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MOLECULAR METABOLISM

Beyond day and night: The importance of ultradian rhythms in mouse physiology

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ABSTRACT

Our circadian world shapes much of metabolic physiology. In mice ~40% of the light and ~80% of the dark phase time is characterized by bouts of increased energy expenditure (EE). These ultradian bouts have a higher body temperature (Tb) and thermal conductance and contain virtually all of the physical activity and awake time. Bout status is a better classifier of mouse physiology than photoperiod, with ultradian bouts superimposed on top of the circadian light/dark cycle. We suggest that the primary driver of ultradian bouts is a brain-initiated transition to a higher defended Tb of the active/awake state. Increased energy expenditure from brown adipose tissue, physical activity, and cardiac work combine to raise Tb from the lower defended Tb of the resting/sleeping state. Thus, unlike humans, much of mouse metabolic physiology is episodic with large ultradian increases in EE and Tb that correlate with the active/awake state and are poorly aligned with circadian cycling.

Keywords Ultradian and circadian rhythms; Contributors to energy expenditure; Body temperature regulation; Sleep/wake; Brown adipose tissue; Physical activity and physical activity energy expenditure

1. INTRODUCTION

Mice are a frequently used animal model for studying human disease, with approximately 100,000 articles published each year, but use of mice is hampered by inconsistent translation into medicines for humans [1–3]. Improving translatability would increase productivity and decrease the time to successful clinical intervention.

Thermal physiology is an area where mice and humans differ. Mice weigh ~3000 times less than humans and have a ~14-fold greater mass-specific surface area and ~7-fold higher mass-specific metabolic rate [4,5]. Since heat transfer is proportional to both thermal conductivity (also greater in mice) and surface area, mice exhibit much higher specific heat loss. Despite these differences, both mice and humans defend similar Tb and have circadian rhythms with a warmer active phase Tb (day for humans, night for mice) [6,7]. However, humans and mice use different mechanisms to maintain Tb. For humans, vasoconstriction/vasodilation and environmental/behavioral interventions suffice so that only byproduct heat from metabolism is typically required. In contrast, mice at usual vivarium ambient temperatures (Ta, ~22 °C) dedicate 1/3 of total energy expenditure (TEE) to cold induced thermogenesis (CIT) [8–10]. Thus, using a thermoneutral Ta where CIT is minimal is suggested to better mimic human physiology [11–13]. The details of how this should be implemented are debated, including the idea that thermoneutrality could be different in the light and dark phases [9,14–17].

We exist in a circadian world synchronized largely by light [18]. Circadian phase affects a large portion of metabolic processes [19] including Tb and EE [8,20]. Thus, mouse results are typically either reported separately for the light and dark phases or performed during one phase. However, a finer-grained analysis is informative. While the average Tb is similar in mice and humans [21], in frequently sampled data mice have transient Tb excursions of 1–2 °C. These short-term Tb changes, referred to as ultradian cycles, occur during both the light and dark phases and are associated with physical activity [22,23]. Importantly, similar ultradian Tb cycles do not occur in humans. There has been no consensus on the underlying cause(s) of the mouse ultradian Tb variability [7]. It has been suggested that they represent a thermoregulatory strategy to reduce TEE [24,25]. Ultradian cycles have also been observed for EE and were thought not to be heat production to defend Tb [14].

The ultradian EE increases account for ~30% of TEE at room temperature [14]. It is crucial to understand the physiology of this

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Abbreviations (see Table S3 for EE model parameters)

BAT	brown adipose tissue
BB	beam breaks
BMR	basal metabolic rate
BW	body weight
CIT	cold induced thermogenesis
k	thermal conductance
Cp	heat capacity
EE	energy expenditure
FI	food intake
PA	physical activity
PA_τ	physical activity transformed using a time constant
PAEE	physical activity energy expenditure
RER	respiratory exchange ratio
RMSD	root mean square deviation
Ta	ambient (environmental) temperature(s)
Tb	core body temperature
TEF	thermic effect of food
WI	water intake

substantial portion of TEE — otherwise we risk designing mouse studies that do not predict human efficacy, for example of anti-obesity drugs. Here we characterize the ultradian EE and Tb cycles. They show increased physical activity, awake time, Tb, and, paradoxically, thermal conductance. Unlike humans, in mice the ultradian cycles occur frequently — multiple times within a single light or dark phase. We propose that the primary driver of the bout EE increase is thermogenesis to achieve the higher defended Tb of the active/awake state.

2. METHODS

2.1. Mice

Mice were housed (including during indirect calorimetry) in Tecniplast 1284 cages (365 × 207 × 140 mm) with a 12:12-h dark:light cycle (lights on at 0600) in a clean, conventional facility with ∼95 g of wood-chip bedding (7090 Teklad sani-chips, Envigo, Indianapolis, IN) without enrichment or nesting materials. Chow (NIH-07 Envigo Inc, Madison, WI, 3.1 metabolizable kcal/g food, food quotient 0.909) and water were provided *ad libitum*. Housing was at 21—23 °C, unless otherwise stated. Experiments were approved by the NIDDK Institutional Animal Care and Use Committee, protocol K016-DEOB-23. C57BL/6 J and *Ucp1*$^{-/-}$ (#003124) [26] mice were obtained from Jackson Laboratory. *Sln*$^{-/-}$ mice were provided by Dr. Gopal Babu [27]. The cohorts of mice are detailed in Table S1.

2.2. Indirect calorimetry and Tb telemetry

Some of the raw data were reported previously [28] with additional data collected using the same protocols. In brief, energy expenditure (EE), respiratory exchange ratio (RER), physical activity (ambulatory infrared beam breaks), food intake (from a hanging feeder), and water intake were measured in a 12-cage 'home cage' Comprehensive Lab Animal Monitoring System (CLAMS-HC using Oxymax v5.52 software, Columbus Instruments, Columbus, OH). Ambient temperature (Ta) inside each cage was monitored continuously. To increase data sampling density, only six cages were used, with each cage sampled every 120 or 140 s (reference air was measured every other cycle). Cages were housed in a single environmental chamber that controlled the ambient temperature. Mice were acclimated for one day, then data were collected for 48 h at each ambient temperature. Temperature settings were changed at 0600, with data omitted (≤1 h) until the temperatures approached the specified target. Food and water were supplied at the start of the indirect calorimetry and did not require replenishment during the experiment, except water replenishment was sometimes needed at 35 °C and then the hour after replenishment was excluded from analysis. Cages were changed immediately before indirect calorimetry and not changed during the experiment. Energy cost of physical activity (PAEE) was calculated from the measured beam breaks and body weight using 0.757 cal/1000 beam breaks/g body weight [28].

G2 E-Mitter (Starr Life Sciences, Oakmont, PA) Tb telemetry sensors were implanted intraperitoneally under isoflurane anesthesia (5% induction, 1.2% maintenance; Baxter Healthcare Corporation, Deerfield, IL) with Prevail (flunixin meglumine) analgesia (2.2 mg/kg sc at operation). Telemetry data were collected using Oxymax v5.52 software. Mice were studied at least two weeks after surgery.

2.3. Detection of bouts of increased EE

Bouts of increased EE were identified with the R Pracma package function 'find peaks' [29] separately in each light or dark phase of each mouse. Bout starts were identified as the start of a peak. Bout ends were initially identified using the additive inverse of the EE, then the last four measurement intervals (8.67 min) were removed to account for the delayed response due to the time constant (8.6 min) of the indirect calorimetry system [30]. See Table S2 for additional details and for the initial function parameters used for each Ta. This was followed by manual curation, generally to remove extra start/end points due to EE variability. For example, the bout identification method does not reliably detect bouts of short (<15 min) duration. The between bout period is defined as the time from the end of one bout (EE minimum) to the start of the next. The baseline for each bout is defined as the average of the EE at bout start and end. Because mice were highly active after Ta was set to 35 °C at 0600 (onset of the light phase) the first 12 h at this temperature were omitted from peak analysis. At 35 °C bout identification may be less accurate since short bouts with small EE changes may be missed, which could lower the percentage of PA, FI, and WI in bouts.

2.4. Infrared thermography

Infrared thermography of the shaved skin over the interscapular and lumbar areas was performed as described [31,32]. During infrared thermography, video tracking (EthnoVision XT, Noldus) was used to capture locomotion activity [33] since EE was not available to determine bouts. Activity initiation was defined using the following criteria with 1-minute intervals: Two consecutive inactive intervals (activity ≤4 cm/min) followed by 3 consecutive active intervals (activity >4 cm/min), with mean activity of the 8 preceding intervals being ≤4 cm/min and the mean activity of the 8 following intervals being >6 cm/min. Termination of activity was defined at the first 2 consecutive inactive points after initiation that also have mean activity ≤4 cm/min over the 8 subsequent intervals.

2.5. Heart rate and blood pressure

Continuous ambulatory intra-arterial blood pressure, heart rate, physical activity, and Tb were measured as described [34] with radio transmitters (HD-X11, Data Sciences International (DSI), St Paul, MN) implanted under ketamine and xylazine anesthesia in the carotid artery in male mice of mixed genetic background by the NHLBI Murine

Phenotyping Core. Mice were studied at least two weeks after transmitter implantation. Data were sampled at 1,000 Hz using a PhysioTel RPC-1 receiver and Ponemah v6.30 (DSI) software, with 1-minute averages used for analysis; EE was not available in this data set. Activity levels were quantified from the HD-X11 data using Ponemah software. Activity initiation was defined as two consecutive inactive 1-minute intervals (activity $\leq$0.01 counts/min) followed by an active interval (activity $>$0.01 counts/min), with at least 2 of the next 4 intervals also being active and mean activity over 10 preceding intervals was $\leq$0.03 counts/min and over 10 following intervals was $>$0.04 counts/min. Initiations also had to be separated by at least 15 intervals. Activity termination was defined as the first 2 consecutive inactive points after the initiation, with activity of the 8 following intervals averaging $\leq$0.02 counts/min.

2.6. Electroencephalogram (EEG) and electromyogram (EMG)

EEG and EMG signals were recorded using radio transmitters (HD-X02, DSI) and Ponemah software. The transmitters were implanted under ketamine and xylazine anesthesia, and the electrodes were placed as described [35]. Mice were studied at least two weeks after transmitter implantation. The sleep-wake states were scored in 10-second periods, categorized as rapid eye movement (REM) sleep, non-REM (NREM) sleep, and awake, using NeuroScore software (DSI, St. Paul, MN). The NeuroScore-defined wake or sleep stages were then visually inspected and corrected if necessary [35].

2.7. Model of EE based on Tb and thermal conductance

From thermodynamics, the change in heat content of a mouse is the difference between the heat produced and heat lost,

$$Cp \cdot dTb = EE \cdot dt - k \cdot (Tb - Ta) \cdot dt \quad (1)$$

where EE is energy expenditure, Tb is body temperature, Ta is ambient temperature, Cp is the mouse heat capacity, k is the whole-body thermal conductance, and t is time [36,37].

A finite difference equation estimate of equation (1) solved for Tb is:

$$Tb(n+1) = Ta(n+1) + \frac{EE(n)}{k} + \left[Tb(n) - Ta(n) - \frac{EE(n)}{k}\right] \cdot e^{-\frac{k \cdot \Delta t}{Cp}} \quad (2)$$

where Δt is the interval duration and n is the interval number.

Equation (2) can be rearranged to

$$EE(n) = \frac{k}{1 - e^{-\frac{k \cdot \Delta t}{Cp}}} \cdot \left([Tb(n+1) - Ta(n+1)] - [Tb(n) - Ta(n)] \cdot e^{-\frac{k \cdot \Delta t}{Cp}} \right) \quad (3)$$

The EE in equation (3) does not depend on prior or subsequent intervals. In contrast, the measured EE lags due to the exhaled gases mixing with air already in the calorimetry chamber. The time constant (τ) for the mixing is the volume of the cage divided by the airflow [30]. Thus, to align the calculated and measured EE values, the calculated EE values were adjusted,

$$EE\tau(n+1) = EE(n+1) + [EE\tau(n) - EE(n+1)] \cdot e^{-\frac{\Delta t}{\tau}} \quad (4)$$

where EEτ represents τ-transformed EE.

Equations (3) and (4) allow one to calculate the EE required to achieve the observed changes in Tb. Since Ta changes are minimal, this tests how well Tb can predict EE.

2.8. Implementation of EE model based on Tb and thermal conductance

A model using equations (3) and (4) was constructed by optimizing the fit (minimizing the sum of squares) between the measured and predicted EE while fitting a constant conductance, k ('optim' function from the 'base' package in R). This was applied to $\sim$48 h of data (omitting $<$1 h before Ta reached its target), sampling every 120–140 s, for each mouse, combining light and dark phases (there were 1261 $\pm$ 12 intervals available per mouse for Ta 23–32 °C and 788 $\pm$ 23 for 35 °C; the first 12 h of data were omitted at 35 °C). The time constant (τ) in our system is 8.6 min. Parameters are summarized in Table S3 and below.

Cp, heat capacity, is estimated from the body weight and fat fraction (m_f) using 0.9100–0.5231 m_f cal/g BW/°C, without correction for the implanted E-Mitter [8,38]. Implanted E-mitters prevent measurement of body composition, so it was estimated from body weight using previously published male C57BL/6J data [32], $m_f = 0.0140 \pm 0.0015 * BW - 0.218 \pm 0.043$, where BW is body weight, n = 28, $r^2 = 0.762$. For each mouse, the Ta changes were minimal, and Ta(n) was used for Ta(n+1).

This initial model held k constant (see **3.8**). However, k was observed to be bimodally distributed, so the model was modified to fit two conductances, k_{high} for intervals with PA(n) $>$ the median 24-h physical activity and k_{low} for intervals with PA(n) $\leq$ the median physical activity (see **3.8**). While other ways of assigning between two conductances were explored, the median PA is biologically relevant and produced the greatest improvement in model fit.

2.9. EE and Tb measurement in human subjects

The EE, Tb, and activity study of healthy men under eucaloric conditions at 24 °C has been reported [39], ClinicalTrials.gov identifier NCT00523627. In brief, subjects resided in a whole-room indirect calorimeter for 23.25 h, with meals provided at 07:00, 11:00, and 16:00 and a snack at 19:00. Exercise was discouraged, but low intensity (self-care) activities were permitted. EE was calculated each minute from the rates of O_2 consumption and CO_2 production, and Tb was measured every 30 s using an ingested capsule (CorTemp®, HQ Inc., Palmetto, FL). All subjects (n = 39) with at least 80% complete EE and Tb data from the 23.25 h session were included. Tb measurements were cleaned by setting consecutive values changing by $\geq$ 1 °C and values $<$ 36 °C or $>$38 °C to missing. Tb was then smoothed with a 21-minute median filter, reducing technical noise (transient drifts and sensor instability) while preserving the prevailing Tb dynamics. The same median filter was applied to the EE measurements, and Pearson's correlation was calculated between the EE and Tb filtered time series for each subject. When indicated, EE was corrected from the measured Tb to 37 °C using a Q_{10} of 2.3 [40–43] as follows:

$$EE_{37°C} = EE \cdot 2.3^{\left(\frac{37 - Tb}{10}\right)}$$

2.10. Statistical analysis and data deposition

Data analysis, model parameter fitting, and statistics were performed using R (version 4.1.1, packages: tidyverse, rio, stats [44]) and Prism (version 8.1.0; GraphPad Software, Inc.). Data are presented as mean $\pm$ SEM, unless indicated otherwise. Data, including curated bout identifications, and computer code are deposited at https://osf.io/9bszy/.

3. RESULTS

3.1. Mouse physiology is episodic, with bouts of increased EE over baseline

In mice, when averaged by photoperiod, energy expenditure (EE), body temperature (Tb), physical activity (PA), food intake (FI), and water intake (WI) are higher during the dark/active phase and depend on ambient temperature (Figs. S1A–E). While EE, Tb, and PA are highly correlated, both FI and WI also correlate with EE, Tb, and PA, but less strongly (Figure 1A). Notably, even within each light or dark phase the EE varies greatly (Figure 1B).

When individual mouse data were examined, there were episodes of increased EE ('EE bouts'), which reached similar maximum levels and returned to an approximately constant between-bout baseline (Figure 1B). We defined the start of the EE bout as the last minimum before EE increases (see 2.3). When the data were aligned to the EE bout start, physical activity began increasing at the bout start (Figure 1C). In contrast, the Tb increase (Figure 1C) occurred after a delay of 3.01 ± 0.22 min (light) or 3.63 ± 0.24 min (dark) at 23 °C, with comparable delays at 26–32 °C and a shorter lag at 35 °C (light, 2.04 ± 0.17; dark, 1.87 ± 0.29 min). The delay in Tb increase may be attributable to an immediate increase in heat loss from locomotion, during which the mouse has a less compact body posture/configuration, increasing convective heat transfer and also from heat loss due to increased blood flow. The initial rates of increase in EE and Tb and the maximum EE and Tb during the bout were greater at lower Ta values. Whole body thermal conductance, k, is a measure of the rate of heat loss from the mouse and is approximated by EE/(Tb-Ta), see 2.7 [37]. The conductance increased in parallel with the EE increase (Figure 1C). It might seem counterintuitive that the conductance would increase during bouts, as greater heat loss requires the mouse to have a higher EE to support Tb. Thus, this result suggests that during bouts, the physiologic processes that increase heat loss (e.g., locomotion with increased convective loss) are prioritized over energy conservation. RER did not consistently change with bout onset. Food intake increased soon after the bouts started.

To examine the end of the bouts, the data were aligned to the EE nadir after each bout (see Methods), revealing that PA had already been minimal for 8–10 min (Figure 1D). The EE nadir preceded the Tb nadir, especially in the light phase.

These results demonstrate that mice have bouts of increased EE accompanied by increased Tb, thermal conductance, and locomotor activity during both the light and dark phases and across a range of Ta values.

3.2. Characteristics of the EE bouts

We next analyzed bout characteristics. At 23 °C, 40% (light) or 74% (dark) of the time was in EE bouts, with similar percentages at 26 °C–32 °C (Figure 2A). The number of EE bouts ranged from four to eight per phase and was not affected by either Ta or light vs dark phase (Figure 2B). Bout durations at 23 °C–32 °C were shorter during the light phase, with medians of 39–45 min vs 54–71 min in the dark phase (Figure 2C).

While the bouts were defined solely from the EE, bout status is a remarkably strong determinant of Tb, conductance, PA, FI, and WI. These were all increased during bouts at all Ta in both phases (Figure 2D–I). For example, at 23 °C bout EE increased by 41.6 ± 2.3% (light, $P = 2 \times 10^{-11}$, t-test) or 50.0 ± 2.2% (dark, $P = 8 \times 10^{-16}$, t-test) over between bout levels, Tb was higher by 0.79 ± 0.07 °C (light, $P = 1 \times 10^{-7}$, t-test) or 0.96 ± 0.06 °C (dark, $P = 1 \times 10^{-7}$, t-test), and conductance was greater by 31.7 ± 3.3 % (light, $P = 9 \times 10^{-19}$, t-test) or 38.7 ± 3.0 % (dark, $P = 3 \times 10^{-18}$, t-test). For all Ta<35 °C, bout status rather than light/dark phase accounted for most of the variance in PA (53%), FI (59%), and WI (55%) (Figure 2J top). While bout status explains more of the variation in Tb (38% at 23 °C), light/dark phase is also a major contributor (21% of variation at 23 °C) (2-way ANOVA, Figure 2J bottom). For example, at 23 °C the mean bout Tb was 36.24 °C (light) and 36.98 °C (dark) while the between bout Tb was 35.43 °C (light) and 36.03 °C (dark). At Ta of 23 °C–32 °C, the vast majority of PA, FI, and WI intake occurred during EE bouts, illustrating the occurrence of this physiology within EE bouts (Figure 2K-M).

At 35 °C mice are heat stressed, with increased Tb, shorter bout durations (28 min in light; 37 min in dark), less time in bouts (25% in light; 44% in dark), and smaller Tb, EE, and conductance differences comparing bouts and between bout intervals.

Taken together, these data suggest that mice have two main physiological states, EE bouts and a between bout baseline. The bouts have a higher Tb and include the vast majority of the PA, FI, and WI. Importantly, light/dark phase correlates less well with Tb, conductance, PA, FI, and WI than does bout status, although phase contributes to Tb. These results support characterization of mouse physiology by bout status.

3.3. EE bouts coincide with the awake state

We next investigated the correlations between sleep-wake measures and the EE bouts. The EE bouts coincide remarkably well with the awake state (Figure 3A). When the data were aligned to the EE bout start, waking up occurred precisely at the bout onset, with a burst of REM sleep and initiation of locomotor activity (Figure 3B, Fig. S2). In the dark phase, as expected, there was less sleep and the EE, Tb, RER, and activity levels were all higher. The end of the EE increase was marked by sleep onset, coincident with reduced activity at 8–10 min prior to the EE minimum (Figure 3C). At 23 °C, over 90% of awake time was during bouts (Figure 3D). The results demonstrate that the EE bouts coincide with the awake state. Thus, the two states identified by EE largely correspond to active/awake (bout) and resting/sleeping (between bout) states.

3.4. Muscle contributes only some of the bout increase in energy expenditure

What contributes to the increased EE and Tb of the active/awake state? Previously, it has been implicitly assumed that muscle activity and attendant metabolism is the chief contributor to the EE bouts, especially at thermoneutrality [8,45]. However, another hypothesis is that there is a lower defended Tb during rest/sleep and a higher defended Tb during active/awake, with increased EE to achieve this. We used a physiology-based model of EE [28] to investigate, quantifying three contributors to the bout EE increase: the energy cost of physical activity (PAEE), the increase in basal metabolic rate (BMR) due to a higher Tb (using a van't Hoff coefficient, Q_{10}, of 3 [46–49]), and the residual EE that is not attributable to either PAEE or the Q_{10} effect.

An example of data at 23 °C from one mouse (Figure 4A) shows that after subtracting the PAEE and Q_{10}-corrected BMR from the EE, much of the bout signal remains, indicating that PAEE only partially contributes to the bout EE increase. Thermic effect of food (TEF) was not included because food intake did not correlate with TEE, with some

A

23 °C	EE	Tb	PA	FI	WI
EE		0.756	0.634	0.185	0.181
Tb	0.737		0.537	0.099	0.139
PA	0.601	0.511		0.135	0.208
FI	0.121	0.026	0.052		0.131
WI	0.171	0.126	0.194	0.143	

26 °C	EE	Tb	PA	FI	WI
EE		0.682	0.546	0.157	0.167
Tb	0.637		0.357	0.055	0.091
PA	0.547	0.449		0.121	0.239
FI	0.129	0.024	0.069		0.086
WI	0.139	0.079	0.169	0.142	

29 °C	EE	Tb	PA	FI	WI
EE		0.634	0.521	0.117	0.174
Tb	0.645		0.345	0.018	0.098
PA	0.563	0.523		0.090	0.230
FI	0.112	0.012	0.034		0.084
WI	0.108	0.043	0.137	0.099	

32 °C	EE	Tb	PA	FI	WI
EE		0.847	0.641	0.084	0.137
Tb	0.828		0.555	0.041	0.121
PA	0.660	0.601		0.066	0.170
FI	0.069	0.035	0.058		0.037
WI	0.147	0.121	0.203	0.036	

35 °C	EE	Tb	PA	FI	WI
EE		0.307	0.661	0.026	0.196
Tb	0.715		0.136	-0.004	-0.043
PA	0.610	0.359		0.038	0.222
FI	0.068	0.029	0.078		-0.004
WI	0.090	0.017	0.125	0.039	

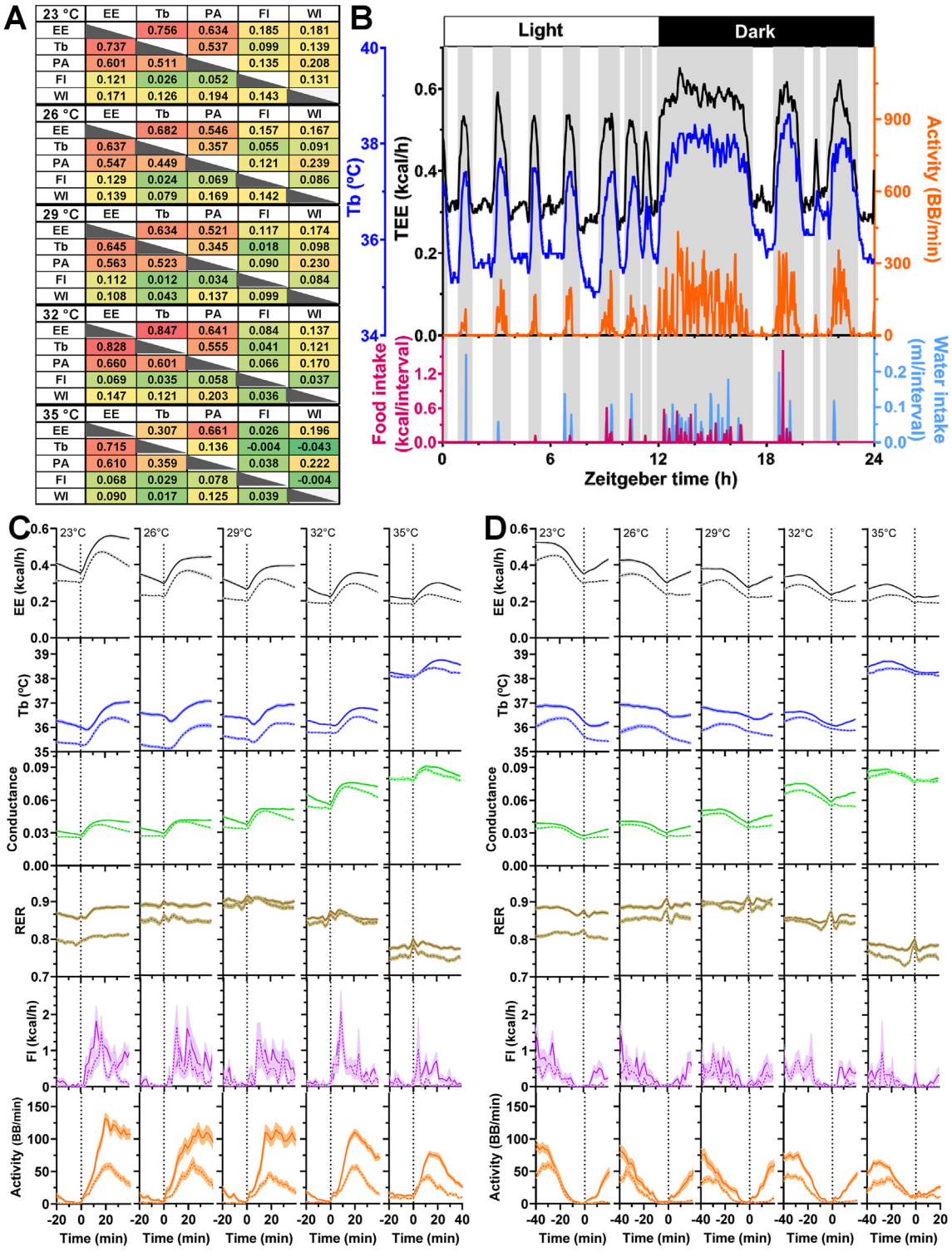
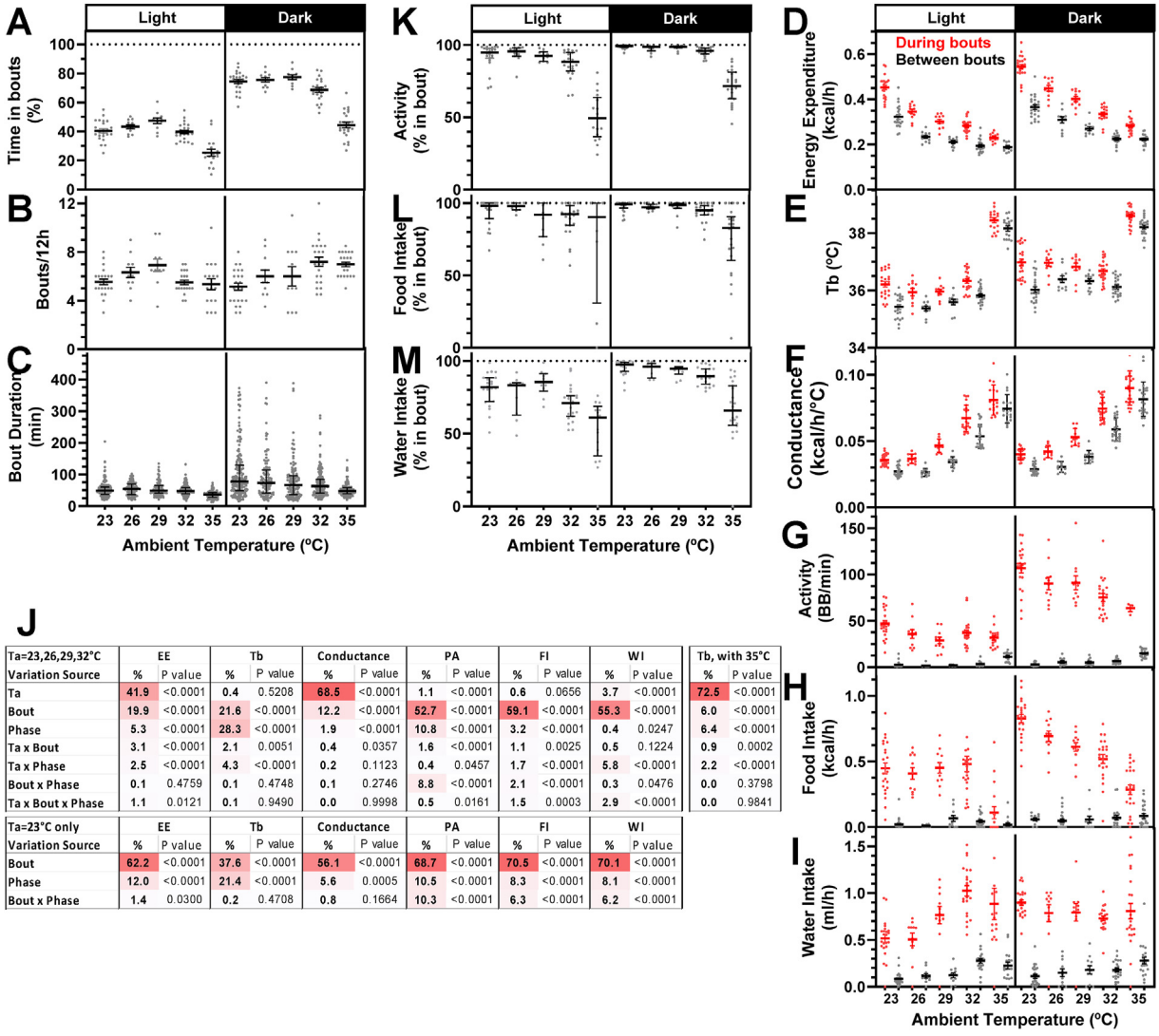


Figure 1: Energy expenditure occurs in bouts (A) Within-mouse Pearson correlations, *r*, between energy expenditure (EE), body temperature (Tb), physical activity (PA), food intake (FI), and water inter (WI) in male C57BL/6J mice at the indicated Ta over 48 h (~1200 intervals/mouse). Light phase data are above the diagonal and dark phase below it. Cells are color-coded green to orange from low to high *r*. Data are mean of the *r* values from n = 11 (26, 29 °C) or n = 23 (23, 32, 35 °C) mice. A subset of this data was reported previously [28]. (B) Example of TEE, Tb, activity, food intake, and water intake at 23 °C of one mouse over 24 h. EE bouts are shaded in gray. (C, D) Characterization of bout transitions. Data were aligned to the EE minimum (time 0) at the start (C) or after the end (D) of the bout. Data are presented for EE, Tb, thermal conductance (calculated as TEE/[Tb-Ta]), respiratory exchange ratio (RER, vCO_2/vO_2), FI, and activity during the dark (solid line) and light (dashed line) phases at a nominal Ta of 23 °C, 26 °C, 29 °C, 32 °C, and 35 °C, as indicated. Data are mean ± SEM. The number of bouts in C,D is: 23 °C, 222 (light), 237 (dark); 26 °C, 139 (light), 132 (dark); 29 °C, 152 (light), 132 (dark); 32 °C, 253 (light), 331 (dark); and 35 °C, 91 (light), 282 (dark).



J

Ta=23,26,29,32°C	EE		Tb		Conductance		PA		FI		WI		Tb, with 35°C	
Variation Source	%	P value	%	P value	%	P value	%	P value	%	P value	%	P value	%	P value
Ta	41.9	<0.0001	0.4	0.5208	68.5	<0.0001	1.1	<0.0001	0.6	0.0656	3.7	<0.0001	72.5	<0.0001
Bout	19.9	<0.0001	21.6	<0.0001	12.2	<0.0001	52.7	<0.0001	59.1	<0.0001	55.3	<0.0001	6.0	<0.0001
Phase	5.3	<0.0001	28.3	<0.0001	1.9	<0.0001	10.8	<0.0001	3.2	<0.0001	0.4	0.0247	6.4	<0.0001
Ta x Bout	3.1	<0.0001	2.1	0.0051	0.4	0.0357	1.6	<0.0001	1.1	0.0025	0.5	0.1224	0.9	0.0002
Ta x Phase	2.5	<0.0001	4.3	<0.0001	0.2	0.1123	0.4	0.0457	1.7	<0.0001	5.8	<0.0001	2.2	<0.0001
Bout x Phase	0.1	0.4759	0.1	0.4748	0.1	0.2746	8.8	<0.0001	2.1	<0.0001	0.3	0.0476	0.0	0.3798
Ta x Bout x Phase	1.1	0.0121	0.1	0.9490	0.0	0.9998	0.5	0.0161	1.5	0.0003	2.9	<0.0001	0.0	0.9841

Ta=23°C only	EE		Tb		Conductance		PA		FI		WI	
Variation Source	%	P value	%	P value	%	P value	%	P value	%	P value	%	P value
Bout	62.2	<0.0001	37.6	<0.0001	56.1	<0.0001	68.7	<0.0001	70.5	<0.0001	70.1	<0.0001
Phase	12.0	<0.0001	21.4	<0.0001	5.6	0.0005	10.5	<0.0001	8.3	<0.0001	8.1	<0.0001
Bout x Phase	1.4	0.0300	0.2	0.4708	0.8	0.1664	10.3	<0.0001	6.3	<0.0001	6.2	<0.0001

Figure 2: Characteristics of EE bouts. (A) Percentage of time spent in bouts for the indicated Ta and phase, (B) average number of EE bouts per 12 h, and (C) bout duration (n = 70 to 257 bouts/condition). (D—I) Energy expenditure, Tb, thermal conductance, physical activity, food intake, and water intake during (red) and between (black) bouts for the indicated Ta and phase. (J) Top is the 3-way ANOVA analysis of D-I, excluding Ta = 35 °C. At the right is 3-way ANOVA for Tb including 35 °C (ANOVA results for TEE, PA, FI, and WI including 35 °C were similar to the analysis excluding 35 °C). Bottom is 2-way ANOVA for Ta = 23 °C. Darker color indicates a higher percentage of the variance explained. (K—M) Percentage of physical activity, food intake, and water intake that occurred during EE bouts for the indicated light/dark phase and ambient temperatures. In A-C, bouts that started in one phase and ended in the other were omitted, which may preferentially remove longer bouts at the beginning of dark phase, while in D-M all time points are used. See Figure 1C,D for the number of EE bouts in B. The first 12 h (light phase) at 35 °C were omitted from analysis in all mice due to increased physical activity. Data are mean ± SEM (median ± interquartile range in C,K-M) for n = 11 (26, 29 °C) or n = 23 (23, 32, 35 °C) male C57BL/6J mice. In H, nine (light) and one (dark) mice were omitted due to no food intake in the interval.

bouts not having any food intake (Figure 1A,B) and because the time lag between food intake and TEF is not known in mice. We refer to the residual EE as arousal thermogenic EE, defined as the EE dedicated to supporting the Tb increase of the active/awake state, that we hypothesize is the basis for the residual EE. We further develop this concept below.

Arousal thermogenic EE was greater at lower ambient temperatures and in the light phase (Figure 4B). At 23 °C the percentage of thermogenic EE was 60 ± 2% in the light phase (PAEE was 32 ± 2% and Q_{10} explained 8.3 ± 0.4%) and 31 ± 2% in the dark phase (PAEE: 62 ± 3% and Q_{10}: 7.2 ± 0.4%) (Figure 4B). Overall, the results show that while PAEE and Q_{10} contribute to the increase in EE during the active/awake state, a large fraction of the EE increase is not due to muscle activity or the BMR increase caused by the higher Tb.

To probe whether locomotor activity is required for the bout EE increase, we examined the EE bouts with the lowest quintile of activity level. The arousal thermogenic EE was similar in the low activity EE bouts (Figure 4C). For example, at 23 °C the PAEE contribution to bout EE was 9.0 ± 0.8% (light) and 22 ± 2% (dark) and the thermogenic energy expenditure fraction increased to 85 ± 4% (light) and 75 ± 4% (dark). Since the low activity bouts were of shorter duration, so we also compared them to bouts of similar duration from the top four quintiles, which confirmed the conclusions (Figs. S3). Thus, EE bouts occur even with only modest locomotor activity, with arousal thermogenesis becoming a larger percentage of bout EE when activity levels are low.

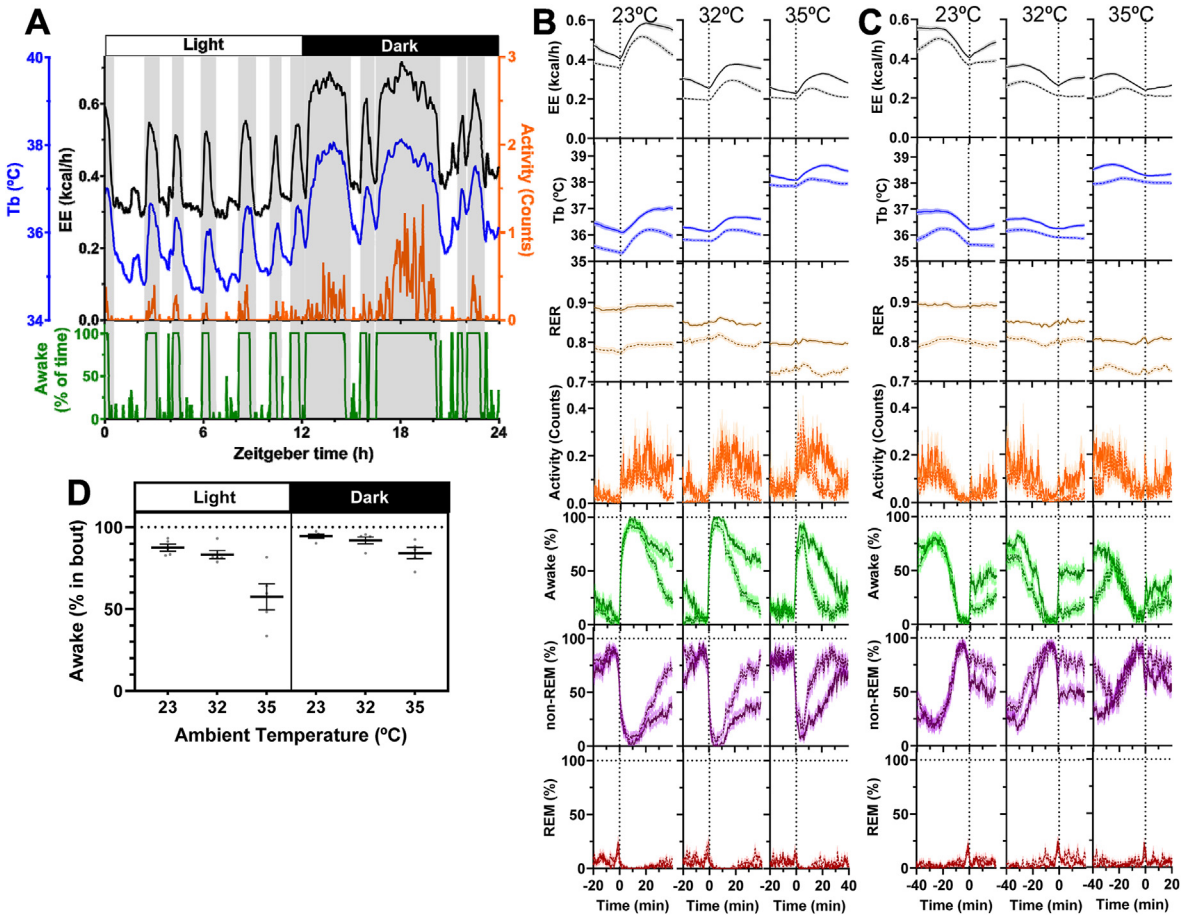


Figure 3: Association of EE bouts with the awake state. (A) Example of 24 h of TEE, Tb, activity, and awake state of a mouse at 23 °C. EE bouts are shaded in gray. Awake is the percentage of 10-sec epochs in each 2-min interval scored as awake (see methods). (B) Data aligned to the EE bout start at time 0, showing EE, Tb, RER, activity, awake state, non-REM sleep, and REM sleep during the dark (solid line) and light (dashed line) phases at nominal Ta of 23 °C, 32 °C, and 35 °C, as indicated. Data are mean ± SEM; SEM is omitted from activity, awake, non-REM, and REM graphs for visual clarity. (C) Data aligned to nadir TEE at time 0 after each bout, otherwise as in (B). The number of EE bouts is: 23 °C, 79 (light), 61 (dark); 32 °C, 63 (light), 80 (dark); and 35 °C, 57 (light), 92 (dark).

3.5. Increased BAT thermogenesis contributes to the bout increase in EE

We hypothesized that PAEE, via production of byproduct heat [28], would reduce the need for BAT thermogenesis, while thermogenesis to increase Tb would activate BAT. We used infrared thermometry to measure interscapular and lumbar skin temperatures (Figure 5A), which reflect BAT and core body temperatures, respectively [32]. With bout onset, both T_{BAT} and T_{Lumbar} increased. Pre-bout, T_{BAT} was 1.01 ± 0.11 °C (light phase) warmer than T_{Lumbar} (Figure 5B). This difference increased to 1.63 ± 0.14 °C at 4 min after the start of activity and returned to baseline by 8–10 min. In *Ucp1*$^{-/-}$ mice with impaired BAT function, the baseline $T_{BAT} - T_{Lumbar}$ difference was smaller, 0.43 ± 0.07 °C ($P = 9 \times 10^{-5}$ vs wild type) and with bout onset, the *Ucp1*$^{-/-}$ $T_{BAT} - T_{Lumbar}$ difference did not change (Figure 5C). In wild type and *Ucp1*$^{-/-}$ mice, at the bout ends, both T_{BAT} and T_{Lumbar} gradually decreased without a distinct reduction in the $T_{BAT} - T_{Lumbar}$ difference (Figs. S4A and D). Dark phase results replicated the light phase results (Figs. S4B and C).

Our interpretation is that there is an increase in thermogenesis to raise Tb, which includes BAT activation coincident with the onset of increased physical activity and this arousal thermogenesis mostly explains the non-PAEE, non-Q_{10} increase in bout EE. Notably, this is the opposite of the expected compensatory decrease in BAT thermogenesis if muscle activity alone was driving the bout increase in EE.

3.6. Increased cardiac work is a minor contributor to bout EE

Heart rate and blood pressure increased at activity onset and decreased with its termination, in both the light and dark phases (Figure 5D, Figs. S4E–G). The increased blood circulation presumably reflects the increased need for fuel supply to and heat dissipation and waste removal from active muscle and BAT. These hemodynamic changes increase cardiac work. The bout increase in heart rate of 120 bpm is expected to cause a 21% increase in cardiac output [50]. Cardiac EE is cardiac output times aortic pressure [51], so the observed 15 mmHg (~15%) rise in mean arterial pressure suggests a (1.21 × 1.15 =) 39% increase in cardiac EE. In resting humans, cardiac oxygen consumption is 7% of body oxygen consumption [52]. Assuming the 7% value applies for mice, the increase in cardiac EE increase is (39% x 0.07 =) 2.7% of BMR. At 23 °C, the increase in bout EE is ~45% of BMR. Thus, the cardiac EE increase is (2.7% ÷ 0.45 =) 6% of the bout EE increase.

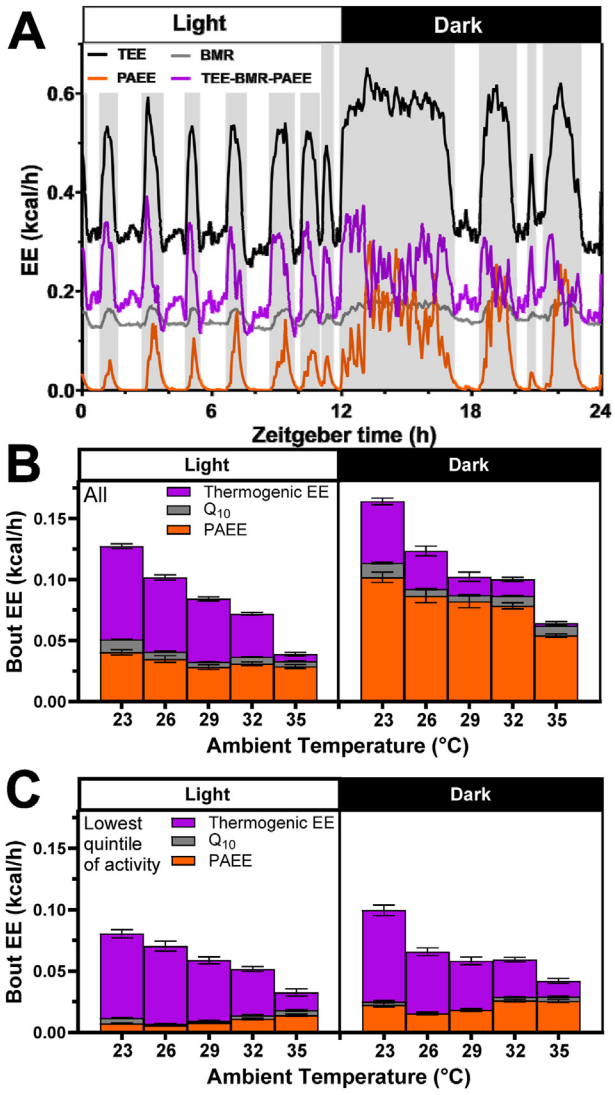


Figure 4: Contributors to bout EE. (A) Example of bout EE contributors, showing the same mouse as in Figure 1B. TEE (black) is measured, PAEE (orange) and BMR (grey) are calculated as described [28], and the arousal thermogenic energy expenditure (violet) is the result of subtracting the PAEE and BMR from the TEE. (B) Contributors to bout EE from 48 h of measurements from n = 11 (26, 29 °C) or n = 23 (23, 32, 35 °C) male C57BL/6J mice; data are mean ± SD. (C) Contributors to bout EE in low activity bouts. Bouts in the bottom quintile of PA level were analyzed as in B.

3.7. Arousal bout physiology is similar in *Ucp1*$^{-/-}$, *Sln*$^{-/-}$, and female mice

We examined the generality of our results in other cohorts of mice at 23 °C and 32 °C (cohort characteristics in Fig. S5). In *Ucp1*$^{-/-}$ mice, the bout number, duration, and time in bouts were comparable to wild type mice (Fig. S6). As seen in wild type mice, the bouts had higher EE, Tb, conductance, and activity. Again, bout status was a better predictor of the increases in these properties than was light/dark phase status. Finally, thermogenic EE was a majority of bout EE (57 ± 4% at 23 °C, light phase) and PAEE was 36 ± 4%, both similar to wild type (58% and 33%, respectively). Thus, the impaired BAT thermogenesis of *Ucp1*$^{-/-}$ mice had no effect on multiple measures of arousal bout physiology, except for the impaired BAT contribution to thermogenesis (Figure 5C); presumably other tissues such as muscle are compensating.

Mice lacking sarcolipin (*Sln*$^{-/-}$) have diminished muscle non-shivering thermogenesis [53]. However, *Sln*$^{-/-}$ exhibited arousal bout physiology indistinguishable from the wild type and *Ucp1*$^{-/-}$ mice (Fig. S6). Female mice have some differences in thermal biology compared to males, such as a higher Tb [10,54,55]. Besides the higher Tb, female C57BL/6 J mice showed no differences from males for the arousal physiology attributes studied (Fig. S6).

3.8. Tb and conductance changes are the drivers of EE variation

A unifying hypothesis that explains our results is that the bouts of increased EE reflect the work to achieve an increased Tb. However, the target Tb alone is not sufficient to determine the EE. For example, a physiologic model of EE variation based on Tb (see Methods) explains only 54 ± 2 % (n = 23, Ta = 23 °C) of the variance in EE (Figure 6A, Fig. S7D). The fit quality is modest because this model assumes a constant conductance while the measured conductance varies, with a bimodal distribution (Figure 6A,B). While some factors that increase bout conductance are known (body position, blood flow changes, body motion that increases convective heat loss, nesting, huddling, etc), they do not permit numerical calculation of k based on first principles. Thus, we used physical activity as a binary marker for the active/awake vs resting/sleeping state. Indeed, when activity was greater than the median activity level, the conductance was greater than the median conductance 86% of the time and when activity was less than the median activity level, the conductance was below the median conductance 85% of the time (Figure 6B). Using this information, EE was modeled from the Tb using activity level to specify between two conductances, namely k_{high} for intervals with activity above the median activity level and k_{low} for intervals with activity below the median. Having two conductance levels greatly improved the fit, now explaining 80 ± 2 % (n = 23, Ta = 23 °C) of the EE variance Figure 6C, Fig. S7E). At all five Ta, the calculated parameters, k_{high} and k_{low}, (Figure 6D) were similar to the measured bout and between bout conductances (Figure 2F).

In the above analysis, activity is an on/off marker of bout status. We also tested Tb, instead of PA, as a binary marker for k_{low} and k_{high}. When the Tb was greater than the median Tb, the conductance was greater than the median conductance 87% of the time and when Tb was less than the median Tb, the conductance was below the median conductance 86% of the time (Fig. S7A). Using the median Tb to switch between two conductances, also yielded an excellent fit to the measured EE, explaining 73 ± 3 % (n = 23, Ta = 23 °C) of the variance (Figs. S7B and F). The k_{high} and k_{low} values from the Tb model were comparable to those from the PA model (Fig. S7C). Since activity changes rapidly, in the PA model the conductance cycled frequently between k_{high} and k_{low}, while in the Tb model there were fewer cycles because Tb changes are gradual, due to the thermal mass of the mouse. Thus, Tb is both a direct contributor to the EE level and a binary marker of arousal state.

These results support the biological hypothesis that the body's Tb and conductance changes are the chief drivers of short term (minutes to hours) changes in mouse EE. It is notable that photoperiod was not needed in the model. Once bout status was captured from the physical activity and two conductance levels were implemented, Tb was sufficient to accurately predict EE changes.

3.9. Comparing human and mouse EE and thermal physiology

We next examined human EE, Tb, and activity patterns in a published dataset [39]. In 39 healthy men consuming a eucaloric (energy balance) diet for 24 h in a room calorimeter, EE, Tb, and activity showed a clear circadian rhythm. All exhibited nadirs during

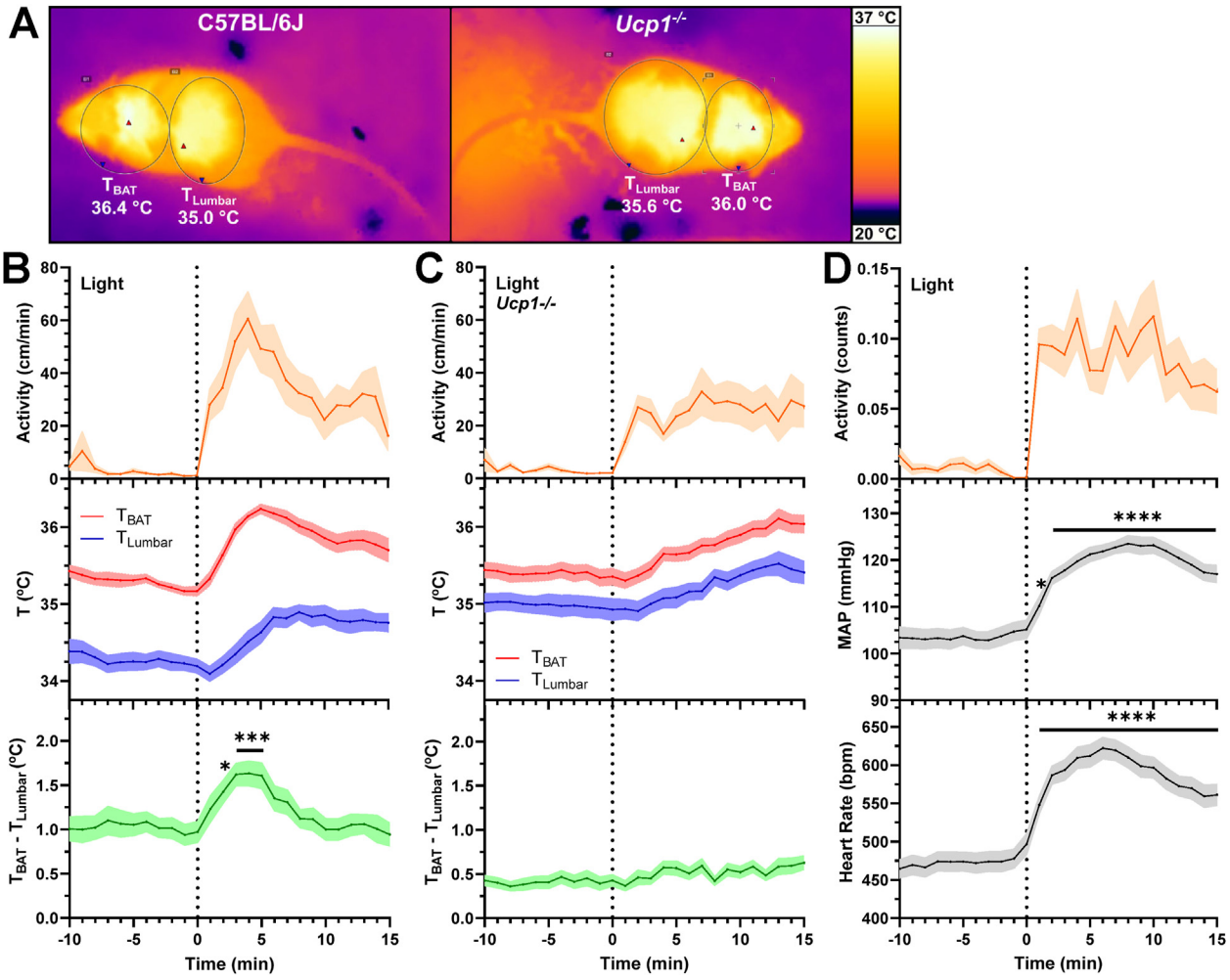


Figure 5: BAT temperature, blood pressure, and heart rate increase at bout initiation. (A) Infrared images of mice showing temperatures of the shaved interscapular (T_{BAT}) and lumbar (T_{lumbar}) areas in male C57BL/6J and *Ucp1*$^{-/-}$ mice. (B,C) Light phase physical activity, T_{BAT}, T_{lumbar}, and the $T_{BAT} - T_{lumbar}$ difference in male C57BL/6J and *Ucp1*$^{-/-}$ mice. (D) Light phase activity, mean arterial pressure (MAP), and heart rate in male mice. Time is relative to bout start, defined by onset of activity (see Methods). Data are mean ± SEM of 15, 14, and 50 bouts from 4, 4, and 9 mice in B, C, and D, respectively. P values are from 1-way ANOVA vs baseline (mean of −5 to −1 min) without multiplicity adjustment, *P < 0.05, ***P < 0.001, and ****P < 0.0001.

sleep, with great inter-individual variation (Figure 7A,B). EE variation over the 24 h period was quantified as the difference between the 95th and 5th percentiles divided by the median. Mouse and human EE variation were similar, with the mean human variation being 12% less than the mean mouse variation (Figure 7C). When the human EE was normalized to a reference Tb of 37 °C using a Q_{10} of 2.3, the EE variation was reduced by 5.7 ± 0.7% (mean ± SEM). Tb variation was estimated as the Tb span, the difference between the 95th and 5th percentiles. Tb variation was 3.2-fold larger in mice than humans ($P < 0.0001$, unpaired t test) (Figure 7D). The Tb-EE Pearson correlations were lower in humans than in mice ($P < 0.0001$, unpaired t test with Welch's correction) and showed greater inter-individual variance ($P < 0.0001$, F test) (Figure 7E). Thus, the major difference between humans and mice is the much smaller Tb variation and the absence of ultradian Tb peaks in humans. Without the ultradian bouts, the underlying circadian rhythm predominates in humans.

4. DISCUSSION

4.1. Ultradian cycles and defended body temperatures

Our results suggest that mice have different defended Tbs depending on arousal state with a lower defended Tb during rest/sleep and a higher one during the active/awake state. The rest/sleep Tb is not the passive consequence of the absence of activity, lack of food intake, and reduced metabolic processes, but rather the regulated defense of a lower target Tb. Arousal state accounts for more of the Tb variation than does light/dark phase.

Humans typically sleep for a single extended period at night and eat and are physically active during the day. At 23 °C, mice ate 77% of their food and got 79% of their activity during the active (dark) phase. Thus, it might seem that mice are similar to humans, albeit with phase-reversed photoperiods. But this is not true. Mouse physiology is fundamentally episodic, with ~4–8 ultradian active/awake bouts during each light and dark phase, encompassing ~40% of the light

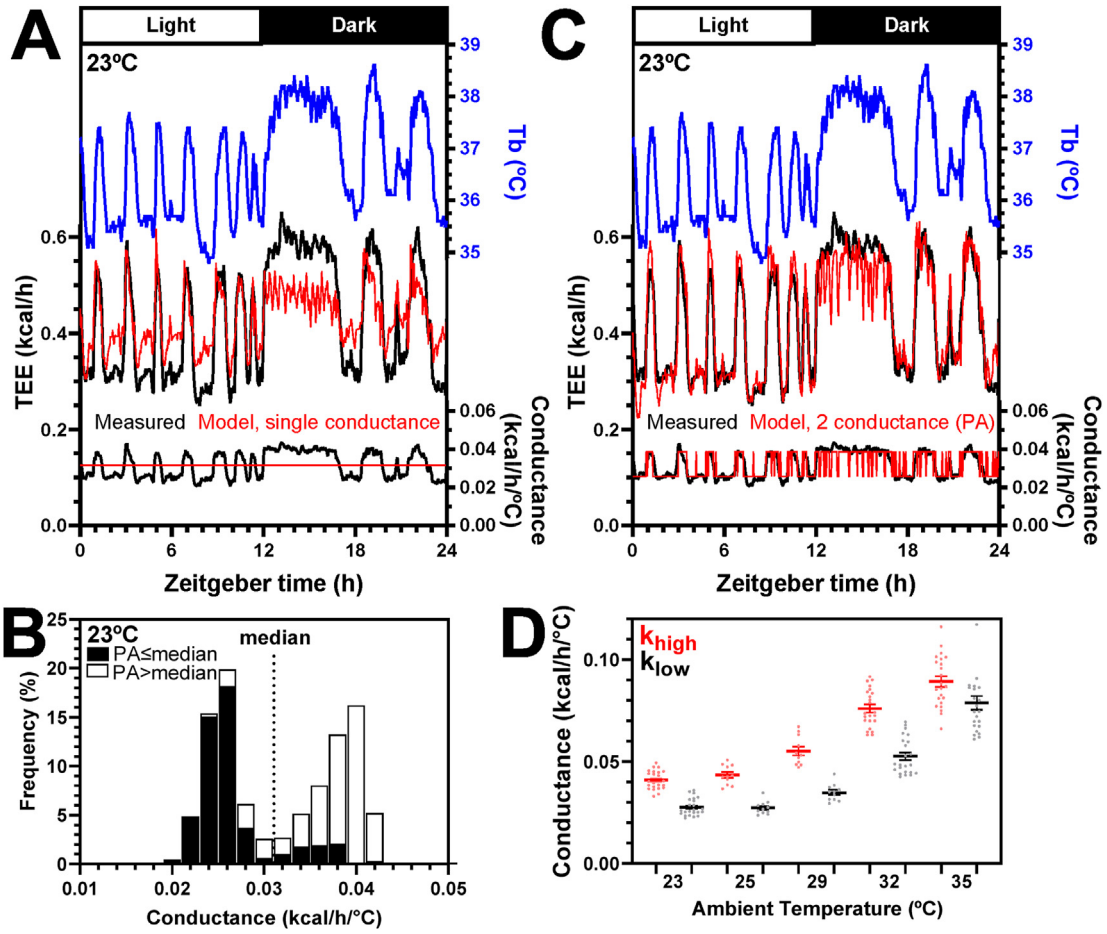


Figure 6: Modeling TEE based on Tb and conductance. (A) Example of analysis of the same mouse as Figures 1B and 4A. Measured TEE (black) and Tb (blue) data were input to a physiology-based constant conductance model (see 2.7 and 2.8), with the output being the model-calculated TEE (red) and conductance (red) vs time. The measured conductance (black, TEE/[Tb-Ta]) is shown for reference. (B) Histogram of the measured conductances from the example in (A). Black indicates values where the activity was $\leq$ the median physical activity and white indicates that activity was $>$ the median activity. (C) Example of the same mouse fit to a physiology-based using two conductances, k_{high} for intervals with activity above the median activity level and k_{low} for intervals with activity below the median activity (see 2.7 and 2.8). Measured TEE (black), Tb (blue), and conductance (black) and model results for TEE (red) and conductance (red) are shown. (D) Model-determined conductances from two days of data, k_{high} (red) and k_{low} (black) at the indicated Ta (data are mean $\pm$ SEM, n $=$ 11–23 mice).

and ~80% of the dark cycle. As an example, at 23 °C in the light/dark phases, 94%/98% of food intake and 92%/99% of physical activity occurs during bouts, bout EE is 42%/50% greater, and bout Tb is 0.78 °C/0.96 °C higher. Thus, bout status is a superior classifier of physiology, with light/dark differences largely secondary to the different percentages of bout time. Tb is an exception, where circadian phase is also a contributor.

The high bout frequency is likely due to the need for frequent food intake to fuel the high metabolic rate. Not eating for 12 h is a major energetic challenge for small homeotherms such as mice. Multiple species (rats, golden hamsters, lemmings, squirrel monkeys) also have ultradian Tb rhythms (reviewed in [7]); presumably bouts of active/awake explain some of these Tb rhythms.

The demonstration of metabolic differences based on bout status means that one must be cautious of single measurements of an analyte. For example, one could handle mice to ensure that they are in an active/awake state before obtaining measurements, but this introduces a systematic bias, excluding the rest/sleep state. When possible, a better approach is to continuously measure across both active/awake and rest/sleep intervals, determine bout status from Tb, activity, and/or EE, and then analyze by bout status. A striking example is the demonstration that mouse blood glucose levels correlate with EE, with glucose being ~40 mg/dl higher during the presumptive active/awake bouts compared to rest/sleep intervals [56]. It seems likely that glucose is one of many analytes that differ by bout status.

4.2. Body temperature drives energy expenditure in mice but not humans

We suggest that the primary driver of the ultradian bouts is a brain-initiated transition to an active/awake state (Figure 8). Possible drivers for this could be spontaneous/stochastic or due to specific environmental stimuli (e.g., noise, odor, demands of offspring, movement in the environment, light) or internal drivers (e.g., hunger, thirst, urination, cold, hot). This changes the lower resting/sleeping defended Tb to the higher active/awake defended Tb (we use the term defended Tb indicating a Tb target, Tb set point [57], or Tb balance point [58]). Energy expenditure is increased in order to achieve the higher Tb via thermogenesis from BAT and other sites. Contributors to the increased bout EE include 54% from arousal thermogenesis, 32% from PAEE, 8% from Q_{10} effect, and 6% from cardiac work (numbers

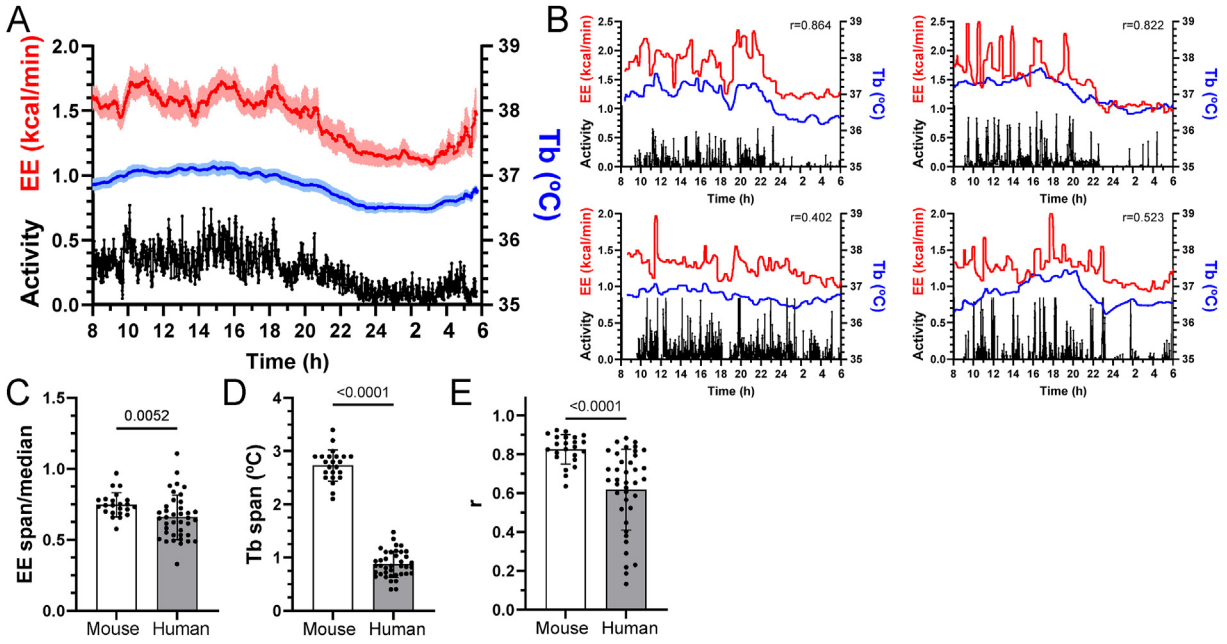


Figure 7: Human EE, Tb, and activity. (A) EE (red), Tb (blue), and activity (black, arbitrary scale) in 39 healthy men, mean and 95% confidence interval [39]. (B) Examples of individual EE, Tb, and activity data. (C) EE variation (95th minus 5th percentile divided by median), (D) Tb variation (95th minus 5th percentile), and (E) Pearson correlations of Tb and EE in mice at 23 °C (n = 23) and humans (n = 39). P values from unpaired t tests in C-E, with E using Welch's correction due to greater inter-individual variance, $P < 0.0001$ by F test.

are for light phase, 23 °C). *Ucp1*$^{-/-}$ mice demonstrate that a BAT contribution to thermogenic EE is not essential; presumably muscle and other tissues contribute in *Ucp1*$^{-/-}$ and wild type mice.

While some have suggested that the lower sleeping Tb is due to a failure or 'suspension of thermoregulation' [59], most agree that the lower Tb is a regulated process, which our results strongly support. Defended Tbs are context dependent, being higher during fever [60] and lower during torpor [61].

There is a fundamental difference in Tb cycling comparing mice and humans. With a large body size, Tb transitions in humans are slower and smoother, approximated by a sinusoid with a 24 h period [62], unlike the short squarer waves of the mouse ultradian bouts.

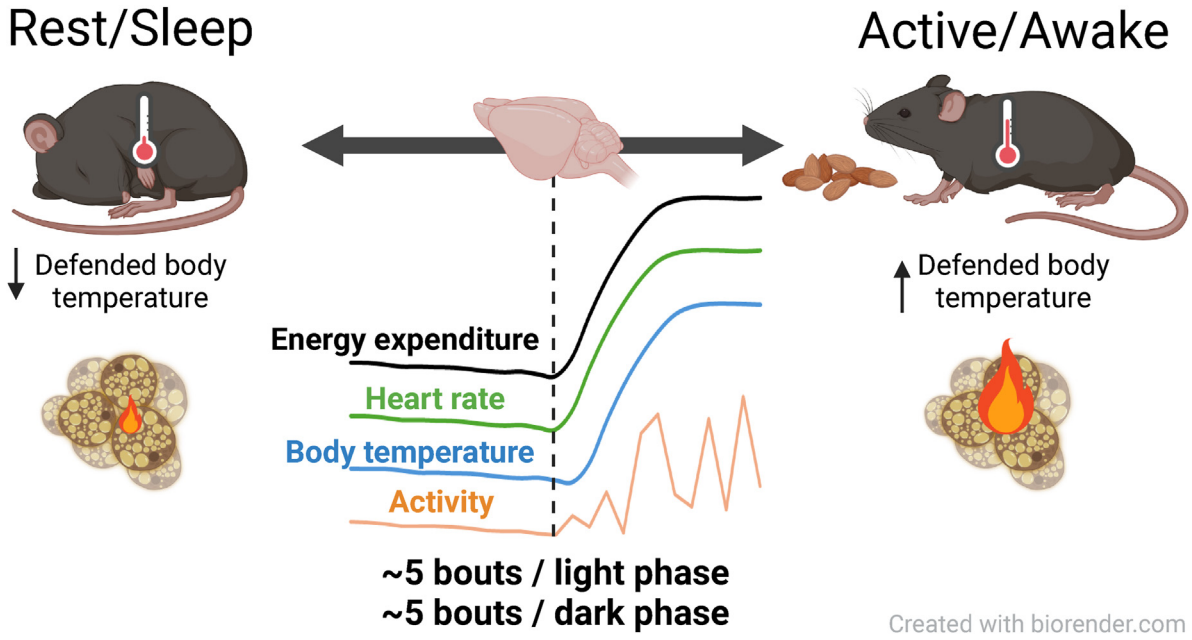


Figure 8: Physiology of energy expenditure bouts. The brain, responding to internal and environmental stimuli, initiates transition between rest/sleep and active/awake states. Active/awake bouts have a higher defended body temperature, physical activity level, and food and water intake. Energy expenditure increases due to the costs of increasing body temperature (e.g., from BAT thermogenesis), physical activity, and heart rate. The increased heart rate is driven by the need to supply fuel and remove waste from tissues with increased metabolic demand.

Homeotherms with intermediate body sizes may have intermediate cycling patterns. Mice require dedicated thermogenesis to reach their defended Tb [5]. In contrast, humans thermoregulate chiefly using vasoconstriction/vasodilation and changes in microenvironment that require no or minimal dedicated EE.

4.3. The physiologic benefits of Tb cycling

The evolutionary conservation of cycling Tb between a warmer active phase and a cooler sleep phase suggests that there are discrete benefits for the warm and cool phases; otherwise it would not be beneficial to cycle between them [63]. The universality of having both cooler resting and warmer active phases in endotherms demonstrates that both are conserved, regulated characteristics and that neither is due to a suspension of thermoregulation.

A warmer Tb during the active phase improves muscle function and is the proposed driver for the evolution of endothermy, benefiting predation avoidance and food acquisition [63]. For example, maximal rates of muscle contraction exhibit a Q_{10} of $\sim$2 [64], so when Tb is increased, either by exercise or passive heating, human athletic performance improves [65,66]. The Tb increase with exercise is a regulated process, not a loss of thermal control [67]. Thus, improved muscle function is a plausible explanation for a warmer active/awake Tb.

Is a lower resting Tb beneficial to the functions of sleep? Sleep is essential, as demonstrated by deprivation experiments. Sleep functions include memory consolidation [68,69] and metabolic processes including waste removal [70,71]. Since sleep-like states are reported in jellyfish and *C. elegans*, before the evolution of endothermy [71,72], the specific contributions of a lower Tb to endotherm sleep physiology are unclear. In mammals, some manipulations can dissociate sleep and thermoregulation [73,74]. If a lower Tb is not essential, why do endotherms have a lower sleeping Tb? One possibility is that it does augment core functions of sleep [75]. Another hypothesis is that the lower Tb evolved to confer ancillary benefits such as energy conservation [71,76,77]. However, Tb cycling and a lower sleeping Tb are still observed in situations where they do not appear to offer an advantage. For example, leptin-deficient *ob/ob* mice, act physiologically as if they are starving, with extreme energy conservation and reduced Tb, yet they maintain Tb cycling despite its cost [9,78,79]. Additionally, Tb cycling persists in mice at 30—33 °C [8] and in large homotherms like humans, where energy conservation is unnecessary. Thus, energy efficiency is not the whole answer for the evolutionary conservation of a lower rest/sleep Tb. A third possible role for Tb cycling might be to augment peripheral synchronization of clock-like rhythms driven by the suprachiasmatic nucleus.

4.4. Thermal conductance

The term thermal conductance as used in this paper [36,37] differs from the way physicists use the term, where it is specifically the heat transferability per unit area, the inverse of the insulation. In our biological context, the conductance provides a numerical measure of the effects of many difficult-to-quantitate contributing factors (e.g., body position, blood flow changes, body motion that increases convective heat loss, nesting, huddling, etc.) to mouse thermal biology.

The observed thermal conductance values are bimodally distributed, higher during EE bouts and lower while resting/sleeping. An interesting question is why the conductance is higher, rather than lower, during the EE bouts since the greater heat loss makes achieving the higher Tb more energetically costly. The observed higher conductance means that processes increasing heat loss are prioritized over energy conservation. We suspect that the increased cardiac output and blood flow is a major contributor to the increased heat loss that is characteristic of the bouts.

4.5. Implications for mouse physiology

The correlations between awake state, physical activity, Tb, and EE inform and complicate interpretation of mouse physiology assays. Mice are typically studied during the light phase when they may be asleep. Gavaging, giving an injection, inserting a rectal temperature probe, or just moving the cage will wake the mouse and initiate awake state physiology. For example, cage-switch, in which a mouse is placed in a cage previously occupied by a different mouse, is considered an assay of psychosocial stress. Cage-switch causes an increase in blood pressure, heart rate, activity, Tb, energy expenditure, corticosterone levels, and other stress responses [80,81], some of which may be due to awakening. Indeed, multiple cage changes have been used to keep mice awake [82].

Humans typically live in a thermoneutral environment, while mice at recommended vivarium temperatures of 20 °C—26 °C [83] are below thermoneutrality. There has been much discussion of how to study mice so that their energy homeostasis is a better model for humans. The advocated solutions are to study the mice just below or at thermoneutrality [14—17]. Most studies use 30 °C as 'thermoneutrality', but we suggested that a non-elevated Tb should be included in the definition of thermoneutrality and then identified a thermoneutral point that was 29 °C in the light phase and 33 °C in the dark [9]. The presence of arousal thermogenesis at 32 °C in both light and dark phases, confirms that 30 °C is below thermoneutrality under some situations. Light and dark results are time-weighted averages of bout and between bout physiology and thus underestimate bout/between bout differences. Since Tb is warmer during bouts and requires thermogenic EE, thermoneutrality is warmer during bouts. Thus, to study mice strictly at thermoneutrality, one would need to cycle Ta rapidly and in synchrony with each mouse's arousal bouts, an impossible task.

In humans, physical activity aids weight loss. Measurement of PAEE in humans and large homeotherms is often via regression of EE vs activity [84,85]. However, in mice, this approach is complicated by activity thermogenesis substituting for CIT, which blunts the measured PAEE; the blunting can be lessened by studying mice at 30 °C to reduce the CIT [8,45]. The current observation that bout thermogenic EE occurs even at 32 °C reinforces the observation that 30 °C is not sufficiently warm to fully neutralize CIT [28]. The arousal model also explains why the apparent cost of activity increases as Tb decreases [28]: increasing the lower initial Tb to the active state level requires more thermogenic EE, which has been mistaken for PAEE. These examples illustrate the care needed to interpret the effect of physical activity on mouse energy balance.

In humans EE is commonly measured as the BMR, defined by the awake, resting, postabsorptive, thermoneutral state in the absence of other metabolic demands [57]. Imposing these conditions on a mouse alters its behavior and energy expenditure, although mouse BMR can be calculated [28]. Consequently, in mice a resting metabolic rate, RMR, is commonly reported, typically from a measurement of the minimum energy expenditure [14,86,87]. Our results indicate that such minimums may actually be sleeping metabolic rates, and not the RMR or BMR, which are defined by the awake state.

4.6. Limitations

A limitation of our analysis is that we did not incorporate the thermic effect of food (TEF), the increase in energy expenditure that occurs after a meal. In humans, TEF spans hours after food intake, with a longer delay after larger meals [88,89]. To our knowledge the kinetics of TEF have not been studied in mice. Since neither the meal size

dependence nor the delay characteristics are known, it is not possible to calculate the mouse TEF time course from the food intake measurements. Also due to the lack of mouse data, we used an assumption based on human physiology for calculation of the cardiac contribution to bout EE.

4.7. Conclusions

While the organization of physiology into active/awake vs resting/sleeping seems obvious (in retrospect), to our knowledge sleep, energy expenditure, and body temperature have not been studied previously in a combined analysis in mice. Mice exhibit ultradian bouts of increased EE, due to a brain-initiated transition to an active/awake state. The bouts have a higher defended Tb, requiring increased energy expenditure, and are further characterized by increased food consumption, water intake, cardiac work, and thermal conductivity. The ultradian bouts also explain variation in mouse glucose levels [56]. Thus, much of mouse physiology is episodic, and is not properly captured using traditional segmentation by photoperiod.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Vojtěch Škop: Writing - review & editing, Writing - original draft, Software, Methodology, Investigation, Conceptualization. **Naili Liu:** Writing - review & editing, Investigation. **Cuiying Xiao:** Writing - review & editing, Investigation. **Emma Stinson:** Writing - review & editing, Investigation. **Kong Y. Chen:** Writing - review & editing, Supervision. **Kevin D. Hall:** Writing - review & editing, Supervision. **Paolo Piaggi:** Writing - review & editing, Software, Investigation. **Oksana Gavrilova:** Writing - review & editing, Supervision, Investigation, Funding acquisition. **Marc L. Reitman:** Writing - review & editing, Writing - original draft, Methodology, Funding acquisition, Conceptualization.

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DECLARATION OF COMPETING INTEREST

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

DATA AVAILABILITY

Data, including curated bout identifications, and computer code are deposited at https://osf.io/9bszy/.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2024.101946.

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