Journal of the American Association for Laboratory Animal Science
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Vol 63, No 2
March 2024
Pages 190–200

Differences in Fluid, Electrolyte, and Energy Balance in C57BL/6J Mice (*Mus musculus*) in Metabolic Caging at Thermoneutral or Standard Room Temperatures

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The *Guide for the Care and Use of Laboratory Animals* recommends mice be pair or group housed and provided with nesting materials. These provisions support social interactions and are also critical for thermoregulatory behaviors such as huddling and burrowing. However, studies of fluid and electrolyte balance and digestive function may involve use of metabolic caging (MC) systems in which mice are housed individually on wire-mesh floors that permit quantitative collection of urine and feces. MC housing prevents mice from performing their typical huddling and burrowing behaviors. Housing in MC can cause weight loss and behavioral changes in rodents. Here, we tested the hypothesis that MC housing of mice at standard room temperature (SRT, 22 to 23 °C) exposes them to cold stress, which causes metabolic changes in the mice as compared with standard housing. We hypothesized that performing MC studies at a thermoneutral temperature (TNT, 30 °C) would minimize these changes. Fluid, electrolyte, and energy balance and body composition were assessed in male and female C57BL/6J mice housed at SRT or TNT in MC, static microisolation cages, or a multiplexed metabolic phenotyping system designed to mimic static microisolation cages (Promethion, Sable Systems International). In brief, as compared with MC housing at SRT, MC housing at TNT was associated with lower food intake and energy expenditure, absence of weight loss, and lower urine and fecal corticosterone levels. These results indicate that housing in MC at SRT causes cold stress that can be mitigated if MC studies are performed at TNT.

Abbreviations and Acronyms: AVP, arginine vasopressin; EE, energy expenditure; FFM, fat-free mass; GLM, general linear modeling; MC, metabolic caging; RER, respiratory exchange ratio; SM, static microisolation caging; SRT, standard room temperature; TNT, thermoneutral temperature

DOI: 10.30802/AALAS-JAALAS-23-000091

Introduction

Metabolic caging (MC) systems are often used to study fluid and electrolyte homeostasis in rodents. However, housing larger animals such as rats on wire flooring for prolonged periods has possible negative effects,[6,17,20,23] which have led to the identification of MC use as a "stressor" that should be avoided. For example, the European Directive 2010/63/EU identifies short-term (less than 24 h) housing in MC to be a procedure of mild severity, housing in MC with moderate restriction of movement for up to 5 d as a procedure of moderate severity, and housing in MC with severe restriction of movement over prolonged periods as a severe procedure.[1]

Submitted: 11 Sep 2023. Revision requested: 16 Oct 2023. Accepted: 15 Dec 2023.
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Although the use of MC may generate concerns about animal welfare, this approach remains necessary for the quantitative collection of urine and fecal samples for studies of total daily fluid and electrolyte flux.[27] Housing rodents in MC systems often leads to weight loss and changes in body composition. To compensate for these effects, researchers may try to acclimate the animals to the cages for several days before initiating a planned experiment.[9] We recently used bomb calorimetric methods to quantify the impact of housing in MC on energy expenditure in C57BL/6J mice. When housed at standard room temperature (SRT, 20 to 22 °C), mice in MC showed a 40% to 60% increase in energy expenditure as compared with mice in static microisolation (SM) caging.[39] Those results together with increased understanding of the interactions between thermoregulatory and cardiovascular control systems prompted us to assess the effects of ambient temperature on fluid, electrolyte, and energy flux in mice.

The body composition and behavioral effects of using MC at SRT are likely the consequence of a combination of psychosocial and thermal stressors, because rodents are thought to prefer cohousing over individual housing. In addition, rodents cannot perform typical thermoregulatory behaviors such as huddling and burrowing when individually housed with no access to nesting materials. To quantify the relative importance

of thermal stress on body composition and fluid, electrolyte, and energy flux effects on mice housed in MC, we examined these end points in male and female C57BL/6J mice housed at SRT or at thermoneutral ambient temperature (TNT, 30 °C) in MC, SM home cages, or a multiplexed metabolic phenotyping system designed to mimic a standard SM cage (Promethion; Sable Systems International). We hypothesized that the thermal stress that mice experience when housed in MC at SRT would account for most or all of the effects of MC housing on energy flux, ingestive behaviors, and energy expenditure, independent of sex.

Materials and Methods

Animal housing and care. Male and female C57BL/6J mice were purchased from the Jackson Laboratories (Stock Number 000664). According to the Jackson Laboratories technical support group, these mice routinely receive ad libitum access to LabDiet 5K52 diet and municipal water that undergoes UV light-based sterilization, filtration, acidification to pH 2.5 to 3.0, and autoclaving. Mice were maintained on a 14:10 light:dark cycle with rooms that are illuminated to approximately 30 foot-candle (≈323 lx) at 21 °C and have 30% to 70% relative humidity.

Mice were shipped to the Medical College of Wisconsin at 10 wk of age. Upon arrival to the institution, mice were group housed (between 2 and 5 per cage) in ventilated microisolation caging on commercial racks (Allentown, Allentown, NJ). Standard housing conditions included hardwood chip bedding (Sani-Chips; P.J. Murphy, Montville, NJ) and nesting material (Enviropak; W.F. Fisher and Son, Branchburg, NJ), at SRT (i.e., 20 to 22 °C), between 35% and 75% relative humidity. Mice were maintained on a 14:10 light:dark cycle with rooms illuminated to approximately 325 lx at the center of the housing rack. Throughout the study, all mice had ad libitum access to the natural ingredient Teklad/Envigo 2920× irradiated soy protein-free diet (Envigo, Indianapolis, IN) in pelleted form unless otherwise noted and municipal water that had undergone reverse osmosis filtering (Avidity Science, Waterford, WI) and chlorination, ultimately supplied to the mice at pH 6.5 with approximately 1.4 mg/L chlorine. Mice were switched among various experimental housing conditions at 14 wk of age, as described below.

All studies were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. The animal care and use program at the Medical College of Wisconsin is accredited by AAALAC International and conforms to the National Research Council's *Guide for the Care and Use of Laboratory Animals, Eighth Edition*.[22]

As previously described in detail,[39] the institutional disease surveillance program consists of a PCR exhaust air dust panel for ventilated cages and serological testing of rodents exposed to soiled bedding in SM cages for detecting excluded pathogens. Excluded murine agents include *Clostridium piliforme*, *Corynebacterium bovis*, cilia-associated respiratory bacillus, ectromelia virus, *Encephalitozoon caniculi*, lymphocytic choriomeningitis virus, minute virus of mice, mouse adenovirus, mouse cytomegalovirus, mouse hepatitis virus, mouse parvovirus, mouse rotavirus, mouse thymic virus, *Mycoplasma pulmonis*, pneumonia virus of mice, polyoma virus, reovirus, Sendai virus, Theiler murine encephalomyelitis virus, pinworms, and fur mites. Murine norovirus and *Helicobacter* spp. are not specifically excluded.

Experimental housing conditions: Promethion. One set of mice ($n = 32$, Figure 1A) was studied using a Promethion multiplexed metabolic phenotyping system (Sable Systems International), as described previously.[27] This system provides continuous or near-continuous assessments of food and water intake, physical activity, body mass, and respiratory gas exchange throughout the light cycle. Individual cages are maintained in 2 separate isolation cabinets ($n = 8$ cages in each cabinet) that each provide experimental control of ambient temperature; cages are designed to mimic the materials (plastic walls and floor, hardwood chip bedding, metal hoppers for water and pelleted food, and filter cage top) used in ventilated microisolation cages. Mice housed in the Promethion did not receive nesting materials, as they would interfere with the photoelectric grid used to detect mouse position in the cage; instead, mice received a small plastic hut that was suspended from a strain gauge and provided both enrichment and the ability to monitor body mass trends whenever the mouse entered the hut. Mice were singly housed when tested in the Promethion system. As a result of this layout, up to 8 individually housed mice could be studied at one ambient temperature in one isolation cabinet, while a simultaneous group of up to 8 individually housed mice could be studied at another ambient temperature in the other isolation cabinet. Separate cohorts of male or female mice were studied in batches at 14 wk of age for 4 overnight periods (e.g., continuously from Monday morning to Friday afternoon), with half of each cohort randomly assigned to SRT (tightly maintained at 22 °C, $n = 8$ each sex) and half assigned to TNT (30 °C, $n = 8$ each sex) conditions. All mice were subsequently returned to ventilated microisolation caging at SRT, although they were singly housed for the remainder of the study to prevent aggression that might occur after reintroduction to group housing. At 17 wk of age, mice were studied again using the Promethion system, but each mouse was assigned to the opposite temperature condition from its assignment at 14 wk of age in a crossover design. Data were acquired using Promethion Live v.21.0.2 software and analyzed using OneClickMacro v.2.50.3 (Sable System International).

Experimental housing conditions: SM and MC. A second set of mice ($n = 80$, Figure 1B) was also studied at 14 wk of age, using SM cages, or MC, as described previously.[27,39] SM cages are identical in dimensions, overall design, and material to ventilated microisolation cages (including filter tops, hardwood chip bedding, nesting material, and pelleted food), but SM cages do not receive active ventilation and are not attached to an automated water supply system. Instead, SM cages were maintained on Metro-style wire shelving and water bottles were used. Commercially produced MC (Nalgene type; Tecniplast, West Chester, PA, model 3600M021) were used for these studies. The food provided in MC was powdered, having been ground from the same lot of pelleted food as that provided in SM cages.

Mice of each sex were randomly assigned to either SRT or TNT conditions for 4 consecutive overnight periods in one of the following 3 configurations. In the first configuration, cages were assigned to either TNT conditions by placement in a Rodent Incubator (model RIS28SSD, Powers Scientific, Pipersville, PA) or to SRT housing by being maintained on racks located in the same room and immediately adjacent to the incubator. Under both temperature conditions, a total of 32 mice were housed in same-sex pairs in an SM cage (hereafter designated as "paired," with 4 cages per sex per temperature and 2 mice in each cage). Another 16 mice were individually housed in similar SM cages (hereafter designated as "single," with 4 mice per sex per temperature). A third group of 32 mice was housed individually in MC (8 mice per sex per temperature).

Body composition. Fat mass and fat-free mass (FFM) were determined at the beginning and end of each experimental testing period using time-domain nuclear magnetic resonance (NMR; Bruker model LF110, Billerica, MA) as described previously.[27,32]

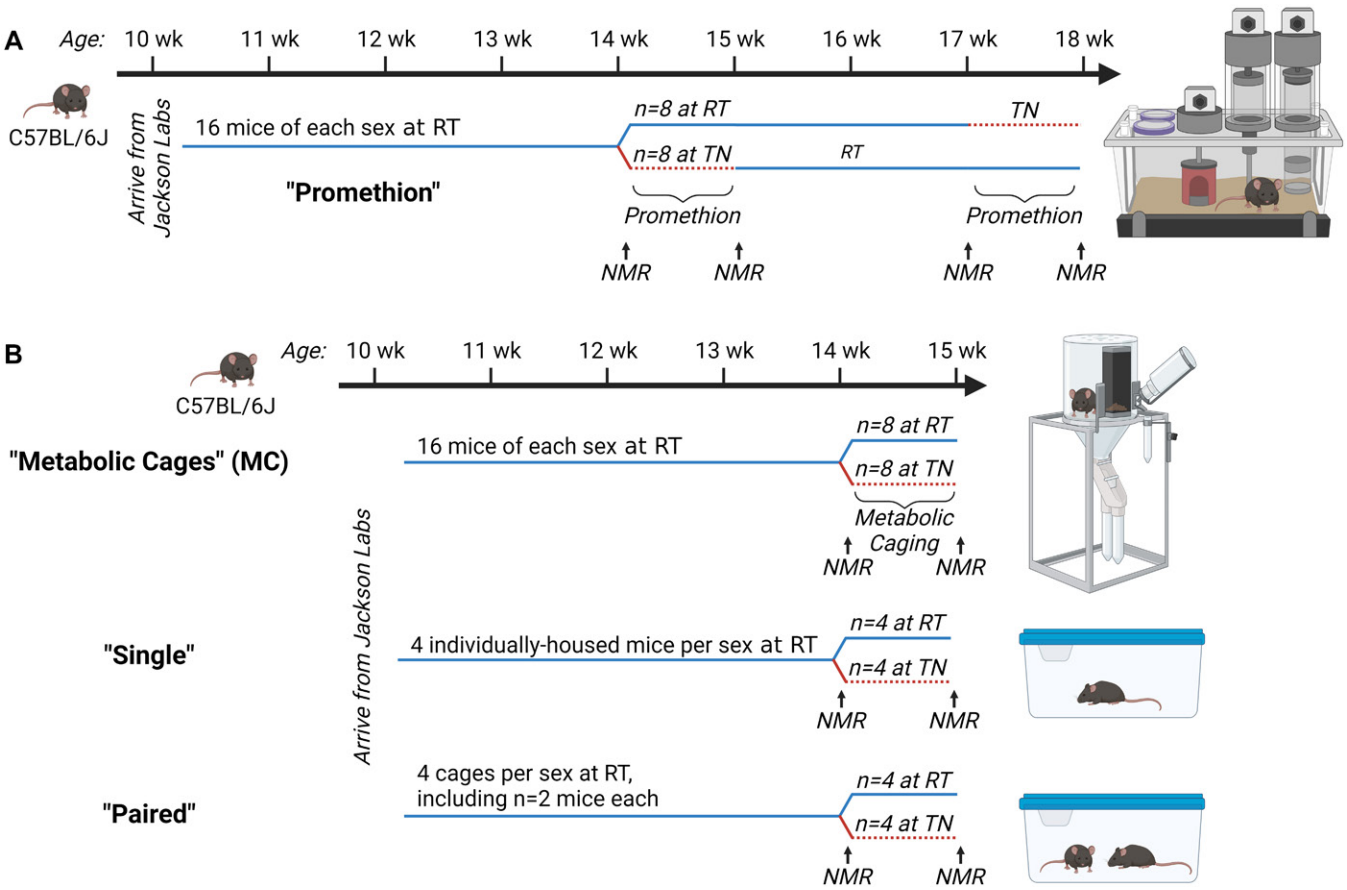

Figure 1. Diagrams illustrating timelines of experimental housing conditions and end-point analyses. (A) Mice housed in Promethion. (B) Mice housed in MC or SM cages. SRT, standard room temperature; TNT, thermoneutral temperature; MC, metabolic caging; SM, static microisolation caging.

In brief, mice were placed into a plastic restraint tube during the scan, which required 2 min. Mice were then immediately returned to the assigned caging conditions. FFM was calculated as the difference between total body mass and fat mass as determined by the NMR. Total body water was calculated by multiplying FFM by 73.2%.[32] Total body hydration was calculated by dividing total body water by total body mass.

Respirometric calculations. Metabolic rate was estimated for mice housed in the Promethion system by using respiratory gas exchange, as described previously.[27] In brief, aerobic energy expenditure (EE) was estimated using the modified Weir equation,[37] with $\dot{V}O_2$ representing the rate of oxygen consumption and $\dot{V}CO_2$ representing the rate of CO_2 production, as follows: (EE) = 3.941 ($\dot{V}O_2$) + 1.106 ($\dot{V}CO_2$). The respiratory exchange ratio (RER) was calculated as the ratio of $\dot{V}CO_2$ to $\dot{V}O_2$.

Bomb calorimetry. Food and fecal samples were analyzed for caloric content by bomb calorimetry as described previously.[9,27] Absorbed calories were calculated as the total calories ingested, minus calories lost as stool. Digestive efficiency was calculated as the ratio of absorbed calories to ingested calories. Energy efficiency, an inverse metric of energy expenditure, was calculated as the ratio of body mass gain per total calories absorbed. For mice housed in MC, total energy expenditure was estimated as the difference between calories absorbed and the calories used for growth, which were estimated from changes in fat mass (at 9 kcal/g) and FFM (at 4 kcal/g). For mice housed in SM cages, bomb calorimetry could not be used because feces could not be quantitatively collected in this cage type. As a result, energy expenditure for mice in SM cages was estimated based on the average digestive efficiency of mice housed in MC.

Urine electrolytes, corticosterone, and copeptin. For mice housed in MC, urine and blood serum were analyzed for electrolyte (sodium, Na, and potassium, K) contents and osmolality using flame atomic absorption spectrophotometry and freezing-point depression osmometry, respectively.[27] Urinary corticosterone concentration was determined via ELISA (Arbor Assays, K014-H1), urine creatinine concentration was determined by colorimetric assay (Arbor Assays, K002-H1), and urine copeptin concentration was determined by ELISA (Cloud-Clone Corporation, CEA365Mu), each according to manufacturer's instructions. Urinary excretion rates were calculated for each mouse by multiplying the total mass of urine produced on a given day (g/d) by the concentration of the analyte measured in that same daily collection (ng or pg/mL), and dividing by the specific gravity of urines in that treatment group (g/mL). Specific gravity was determined by measuring the mass of 20.0 µL of urine from a subset (n = 2 to 7) of mice within each group using urine that remained after hormone and electrolyte analyses. No differences in specific gravity of urines were found among groups (male SRT 1.09 ± 0.01, male TNT 1.10 ± 0.01, female SRT 1.10 ± 0.01, female TNT 1.09 ± 0.01 g/mL; sex P = 0.89, ambient temperature P = 0.72, sex × temperature interaction P = 0.37).

Fecal corticosterone. For mice housed in MC, fecal corticosterone content was determined via ELISA (Arbor Assays, K014-H1) according to manufacturer's instructions. In brief, feces were freeze dried, powdered, and resuspended in 100% ethanol in a 0.1 g feces/1.0 mL ethanol ratio. Samples were

Table 1. Body mass and composition of C57BL/6J mice at 14 wk of age, randomly assigned to various caging types at SRT compared with TNT ambient temperatures

End point	Male SRT	Male TNT	Female SRT	Female TNT	Cage type (P)	Sex (P)	Temperature (P)	Cage × sex interaction (P)	Cage × temperature interaction (P)	Sex × temperature interaction (P)	3-Way interaction (P)
Starting body mass (g)											
Paired (n = 8 each)	30.20 ± 0.43	29.93 ± 0.42	20.84 ± 0.45	20.73 ± 0.48	0.0001	<0.0001	0.4905	<0.0001	0.5161	0.5596	0.9328
Single (n = 4 each)	29.9 ± 0.62	29.41 ± 0.62	21.98 ± 0.49	20.98 ± 0.88							
Metabolic cages (n = 8 each)	26.49 ± 0.43*,†	26.12 ± 0.53*,†	21.91 ± 0.35	21.37 ± 0.48							
Promethion (n = 8 each)	27.25 ± 0.71*	27.85 ± 0.59*	22.04 ± 0.44	22.33 ± 0.29							
Fat-free mass (g)											
Paired (n = 8 each)	22.86 ± 0.45	24.25 ± 0.59	16.63 ± 0.34	16.26 ± 0.41	0.0022	<0.0001	0.7769	<0.0001	0.3166	0.7053	0.1688
Single (n = 4 each)	22.95 ± 1.03	22.40 ± 0.80	17.38 ± 0.27	16.45 ± 0.55							
Metabolic cages (n = 8 each)	20.89 ± 0.46*	20.13 ± 0.47*	17.20 ± 0.19	17.21 ± 0.33							
Promethion (n = 8 each)	21.16 ± 0.51	21.16 ± 0.44*	17.67 ± 0.37	18.12 ± 0.30							
Fat mass (g)											
Paired (n = 8 each)	7.34 ± 0.35	5.68 ± 0.42	4.22 ± 0.25	4.47 ± 0.24	0.1765	<0.0001	0.4526	0.1073	0.1089	0.6753	0.0010
Single (n = 4 each)	6.54 ± 0.47	7.01 ± 0.50	4.60 ± 0.25	4.33 ± 0.36							
Metabolic cages (n = 8 each)	5.60 ± 0.36*	5.99 ± 0.35	4.71 ± 0.20	4.16 ± 0.19							
Promethion (n = 8 each)	6.09 ± 0.26	6.67 ± 0.24	4.37 ± 0.13	4.21 ± 0.16							
Fat mass (%)											
Paired (n = 8 each)	24.3 ± 1.1	19.0 ± 1.4	20.2 ± 0.9	21.6 ± 0.9	0.8406	<0.0001	0.5871	0.1320	0.2446	0.6731	0.0004
Single (n = 4 each)	22.3 ± 2.0	23.9 ± 1.8	20.9 ± 0.7	20.6 ± 0.9							
Metabolic cages (n = 8 each)	21.1 ± 1.3	22.9 ± 1.1	21.5 ± 0.6	19.4 ± 0.5							
Promethion (n = 8 each)	22.3 ± 0.5	24.0 ± 0.6*	19.9 ± 0.4	18.9 ± 0.7							

Data were analyzed by 3-way ANOVA and presented as mean ± SE. *P < 0.05, different from paired-housing group within matched sex and temperature; †P < 0.05 different from single-housing group within matched sex and temperature, by Tukey multiple comparison procedure.

centrifuged, and the supernatant was diluted (1:10) with assay buffer before analysis.

Statistics. Sample sizes were determined using effect size and variance values for total energy expenditure in MC from our previously published data,[39] with $\alpha = 0.05$ and $\beta = 0.2$. All data were analyzed using 2-tailed parametric methods (*t* test, 2-way ANOVA or general linear modeling [GLM], with multiple comparison corrections using the Tukey method). A *P* value of $P < 0.05$ was considered to be statistically significant. Urine corticosterone data were Log_{10} transformed for analytical comparisons. Comparisons were made using SPSS v.27 or GraphPad Prism v.9.5.1.

Results

Body composition. Upon arrival at 10 wk of age, mice were randomly assigned to housing groups. At 14 wk of age, body composition was assessed by NMR before the initiation of experimental housing conditions (Table 1). At this age, female mice were smaller than male mice. A significant effect of assigned caging type on total body mass was noted, as males assigned to paired or single housing in SM cages were larger than males assigned to MC, and males assigned to pair housing in SM cages were larger than males assigned to Promethion. No significant differences within sex or caging type were observed for total body masses of mice assigned to SRT compared with TNT. Observed differences in total body mass between caging types were because of small differences in FFM or fat mass. However, relative body composition expressed as fat mass (%) was similar between caging type and ambient temperature groups within each sex.

Body mass and composition changed over 4 consecutive days (approximately 96 h) depending on the combination of cage type and ambient temperatures. Mice housed in SM cages, including male and female mice housed either as pairs or individually, showed no consistent change in total body mass (Figure 2A), FFM (Figure 2B), or fat mass (Figure 2C). In contrast, male mice housed in MC at SRT showed a reduction in body mass that was primarily because of loss of FFM. However, this effect did not occur when male mice were housed in MC at TNT. Male mice housed in the Promethion system lost weight because of reductions in both FFM and fat masses. Female mice did not show a consistent change in body mass regardless of the ambient temperature.

Studying a subset of mice in a crossover design in the Promethion system permitted additional analyses of potential order effects. For example, mice studied at TNT at 14 wk of age may have altered their behavior or energy balance regulation during the interim before reaching 17 wk of age. Although sex significantly modified FFM ($P < 0.001$), age was not a significant factor in determining FFM between mice studied at 14 compared with 17 wk of age within each sex (all comparisons $P > 0.05$). In addition, no significant interactions were detected among sex, exposure order, or temperature (all interactions $P > 0.05$). As for FFM, fat mass was significantly different between the sexes ($P < 0.001$). However, in contrast to FFM, both sexes showed a small but statistically significant exposure-order effect on fat mass (e.g., an interaction of exposure order and ambient temperature, $P < 0.001$). This effect occurred because mice housed at SRT at first tended to gain more fat mass than did mice housed first at TNT (i.e., the SRT-before-TNT cohort gained 0.69 ± 0.19 g fat between weeks 14 and 17, whereas the TNT-before-SRT cohort gained 0.65 ± 0.19 g fat during this same interval).

Food intake. Food intake was similar in mice of both sexes housed at SRT, regardless of caging type (Figure 3A). However,

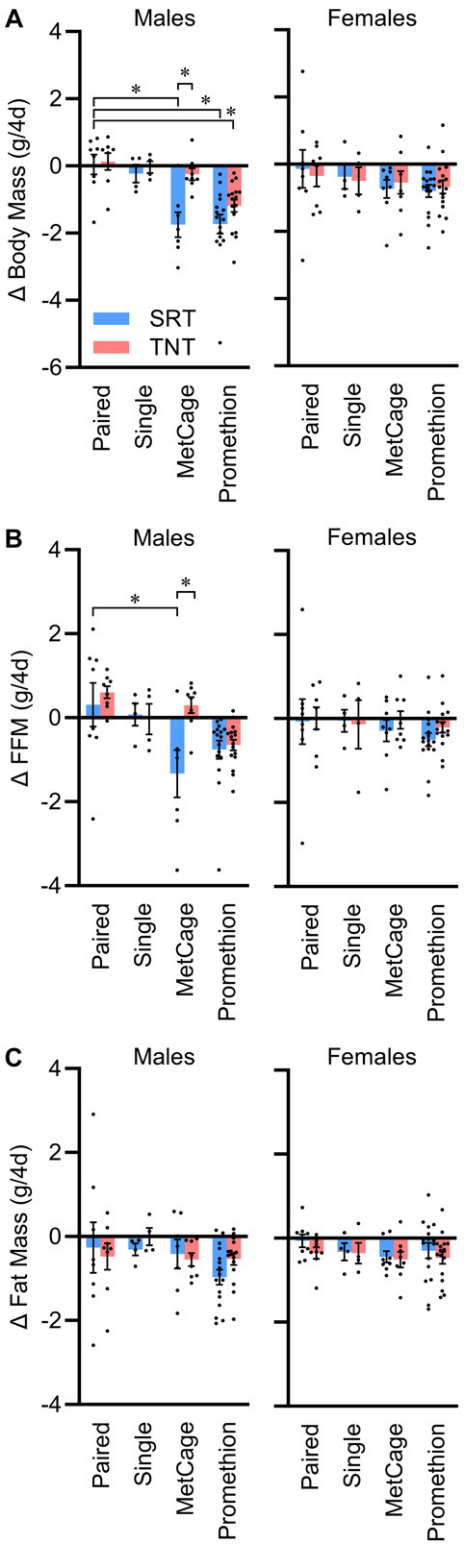

Figure 2. Effect of caging type and ambient temperature on body mass and composition over 4 d of male and female C57BL/6J mice. (A) Change in total body mass. Males: cage $P < 0.0001$, temperature $P = 0.0120$, interaction $P = 0.1089$. Females: cage $P = 0.9863$, temperature $P = 0.3527$, interaction $P = 0.9170$. (B) Change in FFM. Males: cage $P = 0.0460$, temperature $P = 0.0005$, interaction $P = 0.0416$. Females: cage $P = 0.5263$, temperature $P = 0.5586$, interaction $P = 0.9237$. (C) Change in fat mass. Males: cage $P = 0.6704$, temperature $P = 0.2314$, interaction $P = 0.5550$. Females: cage $P = 0.3441$, temperature $P = 0.5866$, interaction $P = 0.9304$. For all panels, data were analyzed by 2-way ANOVA within sex, $^*P < 0.05$ by Tukey multiple comparison procedure, and summary data are presented as mean ± SE. SRT, standard room temperature; TNT, thermoneutral temperature.

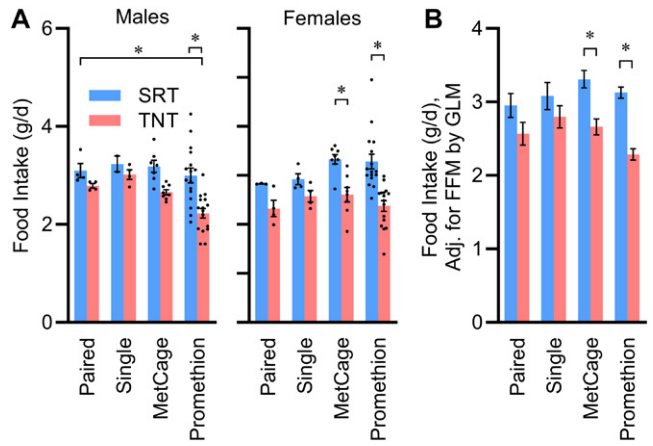

Figure 3. Effect of caging type and ambient temperature on food intake over 4 d in male and female C57BL/6J mice. (A) Food intake. Summary data are presented as mean ± SE. Males: cage $P = 0.0116$, temperature $P = 0.0010$, interaction $P = 0.3093$. Females: cage $P = 0.2545$, temperature $P < 0.0001$, interaction $P = 0.3609$. (B) Food intake adjusted for the covariate of FFM using GLM. Summary data are presented as the estimated marginal mean ± SE, at the covariate FFM of 19.49 g. For all panels, data were analyzed by 2-way ANOVA within each sex, *$P < 0.05$ by Tukey multiple comparison procedure. SRT, standard room temperature; TNT, thermoneutral temperature; FFM, fat-free mass; GLM, general linear modeling.

mice housed at TNT showed reduced food intake in some combinations of caging types, depending upon the sex. In particular, mice housed in MC and the Promethion showed reductions in food intake under TNT conditions.

After showing that body size and composition influence food intake independent of housing conditions, we next examined the effects of housing conditions on food intake while accounting for differences in body composition by using GLM. Food intake was not influenced by the covariate of FFM across this dataset ($P = 0.254$), but correction of the dataset for this covariate showed that caging type ($P = 0.043$) and ambient temperature ($P < 0.001$) each modified food intake, but sex did not ($P = 0.738$) (Figure 3B). In other words, effects of sex were because of sex-based differences in body composition. No pairwise interactions were observed between cage type and sex (interaction $P = 0.133$), cage type and ambient temperature (interaction $P = 0.083$), or ambient temperature and sex (interaction $P = 0.430$), and a 3-way interaction of these variables was not detected (interaction $P = 0.996$). Thus, we conclude that food intake differed based on cage type and that food intake differed between mice housed at SRT and TNT regardless of cage type.

Digestive efficiency of mice housed in MC was not affected by ambient temperature or sex (Table 2). These results indicate that total caloric absorption by the mice parallels total food consumption regardless of ambient temperature or sex.

Post hoc analyses were performed on data from the subset of mice examined by Promethion to confirm that correction of food intake data for body composition sufficiently accounted for any potential order effects of temperature exposures. Order of exposure did not have a significant effect ($P = 0.364$) or an interactive effect with sex or ambient temperature (all $P > 0.05$). Incorporation of exposure order into the GLM did not qualitatively change any conclusions about the effects of other variables.

When mice were housed in Promethion, meal patterning could be evaluated (Table 3). Females ate more meals per day, but their meals were smaller than those of males. Housing at TNT resulted in reduced meal size without changing the duration of individual meals or the total number of meals, indicating that the speed of consumption of any given meal was slowed. No interaction was observed between ambient temperature and sex on any aspect of meal patterning. These findings suggest that ambient temperature modulates some aspects of food intake (e.g., palatability, meal termination, and satiation), but not others (e.g., meal initiation and satiety).

Table 2. Metabolic and behavioral end points assessed in C57BL/6J mice at 14 wk of age, housed in metabolic cages at SRT compared with TNT ambient temperatures

End point	Male SRT ($n = 8$)	Male TNT ($n = 8$)	Female SRT ($n = 8$)	Female TNT ($n = 8$)	Ambient temperature (P)	Sex (P)	Temperature × sex Interaction (P)
Starting body mass (g)	26.49 ± 0.43	26.12 ± 0.53	21.91 ± 0.35†	21.37 ± 0.48†	0.3188	<0.0001	0.8496
Starting FFM (g)	20.89 ± 0.46	20.13 ± 0.47	17.20 ± 0.19†	17.21 ± 0.33†	0.3306	<0.0001	0.3175
Starting fat mass (g)	5.60 ± 0.36	5.99 ± 0.35	4.71 ± 0.20	4.16 ± 0.19†	0.7766	<0.0001	0.1080
Digestive efficiency (%)	84.8 ± 2.5	84.5 ± 2.8	84.2 ± 2.5	83.1 ± 3.6	0.4933	0.3597	0.6755
Caloric absorption (kcal/d)	13.99 ± 0.66	10.60 ± 0.21*	14.32 ± 0.85	10.70 ± 0.51*	<0.0001	0.7289	0.8466
Urine osmolality (mOsm/kg H_2O)	4,726 ± 880	6,777 ± 1142	6,282 ± 543	9,281 ± 792	0.0099	0.0330	0.5984
Urine sodium (mmol/L)	135.7 ± 27.0	234.6 ± 52.3	181.2 ± 18.0	242.1 ± 26.6	0.0163	0.3973	0.5430
Urine sodium (mEq/d)	0.099 ± 0.013	0.087 ± 0.006	0.061 ± 0.012	0.056 ± 0.011	0.5121	0.0112	0.7713
Urine potassium (mmol/L)	398.6 ± 64.1	593.7 ± 96.5	643.7 ± 62.7	775.0 ± 61.6	0.0348	0.0076	0.6641
Urine potassium (mEq/d)	0.300 ± 0.037	0.234 ± 0.027	0.212 ± 0.039	0.186 ± 0.040	0.2479	0.0973	0.6080
Urine corticosterone (ng/mL)	100 ± 21	31 ± 8*	240 ± 39	53 ± 10*,†	<0.0001	<0.0001	0.0916
Fecal corticosterone (ng/d)	9.0 ± 0.8	7.9 ± 0.8	18.3 ± 1.1†	12.3 ± 0.7*,†	0.0004	<0.0001	0.0104
Urine creatinine (mg/dL)	76 ± 11	77 ± 21	96 ± 10	55 ± 21	0.2433	0.9502	0.2170
Urine creatinine (mg/d)	70 ± 10	70 ± 19	88 ± 9	50 ± 19	0.2433	0.9459	0.2320
Urine copeptin (pg/mL)	275 ± 64	169 ± 26	549 ± 115	327 ± 126	0.0962	0.0314	0.5476
Urine copeptin (pg/d)	258 ± 77	60 ± 19	233 ± 94	92 ± 47	0.0124	0.9588	0.6566

Data were analyzed by 2-way ANOVA and presented as mean ± SE. *$P < 0.05$ effect of ambient temperature within sex; †$P < 0.05$ effect of sex within ambient temperature, by Tukey multiple comparison procedure.

Table 3. Metabolic and behavioral end points assessed in C57BL/6J mice at 14 or 17 wk of age, housed in Promethion at SRT compared with TNT ambient temperatures

End point	Male SRT (n = 16)	Male TNT (n = 16)	Female SRT (n = 16)	Female TNT (n = 16)	Ambient temperature (P)	Sex (P)	Temperature × sex Interaction (P)
Starting body mass (g)	28.78 ± 0.58	28.98 ± 0.60	22.91 ± 0.33†	22.85 ± 0.34†	0.8785	<0.0001	0.7890
Starting FFM (g)	21.86 ± 0.36	21.81 ± 0.38	18.18 ± 0.23†	18.22 ± 0.24†	0.9742	<0.0001	0.8768
Starting fat mass (g)	6.91 ± 0.25	7.17 ± 0.28	4.72 ± 0.19†	4.63 ± 0.18†	0.7125	<0.0001	0.4372
Meal count (n/d)	27.9 ± 1.4	27.1 ± 1.1	37.0 ± 1.2†	33.4 ± 1.1†	0.0786	<0.0001	0.2652
Meal size (mg/meal)	108 ± 10	82 ± 7*	88 ± 5	70 ± 3	0.0015	0.0200	0.5615
Meal duration (min/meal)	9.7 ± 0.6	9.8 ± 0.7	9.6 ± 0.5	10.0 ± 0.7	0.6752	0.5930	0.7813
Meal speed (mg/min)	11.2 ± 0.8	8.7 ± 0.7*	9.4 ± 0.6	7.2 ± 0.3*	0.0001	0.0078	0.8274
Drink bout count (n/d)	51.3 ± 3.1	58.6 ± 3.6	77.3 ± 6.5†	66.4 ± 4.5	0.6966	0.0005	0.0532
Drink bout size (µL/bout)	50 ± 3	49 ± 4	49 ± 3	53 ± 4	0.6848	0.7580	0.4561
Drink bout duration (min/bout)	0.77 ± 0.03	0.80 ± 0.02	0.87 ± 0.05	0.89 ± 0.04	0.4308	0.0109	0.8776
Total motion (m/d)	223.3 ± 12.6	223.3 ± 14.5	313.4 ± 18.1†	314.9 ± 21.3†	0.9633	<0.0001	0.9661
Ambulation (m/d)	172.2 ± 10.5	173.0 ± 14.1	274.9 ± 18.7†	273.8 ± 20.5†	0.9908	<0.0001	0.9557
Fine motion (m/d)	51.1 ± 2.8	50.4 ± 1.2	38.5 ± 1.5†	41.1 ± 1.4†	0.5984	<0.0001	0.3769
Sleep (h/d)	13.6 ± 0.6	13.5 ± 0.4	13.6 ± 0.3	13.8 ± 0.3	0.9520	0.7994	0.7195
24-h respiratory exchange ratio	0.895 ± 0.007	0.856 ± 0.010*	0.918 ± 0.009	0.861 ± 0.008*	<0.0001	0.1220	0.2782
Aerobic resting metabolic rate (kcal/h)	0.347 ± 0.009	0.197 ± 0.007*	0.336 ± 0.009	0.194 ± 0.006*	<0.0001	0.4030	0.6553
Maximum aerobic metabolic rate during activity (kcal/h)	0.611 ± 0.012	0.421 ± 0.008*	0.558 ± 0.010†	0.442 ± 0.015*	<0.0001	0.1815	0.0024
Aerobic energy expenditure due to activity (i.e., 24-h average minus resting; kcal/h)	0.105 ± 0.007	0.093 ± 0.007	0.100 ± 0.006	0.093 ± 0.004	0.1475	0.6964	0.7123
Aerobic energy to motion (kcal/km)	11.5 ± 0.8	10.5 ± 0.9	7.9 ± 0.6†	7.5 ± 0.5†	0.3203	<0.0001	0.6993

Data were analyzed by 2-way ANOVA and presented as mean± SE. *P < 0.05 effect of ambient temperature within sex; †P < 0.05 effect of sex within ambient temperature, by Tukey multiple comparison procedure.

Energy expenditure. Energy expenditure was sensitive to both cage type and ambient temperature, and an interaction was observed between these 2 factors (Figure 4A). Regardless of sex, mice housed in MC at SRT showed large increases in energy expenditure as compared with mice housed in SM caging at SRT.

To account for the variance introduced into the dataset by individual differences in body size and composition, energy expenditure rates were analyzed by GLM. Energy expenditure was modified by the covariate of FFM (P < 0.001) (Figure 4B). Correction of the dataset for FFM showed that caging type (P < 0.001), sex (P = 0.019), and ambient temperature (P < 0.001) all affected energy expenditure. However, pairwise interactions were not detected with regard to cage type and sex (interaction P = 0.737) or sex and ambient temperature (interaction P = 0.764). In contrast, a pairwise interaction was observed with regard to cage type and ambient temperature (interaction P < 0.001). A 3-way interaction of cage type, ambient temperature, and sex was not detected (P = 0.268). These findings indicate that energy expenditure was similar in mice housed in SM cages either as pairs or individually, at either SRT or TNT. In the Promethion, mice housed at the TNT had lower energy expenditure than did those housed at SRT. These data indicate that mice housed in MC at SRT showed elevated energy expenditure that was normalized by performing MC studies at TNT.

Post hoc analyses were performed on data from the cohort studied with the Promethion system to determine whether exposure order was a factor. As for food intake, energy expenditure was not influenced by exposure order (P = 0.971), and no significant interactions were detected among exposure order, sex, and temperature (all P > 0.05).

Mice housed in the Promethion system at TNT showed less energy expenditure than occurred with the other housing conditions, with or without adjustment for body composition covariates. This outcome was driven by a suppression of resting metabolic rate in both sexes (Table 3). Energy expenditure due to activity was estimated as the difference between the 24-h average heat production rate and resting metabolic rate and was not affected by ambient temperature or sex. Total motion was greater in females but was not affected by temperature due to sex-dependent differences in both ambulation and fine motions (e.g., grooming, rearing). The energy cost of locomotion,

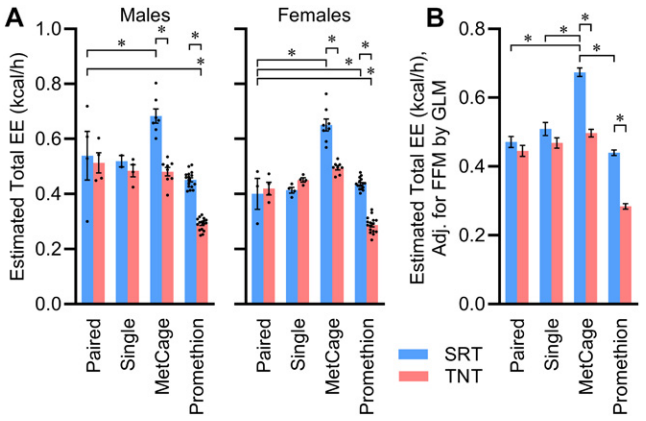

Figure 4. Effect of caging type and ambient temperature upon energy expenditure over 4 d in male and female C57BL/6J mice. (A) Estimated total daily EE. Summary data are presented as mean ± SE. Males: cage $P < 0.0001$, temperature $P < 0.0001$, interaction $P = 0.0017$. Females: cage $P < 0.0001$, temperature $P < 0.0001$, interaction $P < 0.0001$. Data were analyzed by 2-way ANOVA within each sex, and $^*P < 0.05$ by Tukey multiple comparison procedure. (B) Total EE adjusted for the covariate of FFM using GLM. Summary data are presented as the estimated marginal mean ± SE, at the covariate FFM of 19.49 g, and $^*P < 0.05$ by Tukey multiple comparison procedure. SRT, standard room temperature; TNT, thermoneutral temperature; FFM, fat-free mass; GLM, general linear modeling.

estimated by the energy expenditure due to activity divided by total distance traveled per day, was greater in males but was not altered by ambient temperature. Analysis of the influence of differences in FFM by using GLM indicated that FFM did not modify energy expenditure per meter moved ($P = 0.742$), and correction for FFM did not account for the influence of sex on this end point. Thus, regardless of ambient temperature and FFM, males spent more energy to move any given distance, but the sexes ultimately invested similar total amounts of energy toward motion because females moved more total distance than males.

Fluid balance. Water intake was modified by ambient temperature in a complex cage type– and sex-dependent manner (Figure 5A). In contrast, total body hydration was not affected by cage type or ambient temperature (Figure 5B). This discrepancy between fluid flux and fluid accumulation is likely because of differences in sensible and insensible water loss mechanisms.

Urine was collected daily from mice housed in MC, and urine from the third overnight period was used for urinalyses. Urine volume was modified by ambient temperature and by sex, but the effect of temperature was similar between sexes (Figure 5C). In particular, TNT housing reduced urine production in all mice. In contrast, insensible water loss (i.e., exhaled water vapor, evaporation) was increased during TNT housing (Figure 5D). Osmolality, along with sodium and potassium concentrations, was also higher in mice housed at TNT (Table 2). However, total daily electrolyte elimination in the urine not affected by ambient temperature.

Post hoc analyses of water intake were performed on the subset of mice studied in the Promethion to identify possible order effects. An effect of exposure order was not detected ($P = 0.208$), and no interactions were detected with regard to exposure order, sex, and ambient temperature (all $P > 0.05$).

Urine and fecal analyses. Total daily corticosterone elimination in urine was greater in females than in males, and housing at TNT was associated with lower urine corticosterone elimination independent of sex (Figure 5E). Corticosterone elimination per day via feces (though accounting for substantially less corticosterone than elimination in urine) was also greater in females than males, and housing at TNT reduced corticosterone excretion in feces in females but not males (Table 2). These findings indicate that performing MC studies at TNT greatly reduces levels of a biomarker commonly associated with both physiological and psychological stress. Urine creatinine concentrations were similar across groups and thus total daily creatinine elimination in urine followed trends that paralleled total urine volumes.

Total daily elimination of copeptin (a surrogate measurement of the release of arginine vasopressin, AVP) in urine was reduced in mice housed at TNT, regardless of sex (Table 2). Urine osmolality was higher during TNT housing.

Discussion

This study examined energy expenditure and food and fluid intake behaviors in male and female C57BL/6J mice housed in various types of caging types at either SRT or a TNT. Our hypothesis was that the detrimental effects of housing in MC are primarily the result of chronic exposure to an ambient temperature that is below the mouse thermoneutral zone. By extension, the study evaluated whether housing at a TNT could minimize the energy balance changes that occur in mice housed in MC. We also examined how MC studies performed at TNT affect fluid intake and balance in mice. Our data highlight a major, caging-specific effect of ambient temperature on energy intake and expenditure. They also show that ambient temperature has a major modulatory effect on fluid balance physiology and corticosterone concentrations in urine and feces. Collectively, our data support the conclusion that mice housed in MC cages at SRT experience a cold stress that can be ameliorated if MC studies are performed at TNT.

The concept of pathophysiological "stress" is complex, and the evaluation of this ephemeral concept in an experimental and quantitative manner is exceptionally complicated.[10-12] Nonetheless, 24-h urine-free corticosterone excretion is strongly correlated with corticosterone production and secretion rates,[5] and urinary corticosterone elimination rate is a physiological parameter that is commonly used to quantify stress in mice.[2] Cage changes cause increased corticosterone release, which can be interpreted as a marker of psychological stress and can require a period of acclimatization to minimize potentially confounding physiological changes.[25] The concept that MC housing induces a stress response in rodents is widely accepted and supported through measures of urine corticosterone. For example, one previous study used corticosterone levels to assess acclimatization and stress in MC and determined that housing in MC resulted in an approximate 10-fold increase in urine corticosterone.[14] However, the specific factors that induce this increase have not been identified. One possible source is a combination of somatic pain (and potentially foot ulcers in larger rodents) that develops secondary to housing on a metal-rod or wire-mesh floor type and the psychosocial effects of individual housing.[22] Our previous work investigating the modulatory effects of including a plastic platform that allowed mice to avoid the potentially uncomfortable metal floor of MC housing indicated that chow-fed, adult male C57BL/6J mice actively avoid standing on such a plastic floor insert and instead favor the metal-rod flooring, thus providing some evidence against the notion that the floor is aversive.[39] In the current study, increasing the ambient temperature from SRT to TNT reduced urine corticosterone elimination by almost 10-fold. These data therefore indicate that a large fraction of the elevation in urine corticosterone levels

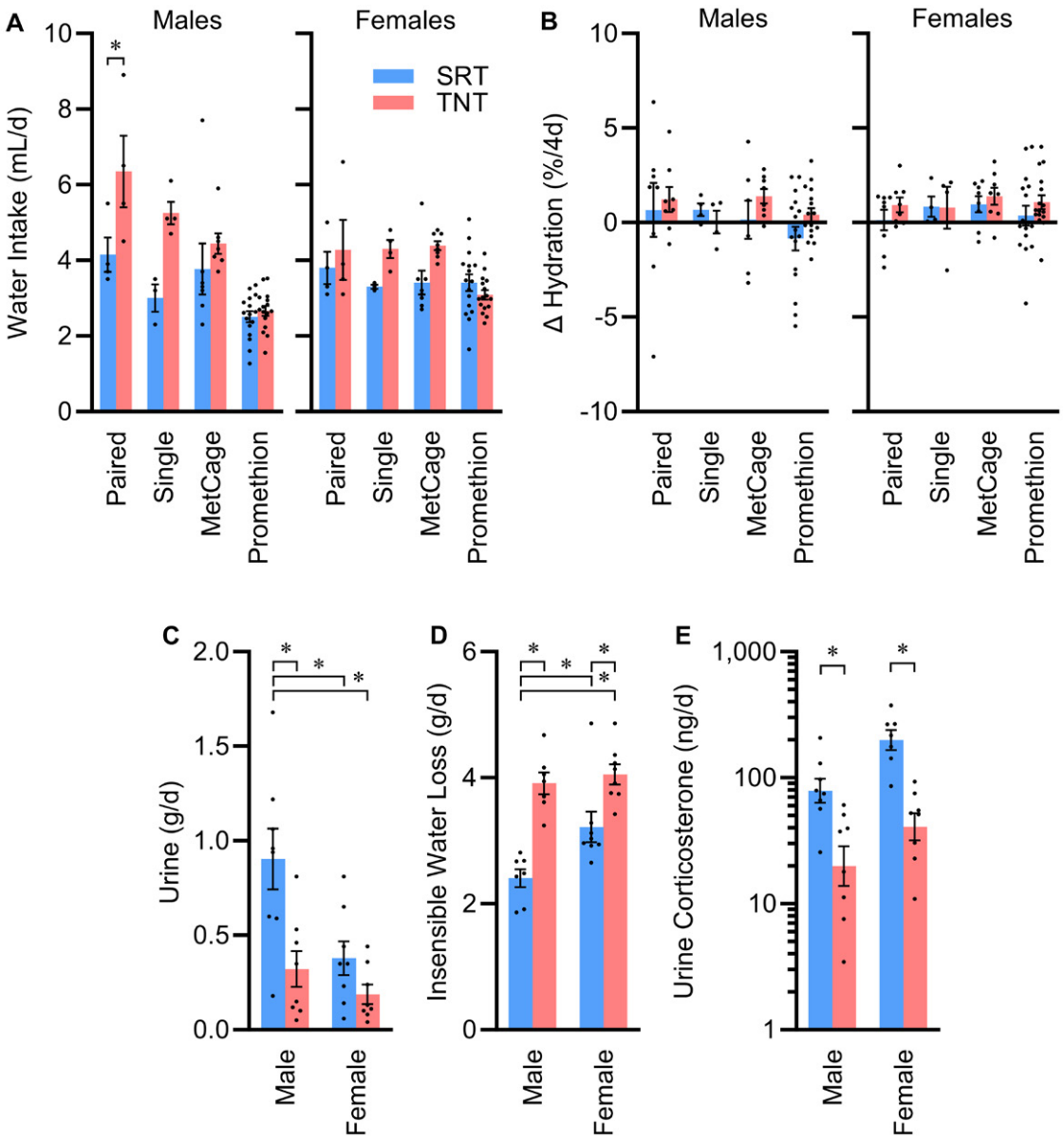

Figure 5. Effect of caging type and ambient temperature on water intake, renal function, and 24-h urine-free corticosterone excretion in male and female C57BL/6J mice. (A) Water intake. Males: cage $P < 0.0001$, temperature $P < 0.0001$, interaction $P = 0.0103$. Females: cage $P = 0.0102$, temperature $P = 0.0240$, interaction $P = 0.0257$. (B) Change in total body hydration (calculated as total body water divided by total body mass). Males: cage $P = 0.3121$, temperature $P = 0.3225$, interaction $P = 0.7226$. Females: cage $P = 0.7050$, temperature $P = 0.2727$, interaction $P = 0.9233$. (C) Urine collected during the third 24-h period from mice housed in MC. Temperature $P = 0.0011$, sex $P = 0.0044$, interaction $P = 0.0765$. (D) Insensible water loss during the third 24-h period. Temperature $P < 0.0001$, sex $P = 0.0170$, interaction $P = 0.0839$. (E) Total daily elimination of corticosterone to urine. Temperature $P < 0.0001$, sex $P = 0.0045$, interaction $P = 0.6786$. For all panels, data are presented as mean ± SE and were analyzed by 2-way ANOVA, and $^*P < 0.05$ by Tukey multiple comparison procedure. SRT, standard room temperature; TNT, thermoneutral temperature; FFM, fat-free mass; GLM, general linear modeling.

(as a metric of stress) is caused by the thermal stress of housing mice in MC, as compared with psychosocial factors that include individual housing.[7,14,21,26] Future studies are needed to carefully identify the relative contribution of psychosocial factors in the corticosterone response to MC housing, and the design of studies that use MC must consider the confounding effects of ambient temperature.

Copeptin is the C-terminal fragment of AVP and is released in a 1:1 molar ratio to AVP. In contrast to the small, biologically active AVP peptide that is rapidly cleared from the blood by enzymatic cleavage (half-life of only minutes) and challenging to measure at low concentrations, copeptin is very stable in plasma and is cleared in the urine (reviewed in previous studies[4,15,19,33]). As such, we hypothesize that urinary copeptin (and more specifically, its elimination per unit time) may be used as a biomarker of AVP release kinetics, although we are not aware of a study that has directly tested or demonstrated this relationship. Increased copeptin in plasma or urine has been associated with various stressors including osmotic stress, infection, sepsis, acute and chronic cardiovascular disorders, and metabolic disorders.[3,13,18,29-31,36] In the current study, TNT housing was associated with a reduction in total daily copeptin excretion despite simultaneous increases in fluid intake and urine osmolality, reduced urine volumes, and no major changes in total body hydration. Therefore, we hypothesize that AVP release does not explain the increases in fluid intake and urine solute concentration that occurs during TNT housing. The observation that TNT housing is associated with a reduction

in urinary copeptin again suggests that, as for urinary corticosterone, MC housing at SRT may result in AVP release as part of a generalized stress response to perceived cold. Consistent with this idea, one recent study demonstrated that exposure to both short-term cold and hot ambient temperatures increased plasma copeptin in human subjects.[35] In contrast, another study found that adult men wearing a cooling vest for 2 h had reduced plasma copeptin.[34] A third study examining causes of increased plasma copeptin in apneic human divers concluded that hypoxia is a dominant stimulant of copeptin, whereas cold stress plays a minor role.[16] Indications that elevated AVP release is involved in the etiology of some clinical disorders (such as preeclampsia[28-30]) support the value of further clarification of interactions among thermal and other stressors, the associated status of other markers and mediators of such stress (e.g., corticosterone), and the effects of such stressors on AVP release.

Previous work has demonstrated that male and female mice housed at various ambient temperatures show distinct thermoregulatory behaviors and physiology responses.[8,38] Thus, we purposefully studied both sexes of C57BL/6J mice. Total daily urinary excretion of both corticosterone and copeptin, urine osmolality, urine sodium and potassium content, meal patterning, drink patterning, and locomotion were all different between the sexes, yet sex did not modify the effects of ambient temperature on these end points. However, sex did modify some of the effects of ambient temperature. For example, housing in MC at SRT upon FFM had much less effect in female mice as compared with males. Ambient temperature had smaller effects on water intake in females as compared with males housed in SM. Daily urine volumes were lower in females housed in MC at SRT as compared with males, thus countering the suppressive effect of TNT on water intake in females. However, some of the apparent effects of sex were secondary to sex-based differences in body composition. For example, correction of food intake (Figure 3B) and total daily energy expenditure (Figure 4B) for body composition accounted for the apparent modulatory effects of sex on these end points. Future studies of the impact of ambient temperature on cardiovascular and metabolic end points must therefore consider both direct and indirect effects of sex upon outcome measures.

Several limitations in our study design should be noted. First, we included only relatively young adult mice (aged 14 to 17 wk). Investigating mice at various ages could be informative. Second, we studied only the C57BL/6J strain of mice, and thus the generalizability of our findings to other mouse strains may be limited. Similar studies in rats, other strains of mice, and rodents with various genetic manipulations are warranted, as various strains and species may have different thermoneutral zones and show different responses to temperatures beyond their TNT range. Indeed, distinct lower (LCT) and upper (UCT) critical temperatures have been documented in albino, BALB/c, OF1, R70, TS, C57, C3H, DBA, FVB, and other mouse strains, and some strains more rapidly increase metabolic rate in response to any given change in ambient temperature.[8,24] Third, we did not evaluate female estrous cycle or include pregnant mice. Consequently, the stage of the female estrous cycle in our study cohorts may introduce heterogeneity, potentially affecting the overall outcomes and interpretation of our findings. Fourth, all mice were fed the same diet (soy-free Teklad/Envigo 2920×), which is a phytoestrogen-free natural-product diet. The effects of ambient temperature on energy and fluid balance in mice fed different diets, including diets with high fat or carbohydrate contents, have not been tested. Fifth, the concept of thermoneutrality continues to evolve. For example, increasing evidence demonstrates that the thermoneutral zone of mice varies by time of day.[8] Therefore, future studies could define and optimize environmental factors that influence outcomes when using MC.

In summary, the current study documents that ambient temperature greatly influences the study of energy and fluid balance in C57BL/6J mice and that the negative consequences of housing mice in MC at SRT can be largely attenuated if ambient temperature is within the thermoneutral zone. Our findings do not suggest that previous research efforts using MC at SRT are fundamentally flawed but rather indicate that MC studies performed at SRT must be interpreted in the appropriate context. Specifically, MC studies performed in mice at SRT inadvertently incorporate a major thermal stress that may mask or exaggerate the effects of experimental manipulations (dietary, genetic, pharmacological, etc.) on end points such as food and fluid intake, energy expenditure, and various renal functions, even though other end points such as digestive efficiency appear to be unaffected. Better understanding of the impact of ambient temperature on mouse physiology should improve the interpretation and translation of experimental outcomes as relevant to human physiology.

Acknowledgments

We gratefully acknowledge the assistance of the Medical College of Wisconsin Biomedical Resource Center, Comprehensive Rodent Metabolic Phenotyping Core, and Engineering Core. This work was supported by grants from the National Institutes of Health (HL134850, DK133121, HL084207, HL072483), American Heart Association (18EIA33890055), the Medical College of Wisconsin Clinical & Translational Science Institute (UL1TR001436), and the Advancing a Healthier Wisconsin Endowment to Medical College of Wisconsin.

References

1. **Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes.** 2010/63/EU.
2. **Balcombe JP, Barnard ND, Sandusky C.** 2004. Laboratory routines cause animal stress. Contemp Top Lab Anim Sci **43**:42–51.
3. **Ding C, Magkos F.** 2019. Oxytocin and vasopressin systems in obesity and metabolic health: mechanisms and perspectives. Curr Obes Rep **8**:301–316. https://doi.org/10.1007/s13679-019-00355-z.
4. **Fenske WK, Schnyder I, Koch G, Walti C, Pfister M, Kopp P, Fassnacht M, et al.** 2018. Release and decay kinetics of copeptin vs AVP in response to osmotic alterations in healthy volunteers. J Clin Endocrinol Metab **103**:505–513. https://doi.org/10.1210/jc.2017-01891.
5. **Fox JG, Barthold S, Davisson M, Newcomer CE, Quimby FW, Smith A.** 2006. The mouse in biomedical research: normative biology, husbandry, and models, 2nd ed, vol **3**. Boston: Academic Press.
6. **Fullerton PM, Gilliatt RW.** 1967. Pressure neuropathy in the hind foot of the guinea-pig. J Neurol Neurosurg Psychiatry **30**:18–25. https://doi.org/10.1136/jnnp.30.1.18.
7. **Giral M, Armengol C, Gavaldà A.** 2022. Physiologic effects of housing rats in metabolic cages. Comp Med **72**:298–305. https://doi.org/10.30802/AALAS-CM-22-000026.
8. **Gordon CJ.** 2012. Thermal physiology of laboratory mice: defining thermoneutrality. J Therm Biol **37**:654–685. https://doi.org/10.1016/j.jtherbio.2012.08.004.
9. **Grobe JL.** 2017. Comprehensive assessments of energy balance in mice. Methods Mol Biol **1614**:123–146. https://doi.org/10.1007/978-1-4939-7030-8_10.
10. **Gurfein BT, Stamm AW, Bacchetti P, Dallman MF, Nadkarni NA, Milush JM, Touma C, et al.** 2012. The calm mouse: an animal model of stress reduction. Mol Med **18**:606–617. https://doi.org/10.2119/molmed.2012.00053.

11. **Herman JP, McKlveen JM, Ghosal S, Kopp B, Wulsin A, Makinson R, Scheimann J, et al.** 2016. Regulation of the hypothalamic-pituitary-adrenocortical stress response. Compr Physiol **6**:603–621. https://doi.org/10.1002/cphy.c150015.
12. **Jacobson L.** 2005. Hypothalamic-pituitary-adrenocortical axis regulation. Endocrinol Metab Clin North Am **34**:271–292. https://doi.org/10.1016/j.ecl.2005.01.003.
13. **Jochberger S, Dorler J, Luckner G, Mayr VD, Wenzel V, Ulmer H, Morgenthaler NG, et al.** 2009. The vasopressin and copeptin response to infection, severe sepsis, and septic shock. Crit Care Med **37**:476–482. https://doi.org/10.1097/CCM.0b013e3181957532.
14. **Kalliokoski O, Jacobsen KR, Darusman HS, Henriksen T, Weimann A, Poulsen HE, Hau J, et al.** 2013. Mice do not habituate to metabolism cage housing–a three week study of male BALB/c mice. PLoS One **8**:e58460. https://doi.org/10.1371/journal.pone.0058460.
15. **Lauar MR, Evans LC, Van Helden D, Fink GD, Banek CT, Menani JV, Osborn JW.** 2023. Renal and hypothalamic inflammation in renovascular hypertension: role of afferent renal nerves. Am J Physiol Regul Integr Comp Physiol **325**:R411–R422. https://doi.org/10.1152/ajpregu.00072.2023.
16. **Marlinge M, Coulange M, Fitzpatrick RC, Delacroix R, Gabarre A, Laine N, Cautela J, et al.** 2019. Physiological stress markers during breath-hold diving and SCUBA diving. Physiol Rep **7**:e14033. https://doi.org/10.14814/phy2.14033.
17. **Mecklenburg L, Kusewitt D, Kolly C, Treumann S, Adams ET, Diegel K, Yamate J, et al.** 2013. Proliferative and non-proliferative lesions of the rat and mouse integument. J Toxicol Pathol **26** 3_Suppl:27S–57S. https://doi.org/10.1293/tox.26.27S.
18. **Möckel M, Searle J.** 2014. Copeptin-marker of acute myocardial infarction. Curr Atheroscler Rep **16**:421. https://doi.org/10.1007/s11883-014-0421-5.
19. **Morgenthaler NG, Struck J, Alonso C, Bergmann A.** 2006. Assay for the measurement of copeptin, a stable peptide derived from the precursor of vasopressin. Clin Chem **52**:112–119. https://doi.org/10.1373/clinchem.2005.060038.
20. **Morrow DT, Robinette LR, Saubert CW IV, Van Hoosier GL, Jr.** 1977. Poditis in the rat as a complication of experiments in exercise physiology. Lab Anim Sci **27**:679–681.
21. **Muta O, Odaka M, Fujii Y, Fushimi T, Sato H, Osakabe N.** 2023. Difference in endocrine and behavior between short-term single- and paired-housing mice in metabolic cage. Neurosci Lett **806**:137246. https://doi.org/10.1016/j.neulet.2023.137246.
22. **National Research Council.** 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press.
23. **Peace TA, Singer AW, Niemuth NA, Shaw ME.** 2001. Effects of caging type and animal source on the development of foot lesions in Sprague Dawley rats (*Rattus norvegicus*). Contemp Top Lab Anim Sci **40**:17–21.
24. **Pennycuik PR.** 1967. A comparison of the effects of a variety of factors on the metabolic rate of the mouse. Aust J Exp Biol Med Sci **45**:331–346. https://doi.org/10.1038/icb.1967.33.
25. **Rasmussen S, Miller MM, Filipski SB, Tolwani RJ.** 2011. Cage change influences serum corticosterone and anxiety-like behaviors in the mouse. J Am Assoc Lab Anim Sci **50**:479–483.
26. **Ravenelle R, Santolucito HB, Byrnes EM, Byrnes JJ, Donaldson ST.** 2014. Housing environment modulates physiological and behavioral responses to anxiogenic stimuli in trait anxiety male rats. Neuroscience **270**:76–87. https://doi.org/10.1016/j.neuroscience.2014.03.060.
27. **Reho JJ, Nakagawa P, Mouradian GC Jr, Grobe CC, Saravia FL, Burnett CML, Kwitek AE, et al.** 2022. Methods for the comprehensive in vivo analysis of energy flux, fluid homeostasis, blood pressure, and ventilatory function in rodents. Front Physiol **13**:855054. https://doi.org/10.3389/fphys.2022.855054.
28. **Sandgren JA, Deng G, Linggonegoro DW, Scroggins SM, Perschbacher KJ, Nair AR, Nishimura TE, et al.** 2018. Arginine vasopressin infusion is sufficient to model clinical features of preeclampsia in mice. JCI Insight **3**:3. https://doi.org/10.1172/jci.insight.99403.
29. **Sandgren JA, Scroggins SM, Santillan DA, Devor EJ, Gibson-Corley KN, Pierce GL, Sigmund CD, et al.** 2015. Vasopressin: the missing link for preeclampsia? Am J Physiol Regul Integr Comp Physiol **309**:R1062–R1064. https://doi.org/10.1152/ajpregu.00073.2015.
30. **Santillan MK, Santillan DA, Scroggins SM, Min JY, Sandgren JA, Pearson NA, Leslie KK, et al.** 2014. Vasopressin in preeclampsia: a novel very early human pregnancy biomarker and clinically relevant mouse model. Hypertension **64**:852–859. https://doi.org/10.1161/HYPERTENSIONAHA.114.03848.
31. **Schwerg M, Slagman A, Stangl K, Stangl V.** 2017. Copeptin, resistant hypertension and renal sympathetic denervation. Biomarkers **22**:311–314. https://doi.org/10.1080/1354750X.2016.1252968
32. **Segar JL, Balapattabi K, Reho JJ, Grobe CC, Burnett CML, Grobe JL.** 2021. Quantification of body fluid compartmentalization by combined time-domain nuclear magnetic resonance and bioimpedance spectroscopy. Am J Physiol Regul Integr Comp Physiol **320**:R44–R54. https://doi.org/10.1152/ajpregu.00227.2020.
33. **Szinnai G, Morgenthaler NG, Berneis K, Struck J, Muller B, Keller U, Christ-Crain M.** 2007. Changes in plasma copeptin, the c-terminal portion of arginine vasopressin during water deprivation and excess in healthy subjects. J Clin Endocrinol Metab **92**:3973–3978. https://doi.org/10.1210/jc.2007-0232.
34. **Thibonnier M, Ghosh S, Blanchard A.** 2022. Effects of a short-term cold exposure on circulating microRNAs and metabolic parameters in healthy adult subjects. J Cell Mol Med **26**:548–562. https://doi.org/10.1111/jcmm.17121.
35. **Timpka S, Melander O, Engstrom G, Elmstahl S, Nilsson PM, Lind L, Pihlsgard M, et al.** 2023. Short-term association between outdoor temperature and the hydration-marker copeptin: a pooled analysis in five cohorts. EBioMedicine **95**:104750. https://doi.org/10.1016/j.ebiom.2023.104750.
36. **Vasile VC, Jaffe AS.** 2014. Emerging biomarkers for acute heart conditions. Curr Opin Cardiol **29**:312–318. https://doi.org/10.1097/HCO.0000000000000077.
37. **Weir JB.** 1949. New methods for calculating metabolic rate with special reference to protein metabolism. J Physiol **109**:1–9. https://doi.org/10.1113/jphysiol.1949.sp004363.
38. **Yamauchi C, Fujita S, Obara T, Ueda T.** 1983. Effects of room temperature on reproduction, body and organ weights, food and water intakes, and hematology in mice. Jikken Dobutsu **32**:1–11.
39. **Ziegler AA, Grobe CC, Reho JJ, Jensen ES, Thulin JD, Segar JL, Grobe JL.** 2022. Short-term housing in metabolic caging on measures of energy and fluid balance in male C57BL/6J mice (*Mus musculus*). J Am Assoc Lab Anim Sci **61**:132–139. https://doi.org/10.30802/AALAS-JAALAS-21-000087.