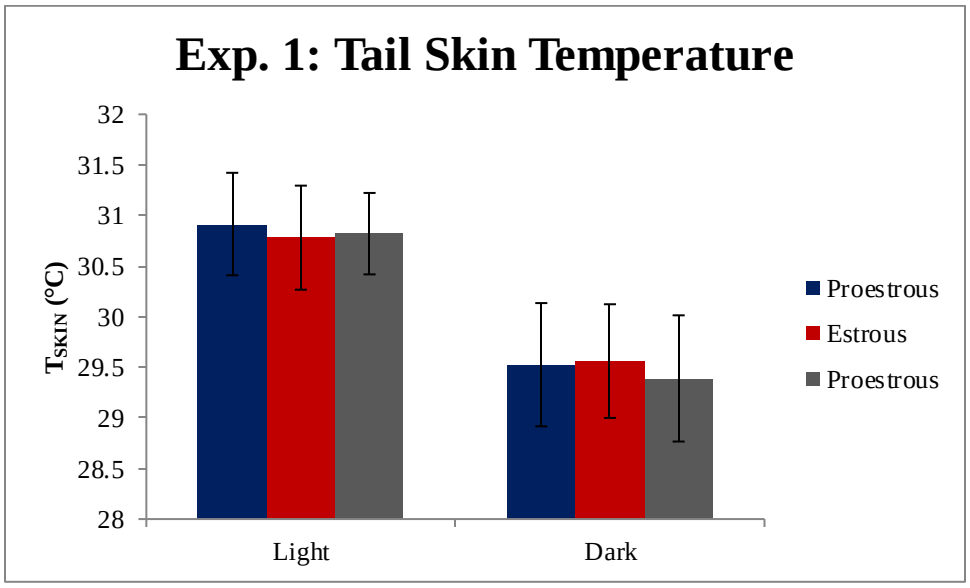
A.



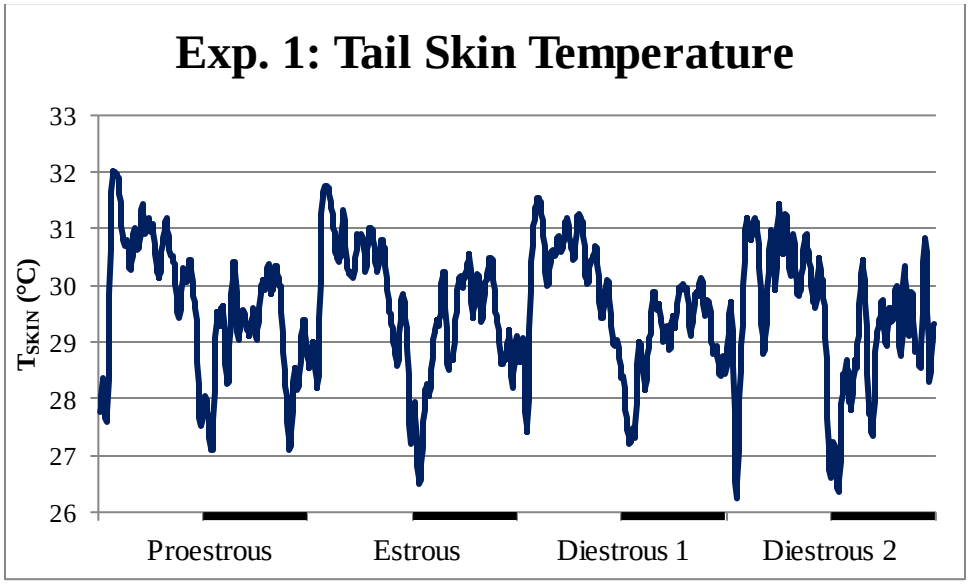
B.



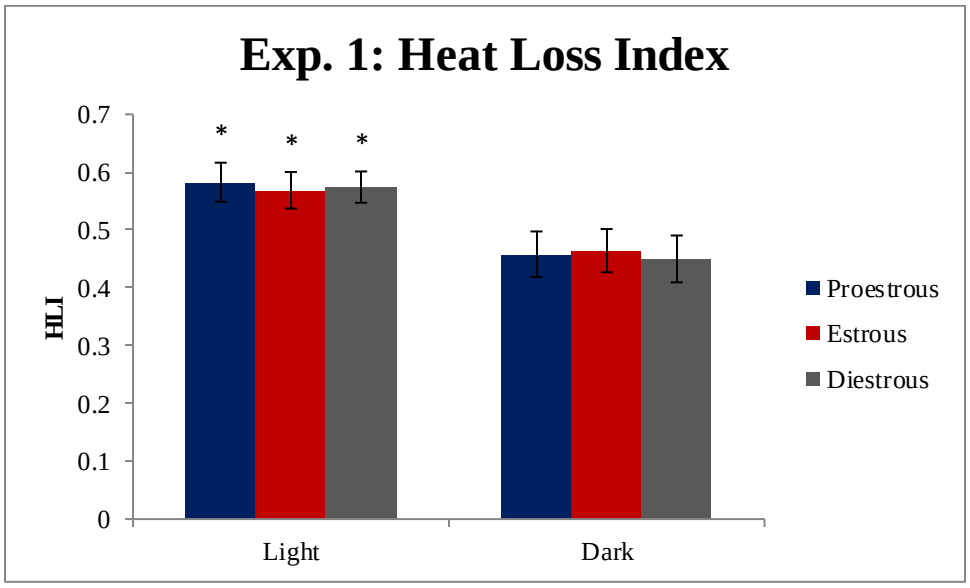
C.


Exp. 1: Tail Skin Temperature
32
31.5
31
30.5
30
29.5
29
28.5
28
TSKIN (°C)
Light
Dark
Proestrous
Estrous
Proestrous

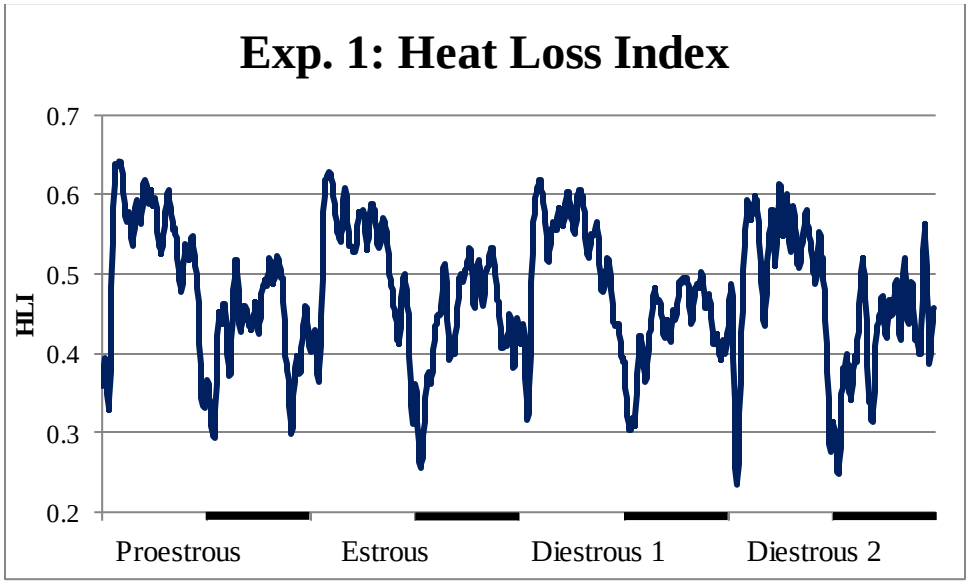
D.


Exp. 1: Tail Skin Temperature
33
32
31
30
29
28
27
26
TSKIN (°C)
Proestrous
Estrous
Diestrous 1
Diestrous 2

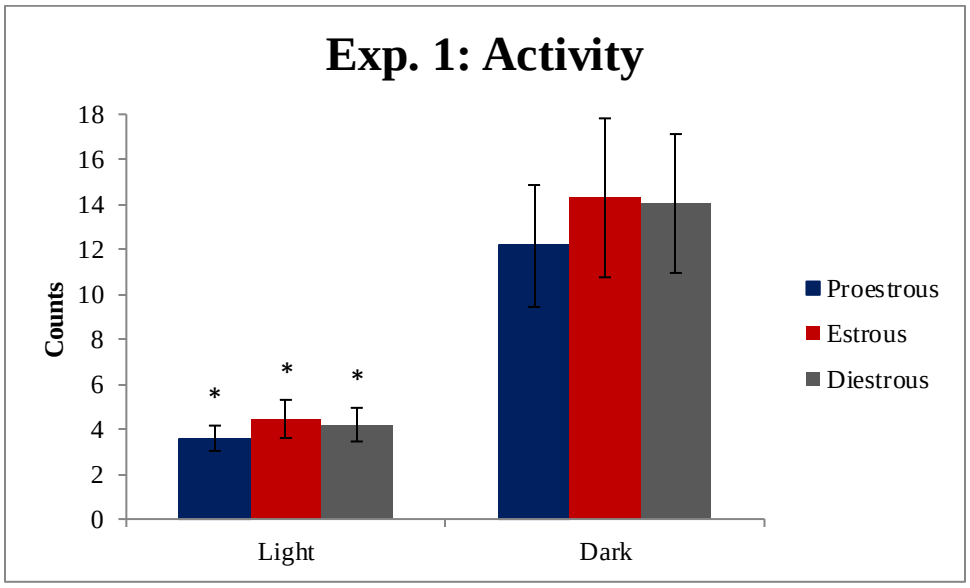
E.


Exp. 1: Heat Loss Index
HLI
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
Light
Dark
Proestrous
Estrous
Diestrous

F.


Exp. 1: Heat Loss Index
HLI
0.7
0.6
0.5
0.4
0.3
0.2
Proestrous
Estrous
Diestrous 1
Diestrous 2

G.



H.

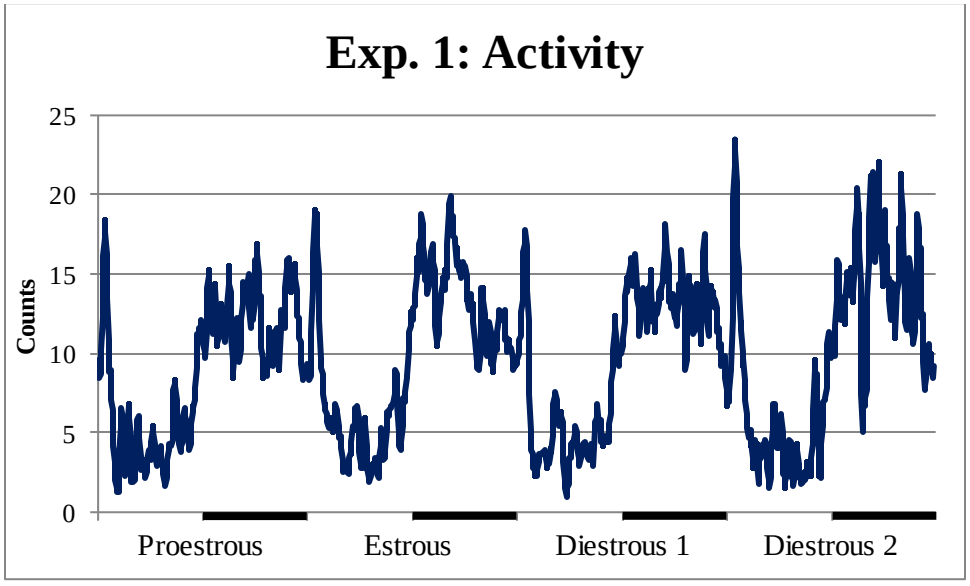
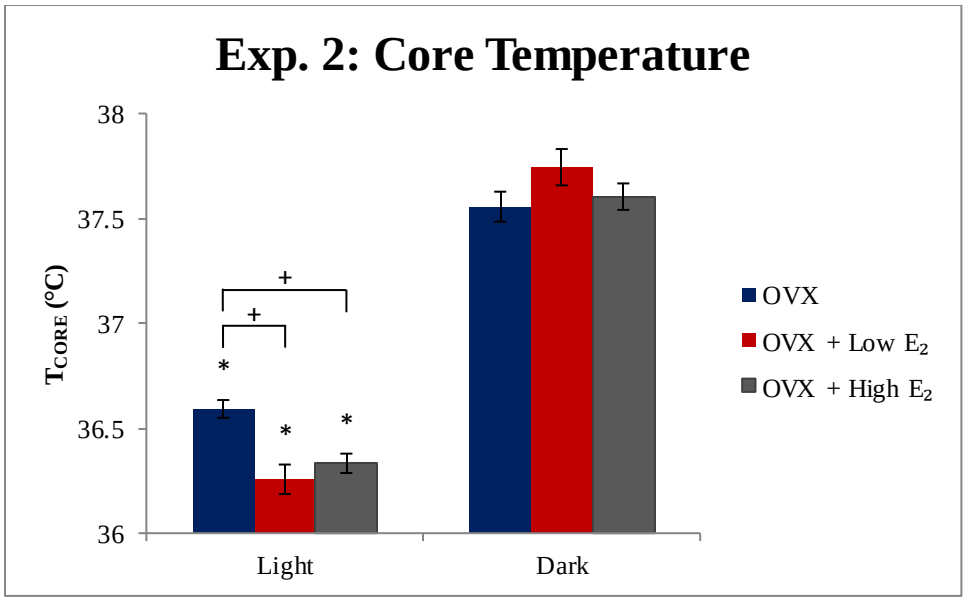


Figure 4: Cycling Animals (n=6; 16 cycles). Average (±SEM) T_{CORE} (A), T_{SKIN} (C), HLI (E), and Activity (G) during the light and dark phases across estrous cycle stages. *, significantly different, light vs. dark within cycle stage. Moving average of T_{CORE} (B), T_{SKIN} (D), HLI (F), and Activity (H) across the estrous cycle, demonstrating circadian rhythms.

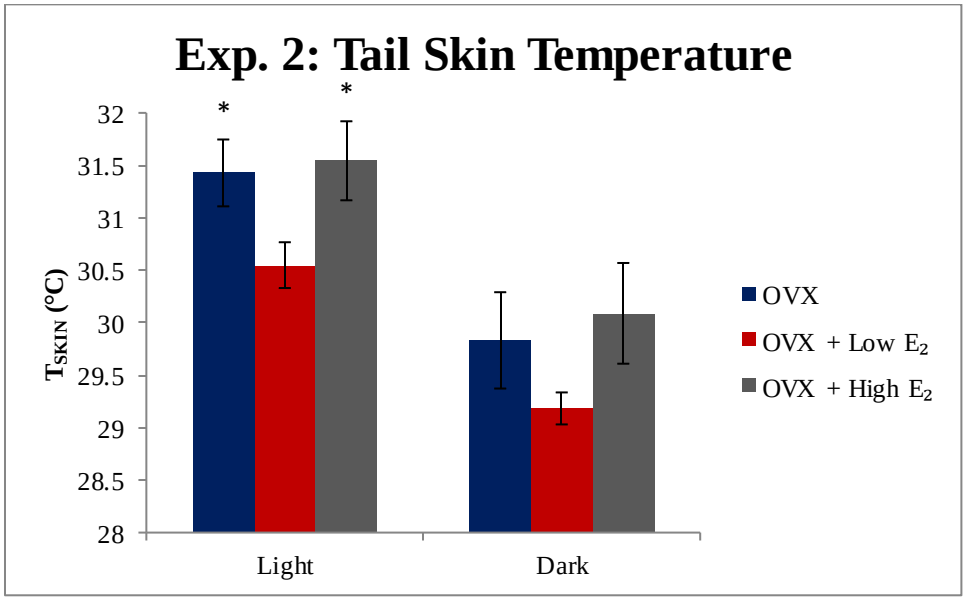
Experiment 2: Effect of E_2 Dose

All treatment groups (OVX, OVX + low E_2, and OVX + high E_2) demonstrated prominent circadian rhythms for T_{CORE} and T_{SKIN}. All groups also demonstrated significant light versus dark phase differences for T_{CORE}, T_{SKIN}, HLI, and Activity (with the exception of low E_2 for T_{SKIN}) (Fig. 5). T_{CORE} was lower in OVX mice treated with both low E_2 and high E_2 in the light phase. There was no significant difference among treatment groups, within the light or dark phases, for T_{SKIN}, HLI, or Activity. Refer to Appendix A for mean ± SEM and p values. Serum estradiol levels were determined to be: low E_2, 36.87 ± 4.97pg/mL; high E_2, 82.67 ± 4.51pg/mL.

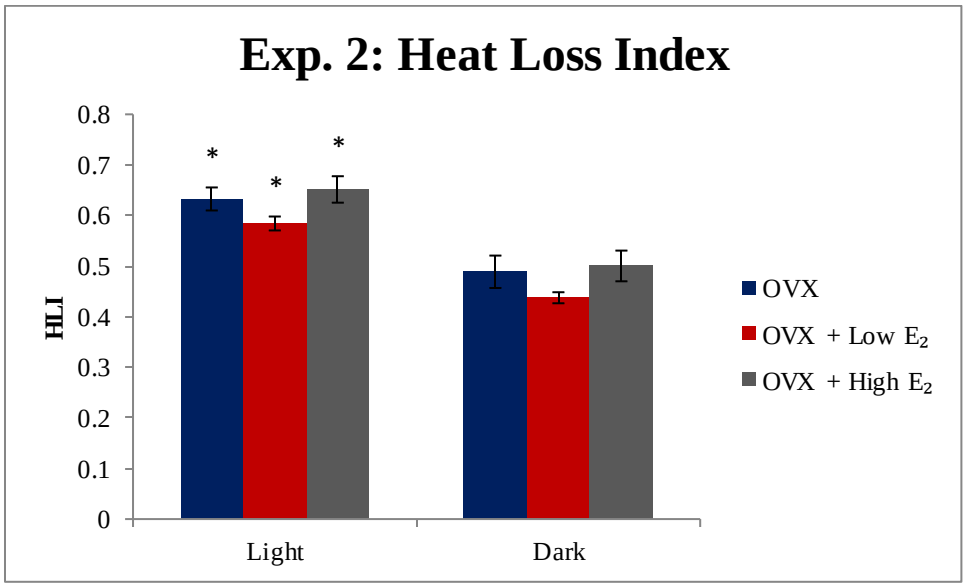
A.



B.



C.



D.

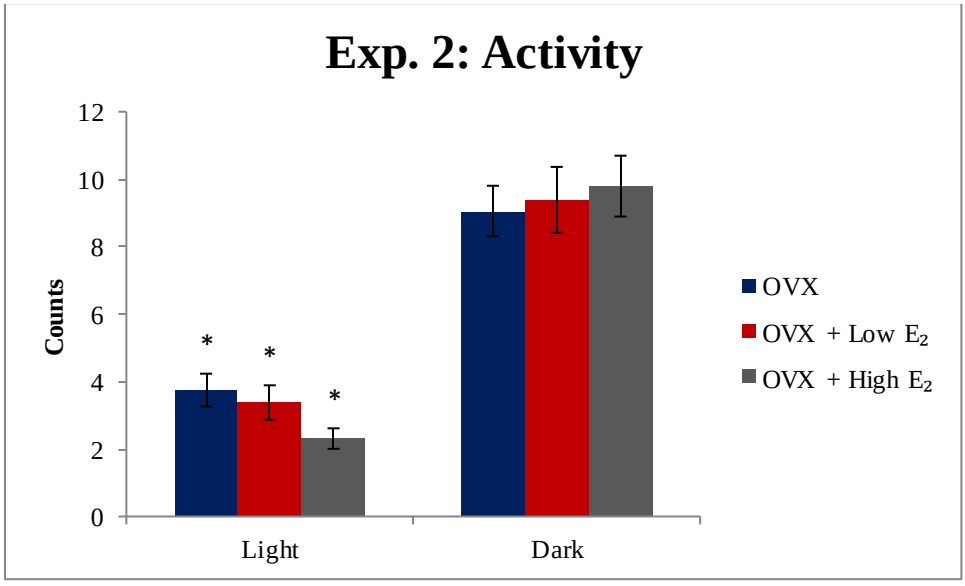


Figure 5: OVX and OVX + E_2 Animals (n=10). Average (±SEM) T_{CORE} (A), T_{SKIN} (B), HLI (C), and Activity (D) of OVX and OVX + E_2 mice during the light and dark phases across 3 days of circadian recording. *, significantly different, light vs. dark within treatment group. +, significantly different, OVX vs. OVX + E_2 within light phase.

Discussion

The rat as an experimental animal model has been well-characterized, but there is less information on the mouse. In our experiments, we found that T_{CORE} and T_{SKIN} in the cycling mouse are unaffected by the fluctuating levels of circulating gonadal hormones present across the cycle stages. The rat, however, demonstrates a decrease in T_{SKIN} during the dark phase of proestrous (Williams, Dacks et al. 2010). Additionally, we demonstrated that estrogen replacement in OVX mice reduces T_{CORE} during the light phase, an effect not observed in the rat. Our studies indicate a species difference between mice and rats in the estrogen regulation of thermoregulation and a lowering of the T_{CORE} set point in estrogen replaced mice.

These data also clearly demonstrate the inverse relationship between T_{CORE} and T_{SKIN}. T_{CORE} is maintained at a higher temperature during the dark phase, while animals are awake and active, and it decreases during the light phase, while they sleep. T_{SKIN}, however, is increased during the light phase, which demonstrates vasodilation to allow for heat loss and a lower T_{CORE}.

This study further validated a novel method for recording tail skin temperature in the mouse and rat that allows for unrestrained, untethered movement for the animal. Using the Star Oddi temperature probe in a plastic housing adhered to the tail caused little to no disturbance to the animal and provided precise, continuous temperature recording. This method will be useful in long-term studies of circadian temperature measurement which require continuous recording for multiple days or weeks.

Characterization of the effects of estrogen on thermoregulation in the mouse is useful given the increased use of transgenic mice in research. We have implicated NKB neurons in the mechanism of flushes, and we can use a transgenic mouse in which neurons expressing the NKB receptor (Tacr3) also express an enhanced green fluorescent protein. This allows for better visualization of the neurons following various thermoregulation experiments.

Interestingly, an antagonist for Tacr3 neurons has been used in clinical trials to treat hot flushes (Prague 2017, Skorupskaite 2017). One trial has currently published through its second phase and is showing positive results. Additionally, an association between the Tacr3 gene and hot flushes has been drawn in a retrospective genetic analysis of the Women's Health Initiative study (Rossouw, Anderson et al. 2002), further adding to the evidence for the thermoregulatory participation of these neurons (Crandall 2016).

While it is encouraging to see supporting data for the role of NKB and the Tacr3 receptor in menopausal hot flushes, elucidating the specific neural pathways involved in this temperature dysregulation is still an important goal. In considering the current neuroanatomical model of thermoregulatory control, it will be important to determine how the projections from arcuate NKB neurons relate to this circuit. With the present characterization of estrogen effects on temperature regulation in the mouse, the transgenic mouse model can be employed to further explore the connection between the reproductive axis and the thermoregulatory control mechanisms.

Appendix A

Experiment 1					
Parameter	*Phase*	*Proestrous*	*Estrous*	*Diestrous*	*p value*
Core (°C)	Light	36.96 ± 0.14*	37.01 ± 0.17*	36.98 ± 0.17*	<0.001*
	Dark	37.86 ± 0.04	37.75 ± 0.04	37.83 ± 0.04	
Tail Skin (°C)	Light	30.92 ± 0.51	30.78 ± 0.51	30.82 ± 0.40	
	Dark	29.53 ± 0.61	29.56 ± 0.56	29.39 ± 0.62	
HLI	Light	0.58 ± 0.03*	0.57 ± 0.03*	0.57 ± 0.03*	<0.05*
	Dark	0.46 ± 0.04	0.46 ± 0.04	0.45 ± 0.04	
Activity (counts)	Light	3.59 ± 0.56*	4.45 ± 0.85*	4.20 ± 0.75*	<0.01*
	Dark	12.15 ± 2.71	14.29 ± 3.53	14.03 ± 3.09	

Table 1: Cycling Animals (n=6; 16 cycles). Average (±SEM) T_{CORE}, T_{SKIN}, HLI, and Activity during the light and dark phases across estrous cycle stages. *, significantly different, light vs. dark within cycle stage.

Experiment 2					
Parameter	*Phase*	*OVX*	*OVX + Low E_2*	*OVX + High E_2*	*p value*
Core (°C)	Light	36.59 ± 0.04*	36.26 ± 0.07*$^+$	36.33 ± 0.05*$^+$	<0.001* <0.02$^+$
	Dark	37.56 ± 0.07	37.74 ± 0.09	37.60 ± 0.06	
Tail Skin (°C)	Light	31.43 ± 0.32*	30.55 ± 0.22	31.54 ± 0.38*	<0.008*
	Dark	29.83 ± 0.46	29.18 ± 0.15	30.09 ± 0.48	
HLI	Light	0.63 ± 0.02*	0.58 ± 0.01*	0.65 ± 0.03*	<0.003*
	Dark	0.49 ± 0.03	0.47 ± 0.01	0.50 ± 0.03	
Activity (counts)	Light	3.75 ± 0.49*	3.38 ± 0.51*	2.31 ± 0.30*	<0.001*
	Dark	9.06 ± 0.75	9.39 ± 0.98	9.79 ± 0.90	

Table 2: OVX and OVX + E_2 Animals (n=10). Average (±SEM) T_{CORE}, T_{SKIN}, HLI, and Activity of OVX and OVX + E_2 mice during the light and dark phases across 3 days of circadian recording. *, significantly different, light vs. dark within treatment group. +, significantly different, OVX vs. OVX + E_2 within light phase.

Acknowledgements

Authors gratefully acknowledge Kenneth Heeg from WB Enterprises for designing our modified tail probe holder for the mouse. This project was supported by National Institute of Health R01 AG-047887.

References

Archer, D. F., D. W. Sturdee, R. Baber, T. J. de Villiers, A. Pines, R. R. Freedman, A. Gompel, M. Hickey, M. S. Hunter, R. A. Lobo, M. A. Lumsden, A. H. MacLennan, P. Maki, S. Palacios, D. Shah, P. Villaseca and M. Warren (2011). "Menopausal hot flushes and night sweats: where are we now?" Climacteric 14(5): 515-528.

Commission for Thermal Physiology, I. U. o. P. S. (2001). "Glossary of terms for thermal physiology: third edition." Jpn J Physiology 51: 245-280.

Crandall C. J., J. E. Manson, C. M. Hohensee, S. Horvath, J. Wactawski-Wende, E. LeBlanc, M. Z. Vitolins, R. Nassir and J. S. Sinsheimer (2017). "Association of genetic variation in the tachykinin receptor 3 locus with hot flashes and night sweats in the Women's Health Initiative Study." Menopause 24(3): 252–261.

Freedman, R. R. (2001). "Physiology of hot flashes." Am. J. Human Biol. 13(4): 453-464.

Hammel, H. T. (1965). "Neurones and temperature regulation. AMRL-TR-65-232." AMRL TR: 1-32.

Krajewski, S. J., M. C. Burke, M. J. Anderson, N. T. McMullen and N. E. Rance (2010). "Forebrain projections of arcuate neurokinin B neurons demonstrated by anterograde tract-tracing and monosodium glutamate lesions in the rat." Neuroscience 166(2): 680-697.

Kronenberg, F. (2010). "Menopausal hot flashes: a review of physiology and biosociocultural perspective on methods of assessment." The Journal of Nutrition 140(7): 1380S-1385S.

Morrison, S. F. and K. Nakamura (2011). "Central neural pathways for thermoregulation." Frontiers in Bioscience 16: 74-104.

Prague J. K., R. E. Roberts, A. N. Comninos, S. Clarke, C. N. Jayasena, Z. Nash, C. Doyle, D. A. Papadopoulou, S. R. Bloom, P. Mohideen, N. Panay, M. S. Hunter, J. D. Veldhuis, L. C. Webber, L. Huson, W. S. Dhillo (2017). "Neurokinin 3 receptor antagonism as a novel treatment for menopausal hot flushes: a phase 2, randomised, double-blind, placebo-controlled trial." The Lancet.

Rance, N. E., N. T. McMullen, J. E. Smialek, D. L. Price and W. S. Young, III (1990). "Postmenopausal hypertrophy of neurons expressing the estrogen receptor gene in the human hypothalamus." Journal of Clinical Endocrinology and Metabolism 71: 79-85.

Rance, N. E. and W. S. Young, III (1991). "Hypertrophy and increased gene expression of neurons containing neurokinin-B and substance-P messenger ribonucleic acids in the hypothalami of postmenopausal women." Endocrinology 128: 2239-2247.

Refinetti, R. and M. Menaker (1992). "The circadian rhythm of body temperature." Physiol Behav 51(3): 613-637.

Romanovsky, A. A., A. I. Ivanov and Y. P. Shimansky (2002). "Selected contribution: ambient temperature for experiments in rats: a new method for determining the zone of thermal neutrality." J. Appl. Physiol 92(6): 2667–2679.

Rossouw, J. E., G. L. Anderson, R. L. Prentice, A. Z. LaCroix, C. Kooperberg, M. L. Stefanick, R. D. Jackson, S. A. Beresford, B. V. Howard, K. C. Johnson, J. M. Kotchen and J. Ockene (2002). "Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial." JAMA 288(3): 321-333.

Santoro, N. (2008). "Symptoms of menopause: hot flushes." Clin. Obstet. Gynecol. 51(3): 539-548.

Skorupskaite K., J. T. George, J. D. Veldhuis, R. P. Millar and R. A. Anderson (2017). "Neurokinin 3 Receptor Antagonism Reveals Roles for Neurokinin B in the Regulation of Gonadotropin Secretion and Hot Flashes in Postmenopausal Women." Neuroendocrinology.

Williams, H., P. A. Dacks and N. E. Rance (2010). "An improved method for recording tail skin temperature in the rat reveals changes during the estrous cycle and effects of ovarian steroids." Endocrinology 151: 5389-5394.