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ORIGINAL PAPER

Effects of short- and long-term cold acclimation on morphology, physiology, and exercise performance of California mice (*Peromyscus californicus*): potential modulation by fatherhood

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Abstract

California mice (*Peromyscus californicus*) differ from most other mammals in that they are biparental, genetically monogamous, and (compared with other *Peromyscus*) relatively large. We evaluated effects of cold acclimation on metabolic rate, exercise performance, and morphology of pair-housed male California mice, as well as modulation of these effects by fatherhood. In Experiment 1, virgin males housed at 5° or 10 °C for approximately 25 days were compared with virgins housed at standard vivarium temperature of 22 °C. Measures included resting metabolic rate (RMR), maximal oxygen consumption ($\dot{V}O_2$max), grip strength, and sprint speed. In Experiment 2, virgin males housed at 22 °C were compared with three groups of males housed at 10 °C: virgins, breeding males (housed with a female and their pups), and non-breeding males (housed with an ovariectomized, estrogen- and progesterone-treated female) after long-term acclimation (mean 243 days). Measures in this experiment included basal metabolic rate (BMR), $\dot{V}O_2$max, maximal thermogenic capacity ($\dot{V}O_2$sum), and morphological traits. In Experiment 1, virgin males housed at 5° and 10 °C had higher RMR and $\dot{V}O_2$max than those at 22 °C. In Experiment 2, 10 °C-acclimated groups had shorter bodies; increased body, fat, and lean masses; higher BMR and $\dot{V}O_2$sum, and generally greater morphometric measures and organ masses than virgin males at 22 °C. Among the groups housed at 10 °C, breeding males had higher BMR and lower $\dot{V}O_2$max than non-breeding and/or virgin males. Overall, we found that effects of fatherhood during cold acclimation were inconsistent, and that several aspects of cold acclimation differ substantially between California mice and other small mammals.

Keywords Biparental care · Cold acclimation · Costs of reproduction · Energetics · Exercise performance · Fatherhood · Morphology · Paternal care · Physiology · Rodent

Introduction

The rodent genus *Peromyscus* (often called 'deer mice') includes ~ 56 species in North and Central America (King 1968; Hill 1983; Bedford and Hoekstra 2015). Although most follow the standard mammalian reproductive pattern

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of exclusively maternal parental care, the California mouse (*P. californicus*) is genetically monogamous and biparental, with pairs forming lifelong bonds and males contributing extensively to offspring care (Gubernick and Alberts 1987b; Ribble and Salvioni 1990; Ribble 1991). California mice produce multiple litters per year, and fathers assist with parturition and exhibit all of the parental behaviors (huddling, grooming, protection, and transportation of offspring) characteristic of mothers except lactation (Dudley 1974; Gubernick and Alberts 1987a; Lee and Brown 2002). Care by fathers increases offspring survival in both field conditions (Gubernick and Teferi 2000) and laboratory environments involving stress (e.g., cold exposure or exercise; Gubernick et al. 1993; Cantoni and Brown 1997). Paternal care also has lasting impacts on offspring development, including social, aggressive, and mating behaviors, neural and endocrine function, and cognitive ability (Braun and Champagne 2014; Bales and Saltzman 2016).

How the intense and sustained investment in offspring affects male California mice is an intriguing question. Although the physiological and endocrine correlates of reproduction in female mammals have been studied intensively (e.g., Gittleman and Thompson 1988; Hammond 1997; Speakman 2008), much less is known about the physiological impacts of parenthood on fathers. In some biparental mammals, including California mice, becoming a father alters neural circuitry and endocrinology (Saltzman and Ziegler 2014; Bales and Saltzman 2016), but effects on energetics, performance, and morphology are less clear. In the biparental common marmoset (*Callithrix jacchus*), cotton-top tamarin (*Saguinus oedipus*), and California mouse, expectant fathers gain body mass during their mate's pregnancy, with subsequent mass loss after parturition (Achenbach and Snowdon 2002; Ziegler et al. 2006; Harris et al. 2011; Saltzman et al. 2015). However, other studies of California mice found no differences in body mass between breeding and non-breeding males, or any consistent effects of fatherhood on fat or lean mass (Andrew et al. 2016; Zhao et al. 2017, 2018).

The latter results suggest that the impact of fatherhood on male physiology is minimal in California mice, but with the important caveat that the animals were housed in benign laboratory conditions. The lab environment (ad libitum food; low thermoregulatory costs; no predation; no requirement for long-distance movements or territorial or mate defense) may not produce enough of an energetic or locomotor challenge to drive extensive physiological changes. Hence, it is not clear if the inference of minimal effects of fatherhood is ecologically, physiologically, and evolutionarily relevant for the more arduous conditions in natural habitats. In a study of adult male California mice, housing at 22 °C under a moderate energy stressor (24-h fasting every third day and having to climb towers to obtain food and water) increased both fat mass and body mass in non-reproductive males but not in fathers (Zhao et al. 2018). One interpretation of these results is that the demands of fatherhood constrained males' ability to obtain, process, or accumulate energy under these artificially stressful conditions.

For many small mammals, cold and its associated thermoregulatory costs are pervasive challenges, as the environmental temperatures they experience—particularly at night, when most small mammals are active—are routinely below thermoneutrality (Hill 1983; Feist and White 1989). This is especially pertinent during winter in temperate and high-latitude habitats. Small body size goes hand-in-hand with high surface/volume ratios and also constrains the ability of small mammals to add insulation (fur or subcutaneous fat), so acclimatization to winter conditions is usually based primarily on increased thermogenic capacity (e.g., Hart 1971; Dawson and Olson 1988), mainly via brown adipose tissue (Heldmaier et al. 1982; Heldmaier et al. 1989; Cannon and Nedergaard 2004). In rodents, including *Peromyscus* species, cold acclimatization (or acclimation) typically increases thermogenic capacity by 30–50% (Hart 1971; Lynch 1973; Heimer and Morrison 1978; Wickler 1980; Heldmaier et al. 1982; Hayes and Chappell 1986; Nespolo et al. 1999; Rezende et al. 2004a). This enhanced thermogenic capacity is often accompanied by elevated resting or basal metabolic rates (RMR or BMR; e.g., Hart 1957; Hayward 1965; Russell and Chappell 2007, Zub 2014). Winter acclimatization or acclimation may also induce substantial alterations in body composition and organ morphology (e.g., Smith and Horwitz 1969; Heldmaier et al. 1982; Konarzewski and Diamond 1994; Hammond and Kristan 2000; Deveci et al. 2001; Brzęk et al. 2007; Rezende et al. 2009; Vaanholt et al. 2009; Zub 2014). These changes may impact both energy budgets (Hayes 1989b) and aspects of performance in addition to cold tolerance (e.g., exercise capacity; Hayes and Chappell 1986).

In its natural range in California and Baja California, the California mouse breeds throughout the year, except for the dry summer (Gubernick 1988), so parents must care for some litters during the cold winter months. Given the apparent impact of an experimental energy stress on fathers in laboratory conditions (Zhao et al. 2018), an understanding of the effects of ecologically relevant thermal conditions—including winter temperatures—may be important for understanding the evolution of the species' monogamous mating system. Accordingly, we performed two experiments to evaluate the effects of cold acclimation on male morphology and physiology. First, we acclimated mice for short periods (~ 1 month) to temperatures typical for winter in the species' natural habitat (5 °C or 10 °C) and measured body composition, hematocrit, energy metabolism (thermoneutral RMR, maximal oxygen consumption in exercise [$\dot{V}O_2max$]), exercise performance (grip strength, sprint speed) and predatory

aggression. Since many small mammals spend much of their lives at environmental temperatures below their thermal neutral zones, we also performed a longer-term (~6 months) cold acclimation at 10 °C. For this experiment we measured body composition, hematocrit, and energy metabolism (BMR, $\dot{V}O_2$max, and thermogenic capacity). To ascertain the effects of pair bonding and fatherhood, we compared virgin males (housed with another adult male), breeding males (housed with an adult female and their first litter) and non-breeding males (housed with an ovariectomized, estrogen- and progesterone-treated female).

We hypothesized that, as is the case for other *Peromyscus* species, cold acclimation would elevate thermogenic capacity in California mice. Additionally, we predicted that cold-acclimated males would have enhanced predatory aggression (related to higher food requirements), elevated BMR and RMR, increased body, fat, and lean masses, changes in organ size, and shifts in exercise performance. Finally, due to the demands of fatherhood, we predicted that the effects of cold acclimation would differ in breeding males compared to non-breeding and virgin males. Findings on effects of fatherhood should be interpreted cautiously, as survival and breeding success were low in cold-housed animals (see below), leading to small sample sizes for breeding males.

Methods

Animals

California mice were born and raised in our colony at the University of California, Riverside (UCR) and were descended from animals purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA; ancestors captured in the Santa Monica Mountains, CA, USA). Animals were housed in polycarbonate cages (44 × 24 × 20 cm) with aspen shavings for bedding but no additional nesting material. Food (Purina 5001 Rodent Chow, LabDiet, Richmond, IN, USA; caloric content—28.5% protein, 13.5% fat, 58.0% carbohydrate) and water were available ad libitum. Lighting was on a 14:10 cycle (lights on at 05:00 hours, off at 19:00 hours), with humidity maintained at approximately 55% and ambient temperature at 22.1 ± 0.9 °C (mean ± SE) except where stated otherwise. Mice were checked twice daily, and cages were cleaned once per week. At weaning age (27-31 days; 28.0 ± 0.3, mean ± SE), animals were ear-punched for identification and placed in same-sex groups of 3–4 related and/or unrelated, age-matched individuals.

All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the UCR Institutional Animal Care and Use Committee. UCR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Experiment 1: short-term cold acclimation

When males reached 60–114 (88.4 ± 1.1, mean ± SE) days of age, they were moved in their virgin groups either to an environmental chamber set at 5 °C (VM5, initial $N = 42$, final $N = 29$; see "Results" for explanation of decreases in sample sizes) or 10 °C (VM10, initial $N = 70$, final $N = 50$) or to a new room with ambient temperature set at 22 °C (VM22, initial $N = 72$, final $N = 61$) (Fig. 1: Experiment 1). Beginning at 14-38 (24.8 ± 1.5) days in their respective temperature conditions, males from each group underwent a series of test procedures over a 7-day period (Table 1). All males remained housed with their cage mates throughout the period of data collection, except where indicated below.

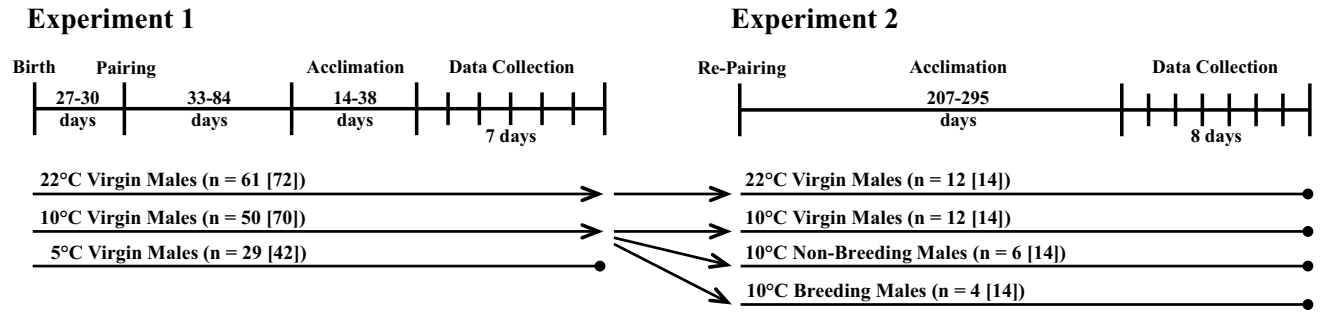

Fig. 1 Timeline and use of male California mice in Experiments 1 and 2. An arrow at the end of a line indicates that males were repaired and used in Experiment 2. A closed circle at the end of a line indicates that males were euthanized and used for morphological measurements, and were not used in subsequent experiments. Numbers of days indicate the minimum and maximum number of days elapsing between procedures. Sample sizes of males that survived through the duration of testing and were used for analysis are in parentheses; total starting sample sizes are in brackets

Table 1 Measures in Experiments 1 and 2, listed by the day and time of day at which they were obtained

Experiment 1			Experiment 2		
Test day	Time	Trait	Test day	Time	Trait
1	9:00–10:30	Hematocrit	1	8:30–16:30	Body mass/basal metabolic rate
1	13:00–14:30	Body mass/fat mass/lean mass			
2/3	9:30–11:00	Maximum grip strength	4/5	11:30–13:30	Body mass/exercise VO_2max
2/3	13:30–15:00	Predatory aggression			
4	8:30–16:30	Body mass/resting metabolic rate	6	12:00–14:00	Body mass/fat mass/lean mass
5/6	10:00–11:30	Body mass/exercise VO_2max	7	11:00–13:00	Body mass/VO_2sum
5/6	14:00–15:30	Maximal sprint speed			
7	9:00–10:30	Body mass/fat mass/lean mass	8	12:30–14:30	Body mass/organ masses
7	13:00–14:30	Hematocrit	8	12:30–14:30	Hematocrit

Experiment 2: long-term cold acclimation

Virgin males from the 10 °C (VM10, initial $N = 14$, final $N = 12$; see "Results") and 22 °C (VM22, initial $N = 14$, final $N = 12$) conditions [age 126–198 days (147.0 ± 5.1)] in Experiment 1 were pair-housed with a virgin male cage mate from their original same-sex group and maintained at their respective housing temperatures until 346–421 (381.5 ± 4.8) days of age (Fig. 1: Experiment 2). Additional virgin males that had been housed at 10 °C in Experiment 1 [age 123–173 days (142.7 ± 4.4)] were randomly paired with an age-matched virgin female in one of two conditions (Fig. 1). Breeding males (BM10, initial $N = 14$, final $N = 4$) were paired with a sham-ovariectomized female, and non-breeding males (NB10, initial $N = 14$, final $N = 6$) were paired with an ovariectomized female treated periodically with estradiol benzoate and progesterone (see below) to induce estrous behavior. After 207–295 days (243.0 ± 7.4), all virgin males, breeding males, and non-breeding males underwent an 8-day testing period (Table 1), after which they were euthanized and dissected (see below). BM10 [age 342–380 days (357.3 ± 6.8)] and NB10 [age 340–363 days (346.5 ± 5.5)] were compared with the age-matched VM10 [age 381–392 days (387.8 ± 1.2)] and VM22 [age 374–401 days (389.5 ± 2.9)].

Ovariectomies and estrogen/progesterone treatment

Females in Experiment 2 underwent bilateral ovariectomies prior to being paired with NB10 (to allow mating but prevent pregnancy) or sham-ovariectomies prior to being paired with BM10. Females were anesthetized with isoflurane, and surgeries were performed under aseptic conditions using standard procedures as previously described (Zhao et al. 2018). Females were then housed individually for 2 weeks to recover before being paired with males. Forty-eight hours prior to pairing, NB10 females were injected with estradiol benzoate (0.072 mg, s.c.; suspended in sesame oil, Sigma-Aldrich, St. Louis, MO, USA). At the time of pairing, they were injected with progesterone (0.48 mg, s.c.; suspended in sesame oil, Sigma-Aldrich, St. Louis, MO, USA) (Zhao et al. 2018). A pilot study (unpub.) found that this treatment usually led to mating behaviors in ovariectomized females ~ 13 h after progesterone injection, whereas untreated ovariectomized females were never observed to copulate. At the end of the experiment, ovariectomized females were euthanized by CO_2 inhalation and dissected to check for the presence of fetuses in the uterine canal; no females had visible fetuses.

Body mass

Males in Experiments 1 and 2, as well as breeding females in Experiment 2, were weighed to ± 0.1 g twice per week (13:00-15:00 hours) at approximately 3- to 4-day intervals from pairing until the beginning of their test period. This procedure was used to monitor pregnancies in breeding females (Experiment 2) and animal health, and to habituate animals to handling.

Body composition

Body composition was measured in Experiment 1 on test days 1 (13:00–14:30 hours) and 7 (9:00–10:30 hours) and in Experiment 2 on test day 6 (12:00–14:00 hours) (Table 1). Males were weighed and then scanned with a magnetic resonance whole-body analyzer (EchoMRI-100; Echo Medical Systems, Houston, TX, USA) to assess fat mass, lean mass, free water mass, and total water mass that was calibrated in our lab for this species (Zhao et al. 2017, 2018). Scans lasted ~ 90 s and did not require anesthesia or sedation. Here, we report fat and lean masses only (unaltered and as percentages of total body mass).

Hematocrit

Blood samples (~200 µl) were collected in Experiment 1 on test days 1 (9:00–10:30 hours) and 7 (13:00–14:30 hours) and in Experiment 2 on test day 8 (12:30–14:30 hours) (Table 1). Mice were anesthetized with isoflurane and blood was collected from the retro-orbital sinus into heparinized microhematocrit capillary tubes (Chauke et al. 2011; Harris et al. 2011; Andrew et al. 2016). Blood was centrifuged at 4 °C and 1300 RPM (~1900 *g*) for 12 min (Sorvall Legend Micro 21R; Thermo Scientific), and hematocrit was recorded.

Predatory aggression

Mice were tested for predatory aggression (Gammie et al. 2003; Zhao et al. 2017), without prior fasting, in Experiment 1 (test days 2 and 3, 13:30–15:00 hours). Briefly, the mouse was placed singly in a clean cage with a thin layer of aspen shavings covering the floor and no food or water. After a 15-min habituation period, a live adult cricket (0.2–0.5 g) was dropped into the cage on the side opposite the mouse. Behavior was video recorded until either the cricket was killed or 7 min had elapsed. Videos were scored for latency to attack and latency to kill the cricket. If the mouse did not kill the cricket, it was assigned a latency of 7 min. Predatory aggression was tested on two successive days to determine repeatability. The shorter latency of each animal's two tests was used for comparisons among temperature conditions. California mice are omnivorous, with a diet that includes arthropods (Merritt 1974; Reid et al. 2013).

Resting metabolic rate (RMR)

RMR was measured in Experiment 1 on test day 4 (8:30–16:30 hours) as previously described (Andrew et al. 2016). Males were separated from their cage mates, weighed before testing, and placed in a Plexiglas metabolic chamber with bedding (volume 525 mL), inside an environmental cabinet maintained at 28–30 °C (within the thermal neutral zone of these mice) for the 8-h experimental period. Subsampled excurrent air was dried (soda lime and Drierite) and sent through an oxygen analyzer (Sable Systems Oxzilla; Las Vegas, NV, USA). Oxygen concentration, temperature, and flow rate were measured every 5 s, and 3-min reference readings were taken every 42 min (Warthog LabHelper software; http://www.warthog.ucr.edu). Rates of oxygen consumption ($\dot{V}O_2$) were computed in Warthog LabAnalyst using the Mode 1 equation. RMR was computed as the lowest 10-min average $\dot{V}O_2$ (ml O_2/h) during the 8-h period.

Basal metabolic rate (BMR)

BMR was measured in Experiment 2 on test day 1 (8:30–16:30 hours). The procedure for measuring BMR was identical to the method for obtaining RMR, except that food was removed 8 h before testing began.

Forced-exercise maximal oxygen consumption ($\dot{V}O_2$ max)

$\dot{V}O_2$max was measured in Experiment 1 (test days 5 and 6; 10:00–11:30 hours) and in Experiment 2 (test days 4 and 5; 11:30–13:30 hours), using a running-wheel respirometer (circumference: 51.8 cm; effective volume: 900 mL) as previously described (Dlugosz et al. 2012; Andrew et al. 2016). Air flow through the wheel was 2400 mL/min, and ambient temperature was 22.0 ± 0.2 °C (mean ± SE). Excurrent air was subsampled (~150 mL/min) and dried with soda lime and Drierite prior to oxygen measurements (Applied Electrochemistry S-3A; Sunnyvale, CA, USA). Mice were weighed, placed in the wheel, and given ~2 min to acclimate. We then started rotation and gradually increased rotation speed approximately every 30 s until either oxygen concentration did not change or mice could no longer maintain position. Flow rate and O_2 concentration were measured every second using LabHelper. Reference air was taken at the beginning and end of trials, and a baseline was computed by linear regression. We calculated $\dot{V}O_2$ in LabAnalyst using the Mode 1 equation, and computed $\dot{V}O_2$ max as the highest $\dot{V}O_2$ averaged over 1 min (ml O_2/h). We measured $\dot{V}O_2$ max on each of the two test days to assess repeatability, and the higher of the two values for each animal was used for further statistical analyses.

Maximal thermogenic capacity

The maximal $\dot{V}O_2$ during thermogenesis (summit metabolism; $\dot{V}O_2$sum) was only measured in Experiment 2 on test day 7 (11:00–13:00 h), using acute cold exposure in heliox (21% O_2: 79% He by volume) to quickly elicit maximal thermogenic capacity without use of dangerously low temperatures and attendant risk of frostbite injury (Rosenmann and Morrison 1974; Chappell et al. 2003). Males were separated from their cage mates, weighed, and placed in a Plexiglas metabolic chamber (volume 850 mL) with a small amount bedding, inside an environmental cabinet. Excurrent air was subsampled (~150 mL/min) and dried with soda lime and Drierite prior to oxygen analysis (Applied Electrochemistry S-3A). Reference air was taken at the beginning and end of trials, and a baseline was computed by linear regression. Flow rates (1700 mL/min), temperature, and O_2 concentrations were measured every second using LabHelper. Mice

were placed in the chamber at a moderately low temperature (0 to − 5 °C), after which temperature was reduced by ~ 1 °C/min until it stabilized or declined despite decreasing ambient temperature. At this point, mice were removed from the chamber and a temperature probe was inserted into the anus to determine the final body temperature. We computed $\dot{V}O_2$ in LabAnalyst using the mode 1 equation, and $\dot{V}O_2$sum was determined as the highest $\dot{V}O_2$ averaged over 1 min (ml O_2/h). We did not test for $\dot{V}O_2$sum in Experiment 1 because the duration of cold exposure was variable and insufficient to achieve stable cold acclimation responses (Rezende et al. 2004b).

Grip strength

Grip strength was tested in Experiment 1 on test days 2 and 3 (9:30–11:00 hours). Mice were suspended by their tail over a horizontal wire-mesh surface (0.25-in. grid) attached to a force gauge (HF-10N, M&A Instruments Inc., Arcadia, CA, USA). The mouse was lowered until both the forelimbs and hindlimbs were touching the mesh without pulling on the force gauge. Once the mouse had relaxed on the mesh, the end of its tail was gently pulled horizontally until it released its grip (Meyer et al. 1979; Maurissen et al. 2003). Peak force value was recorded and the test was repeated once. The higher value from each day was used to assess repeatability, and the higher of the two values was used for analysis comparing groups.

Sprint speed

Maximum sprint speed (Djawdan and Garland 1988) was measured in Experiment 1 on test days 5 and 6 (14:00–15:30 h) using a 'racetrack' (8 m long by 10 cm wide, with 30 cm high walls) equipped with 12 sets of aligned photocells at 50-cm intervals (Andrew et al. 2016). A mouse was placed near the start of the track and encouraged to walk or run down the track 2–4 times for familiarization at the start of each test. The male was then returned to the starting area, the photocells were activated, and the mouse was chased down the track with a padded strip of plastic (~ 10 cm wide by ~ 30 cm long), tripping successive photocells as it ran. Sprint speed was measured 5 times on each of the 2 days, yielding a total of 10 trials per individual; the fastest 1.0-m interval on each day was recorded. Trials were scored subjectively as 'poor', 'fair', 'okay', 'good' or 'excellent' depending on mouse cooperation. Trials with scores of poor or fair were excluded from analysis. The highest values from each day were used to determine repeatability, and the single highest value for each individual was used as its maximum sprint speed.

Euthanasia and organ collection

On the final day of testing in Experiment 2 (day 8; 12:30–14:30 hours), males were anesthetized with isoflurane and euthanized by CO_2 inhalation. Morphometric measurements were taken [body length, head length, head width, right foot length (tip of phalanges to tibia/fibula), and baculum length]. Organs [brain, all subcutaneous fat (white adipose only), heart ventricles, lungs, spleen, pancreas, liver, stomach (emptied), small/large intestines (emptied), caecum (emptied), adrenals (left and right), kidneys (left and right), testes (left and right)], and muscles (left thigh and left gastrocnemius) were then removed, blot dried, and weighed.

Statistical analysis

In both experiments, we examined repeatability for all measures taken on two trials or two paired organs, using Pearson's correlations and paired *t* tests. We used single values (e.g., mean or maximum) for comparisons of group means for these measures. Repeatability analysis was conducted in the same manner as in previous studies (Andrew et al. 2016; Table S1). We also computed multiple linear regressions of the performance measures (BMR, $\dot{V}O_2$max, and $\dot{V}O_2$sum) for Experiment 2 on relevant organ masses and hematocrit (Table S2). For all measures, we used analysis of covariance (ANCOVA) in SPSS 24.0 (see below for covariates used) to compare traits among groups (Experiment 1: VM5, VM10, VM22; Experiment 2: VM22, VM10, NB10, BM10). Data were $\log_{10}$- or rank-transformed prior to analysis where appropriate (noted on Tables 2, 3); results are presented in untransformed units (as estimated marginal mean ± standard error unless otherwise noted).

For Experiment 1, male age and testing cohort differed among the three groups (both $P < 0.001$), whereas the number of days between relocation to new housing and testing did not ($P = 0.318$). All three variables were used as covariates in analysis. For Experiment 2, age, testing cohort, and days between relocation to new housing and testing differed significantly among the four groups (all $P < 0.006$), but for reasons explained below, were not used as covariates in analysis. We also used body mass, body length, and cricket mass as covariates where appropriate (denoted in Tables 2, 3). We performed the overall *F* test for group differences (Tables S3 and S4) and a priori contrasts among all of the groups for Experiments 1 and 2. We discuss only the a priori contrasts between groups.

Excluding nuisance variables such as age, cohort, and duration of acclimation, Experiment 1 generated 60 *P* values, 15 of which were < 0.05 (underlined values in Table 2), and Experiment 2 generated 258 *P* values, 61 of which were < 0.05 (underlined values in Table 3). These tests include a substantial amount of non-independence

Table 2 Experiment 1 results of a priori contrasts comparing virgin males housed at 5, 10, and 22 °C

Traits	Units	Trans.	Covar.	*P* of a priori contrasts			5 °C VM			10 °C VM			22 °C VM		
				5° *vs.* 10°	5° *vs.* 22°	10° *vs.* 22°	*N*	EMM	SE	*N*	EMM	SE	*N*	EMM	SE
Body mass (day 1)	g	None	NC	0.176	0.036	0.634	29	37.92	1.49	50	40.71	0.97	61	41.32	0.77
Body mass (day 4)	g	None	NC	0.325	0.102	0.634	28	38.41	1.52	49	40.47	0.99	61	41.10	0.77
Body mass (day 7)	g	None	NC	0.181	0.024	0.461	26	37.38	1.52	49	40.15	0.96	61	41.09	0.75
Fat mass (day 1)	g	None	NC	0.041	0.055	0.374	29	5.50	0.95	49	8.22	0.62	61	7.49	0.49
Percent fat mass (day 1)	%	None	NC	0.034	0.169	0.098	29	14.38	1.84	48	19.83	1.21	60	17.13	0.96
Fat mass (day 7)	g	Log_{10}	NC	0.175	0.101	0.919	26	5.49	0.90	50	7.25	0.57	61	7.46	0.45
Percent fat mass (day 7)	%	None	NC	0.162	0.109	0.823	26	14.38	1.70	49	17.63	1.08	61	17.31	0.85
Lean mass (day 1)	g	None	NC	0.876	0.146	0.040	29	29.91	0.79	51	29.74	0.51	60	31.15	0.41
Percent lean mass (day 1)	%	None	NC	0.008	0.073	0.043	29	79.66	1.73	49	73.25	1.13	59	76.29	0.89
Lean mass (day 7)	g	None	NC	0.972	0.253	0.125	26	29.87	0.85	49	29.83	0.54	60	30.91	0.42
Percent lean mass (day 7)	%	None	NC	0.033	0.039	0.400	26	79.84	1.64	49	75.02	1.04	60	76.17	0.81
Resting metabolic rate	ml O_2/h	None	B	**0.002**	$\mathbf{3.00 \times 10^{-6}}$	0.317	19	1.74	0.32	34	1.48	0.25	40	1.12	0.20
Hematocrit (day 1)	%	None	NC	0.071	0.785	0.010	27	47.94	0.66	49	49.59	0.42	60	48.13	0.33
Hematocrit (day 7)	%	None	NC	0.265	0.079	0.671	26	47.28	0.70	45	46.20	0.46	61	45.94	0.35
Exercise VO_2max	ml O_2/h	None	B	0.316	**0.001**	**0.004**	27	6.28	0.14	50	6.09	0.09	61	5.76	0.07
Maximum sprint speed	m/s	None	NC	0.874	0.125	0.089	27	1.61	0.14	50	1.64	0.09	61	1.85	0.07
Maximum sprint speed	m/s	None	B	0.614	0.041	0.068	27	1.56	0.14	50	1.65	0.09	61	1.87	0.07
Maximum grip strength	N	None	B	0.401	0.090	0.415	29	4.43	0.20	50	4.67	0.13	61	4.81	0.10
Predatory aggression: latency to first attack cricket	s	None	C	0.279	0.393	0.502	27	14.79	6.33	50	24.21	4.01	58	20.66	3.19
Predatory aggression: latency to kill cricket	s	Rank	C	0.791	0.380	0.142	25	63.88	8.59	40	69.96	5.66	49	55.92	4.48

Units, transformation, covariates, a priori contrasts, sample sizes (*N*), untransformed estimated marginal means (EMM), and associated standard errors (SE) from 1-way ANCOVAs are reported. Cohort, age, and duration of acclimation were used as covariates in all analysis

Significant *P* values ($P \leq 0.004$, when modified for adaptive false discovery rate) are both bold and underlined. Nominally significant *P* values ($0.004 \leq P \leq 0.05$) are underlined but not bold

NC no covariate, *B* body mass, *C* cricket mass

Table 3 Experiment 2 results of a priori contrasts comparing 22 °C virgin (VM), 10 °C virgin, 10 °C non-breeding (NB), and 10 °C breeding males (BM)

Traits	Units	Trans.	Covar.	*P* of a priori contrasts						10 °C BM			10 °C NB			10 °C VM			22 °C VM		
				10 °C BM *vs.* 22 °C VM	10 °C NB *vs.* 22 °C VM	10 °C VM *vs.* 22 °C VM	10 °C BM *vs.* 10 °C NB	10 °C BM *vs.* 10 °C VM	10 °C NB *vs.* 10 °C VM	*N*	EMM	SE	*N*	EMM	SE	*N*	EMM	SE	*N*	EMM	SE
Body mass (day 1)	g	None	NC	0.165	0.849	0.142	0.102	0.519	0.096	4	41.00	4.45	6	50.70	3.64	12	44.36	2.57	12	49.85	2.57
Body mass (day 1)	g	None	L	0.344	**0.015**	0.082	0.099	0.295	0.536	4	45.20	3.63	6	52.96	2.91	12	49.52	2.34	12	42.15	2.67
Body mass (day 4)	g	None	NC	0.098	0.429	0.275	0.085	0.612	0.204	4	42.66	4.67	6	53.40	3.81	12	45.42	2.69	12	49.66	2.69
Body mass (day 4)	g	None	L	0.190	**0.001**	**0.010**	0.061	0.325	0.149	4	47.57	3.48	6	56.04	2.78	12	51.46	2.24	12	40.66	2.56
Body mass (day 8)	g	None	NC	0.159	0.422	0.446	0.101	0.527	0.245	4	42.87	4.67	6	53.06	3.81	12	46.31	2.69	12	49.26	2.69
Body mass (day 8)	g	None	L	0.335	**0.001**	**0.005**	0.082	0.255	0.131	4	47.71	3.52	6	55.66	2.82	12	52.27	2.27	12	40.38	2.59
Fat mass	g	None	NC	0.377	0.580	0.683	0.194	0.484	0.325	4	6.83	1.98	6	10.23	1.62	12	8.45	1.14	12	9.12	1.14
Fat mass	g	None	L	0.753	**0.007**	**0.012**	0.223	0.274	0.198	4	8.66	1.64	6	11.21	1.31	12	10.70	1.06	12	5.77	1.21
Percent fat mass	%	None	NC	0.762	0.858	0.879	0.296	0.362	0.309	4	14.75	2.70	6	18.46	2.21	12	17.64	1.56	12	17.98	1.56
Percent fat mass	%	None	L	0.802	0.064	0.037	0.374	0.227	0.427	4	16.82	2.44	6	19.58	1.96	12	20.18	1.57	12	14.18	1.80
Lean mass	g	Log_{10}	NC	0.108	0.512	0.233	0.096	0.629	0.188	4	33.38	2.67	6	39.40	2.18	12	34.74	1.54	12	37.27	1.54
Lean mass	g	Log_{10}	L	0.218	**0.002**	0.026	0.083	0.371	0.237	4	35.98	2.13	6	40.79	1.70	12	37.93	1.37	12	32.51	1.57
Percent lean mass	%	Log_{10}	NC	0.377	0.589	0.671	0.191	0.478	0.315	4	79.11	2.70	6	74.35	2.21	12	76.82	1.56	12	75.86	1.56
Percent lean mass	%	Log_{10}	L	0.725	**0.015**	0.025	0.227	0.292	0.281	4	76.80	2.33	6	73.11	1.87	12	73.98	1.50	12	80.09	1.72
Basal metabolic rate	ml O_2/h	None	B	$\mathbf{3.00\times10^{-6}}$	$\mathbf{1.00\times10^{-6}}$	0.764	**0.003**	$\mathbf{1.34\times10^{-7}}$	$\mathbf{9.40\times10^{-8}}$	2	1.62	0.03	4	1.47	0.02	6	1.21	0.02	6	1.21	0.02
Exercise VO_2max	ml O_2/h	None	B	**0.018**	0.324	0.062	0.781	**0.014**	0.249	4	6.27	0.33	6	6.39	0.27	12	7.23	0.19	12	6.72	0.19
VO_2sum	ml O_2/h	None	B	0.088	0.681	**0.011**	0.555	0.389	0.308	4	7.37	0.37	6	7.08	0.30	12	7.74	0.21	12	6.93	0.21
Hematocrit	%	Log_{10}	NC	0.510	0.850	0.564	0.821	0.411	0.677	4	46.33	1.45	6	45.83	1.18	12	44.96	0.84	12	45.54	0.84
Body length	mm	None	NC	0.311	**0.001**	$\mathbf{5.00\times10^{-6}}$	0.598	0.769	**0.001**	4	102.88	2.25	6	104.43	1.84	12	102.11	1.30	12	112.37	1.30
	mm	Log_{10}	NC	0.051	0.073	0.835	0.595	0.257	0.322	4	34.41	0.99	6	35.16	0.81	12	33.15	0.57	12	33.27	0.57
	mm	Log_{10}	L	0.034	0.353	0.338	0.520	0.231	0.842	4	34.09	1.02	6	34.99	0.81	12	32.76	0.66	12	33.85	0.75
Head length	mm	Log_{10}	NC	**0.004**	0.046	0.222	0.217	0.212	0.698	4	15.75	0.60	6	14.82	0.49	12	16.67	0.35	12	16.02	0.35
Head length	mm	Log_{10}	L	**0.002**	0.405	0.037	0.151	0.168	0.519	4	16.03	0.60	6	14.97	0.48	12	17.01	0.39	12	15.51	0.44
Right foot length	mm	None	NC	**0.002**	**0.007**	0.509	0.969	**0.005**	**0.015**	4	24.61	0.54	6	24.58	0.44	12	22.70	0.31	12	22.99	0.31
Right foot length	mm	None	L	**0.003**	**0.001**	0.360	0.817	**0.004**	**0.002**	4	24.89	0.54	6	24.73	0.43	12	23.04	0.35	12	22.48	0.40
Baculum length	mm	None	NC	**0.015**	0.160	0.168	0.724	**0.013**	0.110	4	15.17	0.47	6	14.95	0.38	12	13.73	0.27	12	14.28	0.27

Table 3 (continued)

Traits	Units	Trans.	Covar.	*P* of a priori contrasts						10 °C BM			10 °C NB			10 °C VM			22 °C VM		
				10 °C BM *vs.* 22 °C VM	10 °C NB *vs.* 22 °C VM	10 °C VM *vs.* 22 °C VM	10 °C BM *vs.* 10 °C NB	10 °C BM *vs.* 10 °C VM	10 °C NB *vs.* 10 °C VM	*N*	EMM	SE	*N*	EMM	SE	*N*	EMM	SE	*N*	EMM	SE
Baculum length	mm	None	L	0.027	0.057	0.985	0.625	**0.014**	0.039	4	15.34	0.48	6	15.05	0.39	12	13.95	0.31	12	13.96	0.35
Brain mass	g	None	B	0.337	0.795	0.133	0.417	0.930	0.253	4	0.838	0.027	6	0.867	0.022	12	0.841	0.015	12	0.874	0.015
Subcutaneous fat mass	g	Log_{10}	B	0.247	0.405	0.659	0.488	0.072	0.138	4	2.700	0.508	6	2.524	0.417	12	3.553	0.289	12	3.129	0.288
Heart mass	g	None	B	0.519	**0.002**	$\mathbf{2.40\times10^{-5}}$	0.054	**0.007**	0.532	4	0.166	0.006	6	0.183	0.005	12	0.188	0.004	12	0.162	0.004
Lung mass	g	Log_{10}	B	0.176	0.166	0.997	0.663	0.091	0.096	4	0.323	0.026	6	0.301	0.022	12	0.265	0.015	12	0.268	0.015
Liver mass	g	Log_{10}	B	0.198	0.185	0.989	0.322	0.999	0.992	4	2.838	0.388	6	2.391	0.319	12	2.651	0.221	12	2.662	0.220
Spleen mass	g	Log_{10}	B	0.751	0.703	0.943	0.748	0.927	0.968	4	0.087	0.012	6	0.075	0.010	12	0.079	0.007	12	0.079	0.007
Pancreas mass	g	None	B	0.417	0.212	0.599	0.486	0.930	0.778	4	0.171	0.025	6	0.194	0.020	12	0.173	0.014	12	0.163	0.014
Kidney mass	g	None	B	0.057	0.120	$\mathbf{1.15\times10^{-4}}$	0.845	0.135	0.121	4	0.362	0.032	6	0.353	0.027	12	0.418	0.018	12	0.302	0.018
Adrenal mass	g	Log_{10}	B	0.173	0.826	0.052	0.042	0.230	**0.014**	4	0.005	0.002	6	0.011	0.002	12	0.008	0.001	12	0.011	0.001
Stomach mass	g	Log_{10}	B	0.701	**0.014**	**0.011**	0.566	0.744	0.128	4	0.722	0.037	6	0.746	0.031	12	0.722	0.021	12	0.643	0.021
Small + large intestine mass	g	Log_{10}	B	0.419	**0.002**	$\mathbf{1.50\times10^{-5}}$	0.756	0.719	**0.003**	4	1.676	0.119	6	1.562	0.098	12	1.682	0.068	12	1.145	0.067
Caecum mass	g	Log_{10}	B	0.049	0.061	0.854	0.937	0.094	0.126	4	0.705	0.123	6	0.754	0.101	12	0.518	0.070	12	0.582	0.070
Testis mass	g	Log_{10}	B	0.296	0.963	0.175	0.601	0.129	0.576	4	0.269	0.037	6	0.250	0.030	12	0.215	0.021	12	0.253	0.021
Baculum mass	g	Log_{10}	B	**0.001**	0.038	0.073	0.588	**0.015**	0.226	4	0.011	0.001	6	0.013	0.001	12	0.008	0.001	12	0.010	0.001
Left thigh muscle mass	g	Log_{10}	B	0.839	0.069	0.049	0.224	0.114	**0.005**	4	0.902	0.073	6	0.996	0.060	12	1.007	0.041	12	1.139	0.041
Left gastrocnemius mass	g	Log_{10}	B	0.150	$\mathbf{3.63\times10^{-4}}$	**0.004**	0.616	0.067	$\mathbf{3.65\times10^{-4}}$	4	0.220	0.028	6	0.236	0.023	12	0.272	0.016	12	0.346	0.016

Units, transformation, covariates, a priori contrasts, sample sizes (*N*), untransformed estimated marginal means (EMM), and associated standard errors (SE) from 1-way ANCOVAs are reported. Cohort, age, and duration of acclimation were not used as covariates in analysis

Significant *P* values ($P \leq 0.018$, when modified for false discovery rate) are both bold and underlined. Nominally significant *P* values ($0.018 \leq P \leq 0.05$) are underlined but not bold

NC no covariate, *B* body mass, *L* body length

because the same individuals were measured for all traits, some traits were correlated, and many were interrelated. Numerous methods to compensate for non-independence in multiple related tests, and hence control the number of false positives, are available. No single procedure performs best in all situations, and indeed multiple types of error rates can be defined, where "each of them might be appropriate and useful for some inferential situation" (Benjamini 2010). Moreover, some workers argue that such corrections are often unnecessary and undesirable. Given such controversies, we computed both the false discovery rate (FDR) and adaptive false discovery rate procedures in PROC MULTTEST in SAS 9.4 (SAS Inc., Cary, NC, USA). For Experiment 1, the smallest four values would have adjusted P values < 0.05 (0.034 being the highest) using the adaptive FDR procedure, while no values would have adjusted P values < 0.05 using the FDR procedure. We used the adaptive FDR procedure for Experiment 1. For simplicity, all P values are reported in the text and tables as raw values, not adjusted for multiple comparisons; however, we refer to P values ≤ 0.034 as "significant" (bold and underlined in Table 2) and those between 0.034 and 0.05 as "nominally significant" (underlined but not bold in Table 2). For Experiment 2, the smallest 34 values would have adjusted P values < 0.05 (0.007 as the highest) using the adaptive FDR procedure, whereas the smallest 49 values would have adjusted P values < 0.05 (0.018 as the highest) using the FDR procedure. We used the FDR procedure for Experiment 2. Similar to Experiment 1, we refer to P values ≤ 0.018 as "significant" (underlined in Table 3) and those between 0.018 and 0.05 as "nominally significant" (bolded and underlined in Table 3).

Results

Experiment 1: short-term cold acclimation

In Experiment 1, mortality did not differ significantly among groups. Twenty-nine of 42 (69.0%) virgin males acclimating to 5 °C survived to the last day of testing compared to 50 of 70 (71.4%) virgin males acclimating to 10 °C and 61 of 72 (84.7%) virgin males housed at 22 °C ($\chi^2 = 4.9$, $P = 0.085$). Deaths occurred either during testing procedures ($N = 3$ VM5, 6 VM10, 5 VM22) or of unknown causes under undisturbed conditions in the home cage (10 VM5, 14 VM10, 6 VM22).

With body mass as a covariate, RMR was significantly higher in 5 °C males than in males housed at higher temperatures (VM10: $P = 0.002$, VM22: $P = 3.00 \times 10^{-6}$; Table 2). Forced-exercise $\dot{V}O_2$max (with body mass as a covariate) was slightly higher (by 6–9%) for VM5 ($P = 0.001$) and VM10 ($P = 0.004$) than for VM22. No other measures (i.e., body mass, fat mass, lean muscle mass, organ masses, hematocrit, predatory aggression, sprint speed) differed significantly between virgin males housed at the three temperatures (Table 2).

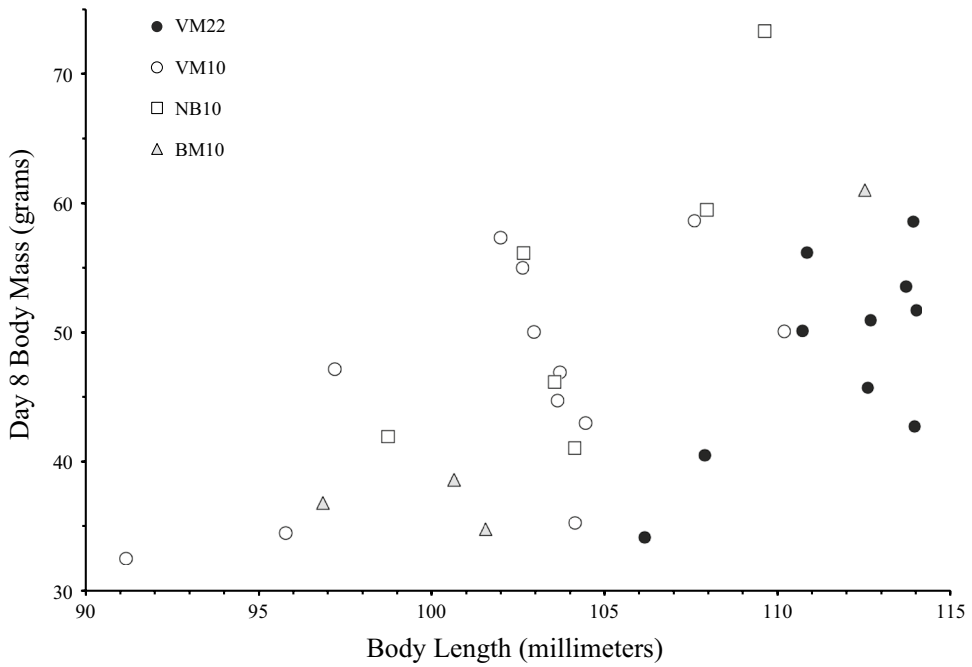

Fig. 2 Body length versus day 8 body mass of mice in Experiment 2 on the y-axis. Closed circles—virgin males (VM22) housed at 22 °C ($n = 12$), open circles—VM housed at 10 °C (VM10, $n = 12$), open squares—non-breeding males (NB10) housed at 10 °C ($n = 6$), grey triangles—breeding males (BM10) housed at 10 °C BM ($n = 4$). Body length was lower in NB10 ($P = 0.001$) and VM10 ($P = 5 \times 10^{-6}$) than in VM22 and higher in NB10 than in VM10 ($P = 0.001$)

Experiment 2: long-term cold acclimation

Mortality differed among groups in Experiment 2 ($\chi^2 = 8.8$, $P = 0.031$). Survival rates of males to the final day of testing were 85.7% (12 of 14) for VM22, 85.7% (12 of 14) for VM10, 42.9% (6 of 14) for NB10, and 57.1% (8 of 14) for BM10. Again, deaths occurred either during testing ($N = 2$ VM22, 1 VM10, 2 NB, 1 BM) or in the home cage for unknown reasons ($N = 0$ VM22, 1 VM10, 6 NB, 5 BM). Of the 8 surviving breeding pairs, two did not produce offspring; thus, the survival rate for pairs that bred was 42.9% (6 of 14). When these latter two pairs were removed from analysis, the difference in survival rate among groups was even more highly significant ($\chi^2 = 11.2$, $P = 0.011$).

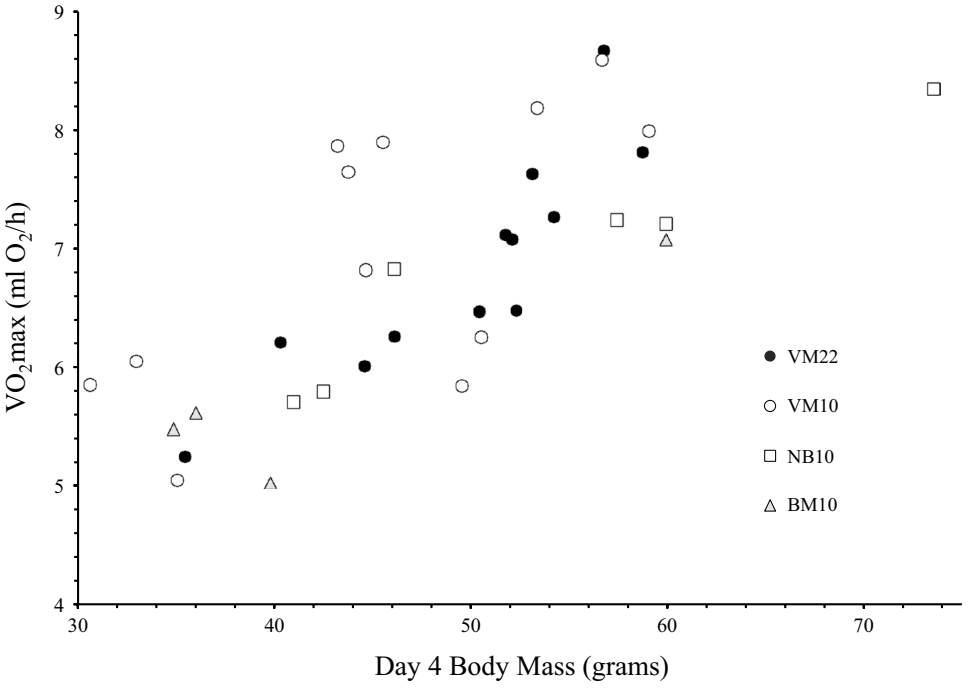

Fig. 3 $\dot{V}O_2$max versus day 4 body mass of mice in Experiment 2. Closed circles—virgin males (VM22) housed at 22 °C ($n = 12$), open circles—VM housed at 10 °C (VM10, $n = 12$), open squares—non-breeding males (NB10) housed at 10 °C ($n = 6$), grey triangles—breeding males (BM10) housed at 10 °C BM ($n = 4$). $\dot{V}O_2$ max was 7% lower in BM10 compared to VM22 ($P = 0.018$) and 15% lower in BM10 than in VM10 ($P = 0.014$)

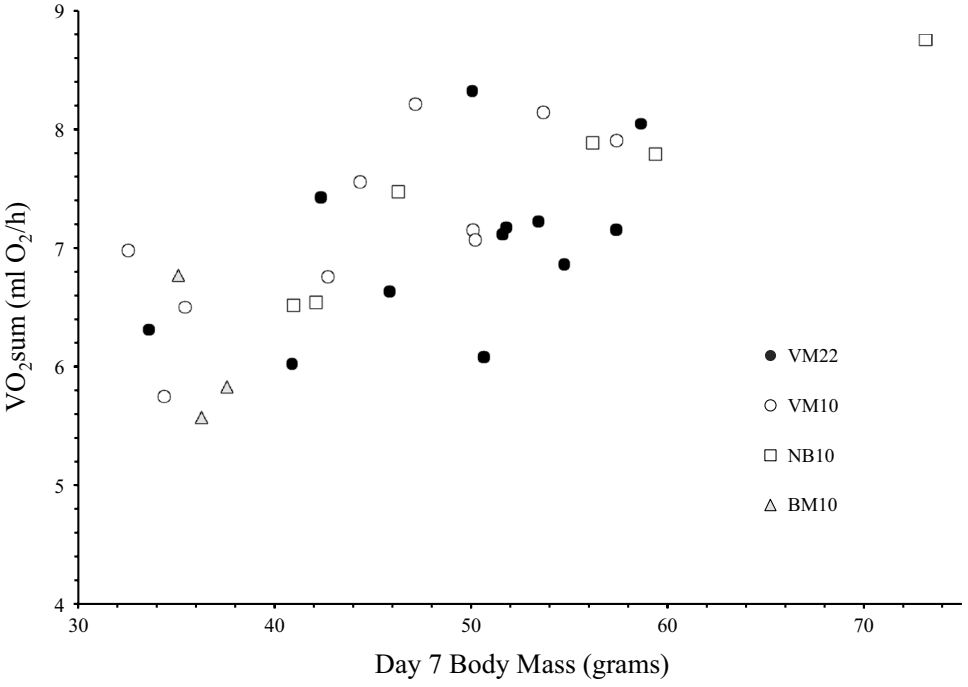

Fig. 4 $\dot{V}O_2$sum versus day 7 body mass for mice in Experiment 2. Closed circles—virgin males (VM22) housed at 22 °C ($n = 12$), open circles—VM housed at 10 °C (VM10, $n = 12$), open squares—non-breeding males (NB10) housed at 10 °C ($n = 6$), grey triangles—breeding males (BM10) housed at 10 °C BM ($n = 4$). $\dot{V}O_2$sum was 12% higher in VM10 than VM22 ($P = 0.011$)

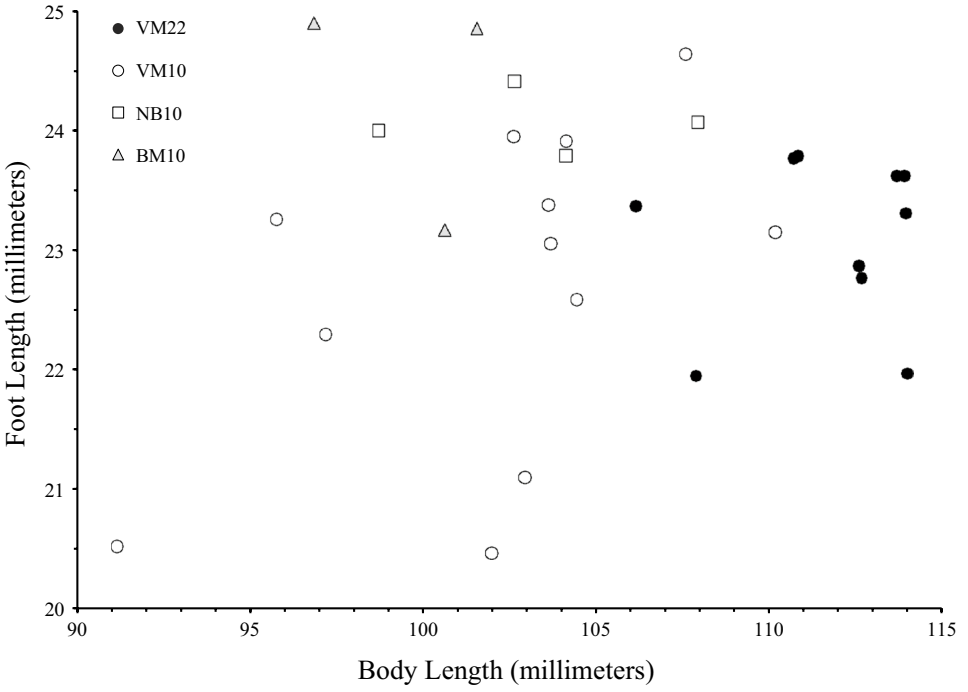

Fig. 5 Foot length versus body length of mice in Experiment 2. Closed circles—virgin males (VM22) housed at 22 °C ($n=12$), open circles—VM housed at 10 °C (VM10, $n=12$), open squares—non-breeding males (NB10) housed at 10 °C ($n=6$), grey triangles—breeding males (BM10) housed at 10 °C BM ($n=4$). Both BM10 (both $P \leq 0.005$) and NB10 (both $P \leq 0.015$) had longer feet than VM22 or VM10, regardless of whether body length was used as a covariate

Long-term acclimation to 10 °C

To determine possible interactions of reproductive/mating status and cold acclimation, we compared each group of males housed at 10 °C (BM10, NB10, VM10) with the control group of VM22. Body length, measured at euthanasia, was lower in NB10 ($P=0.001$) and VM10 ($P=5\times10^{-6}$) than in VM22 (Fig. 2; Table 3). Body mass did not differ significantly between VM22 controls and any of the three groups housed at 10 °C on test days 1, 4 or 8, but when body length was used as a covariate, NB10 were heavier than VM22 on all three test days ($P=0.015$, $P \leq 0.001$, and $P \leq 0.001$, respectively) and had higher lean mass ($P=0.002$). With body length as a covariate, both NB10

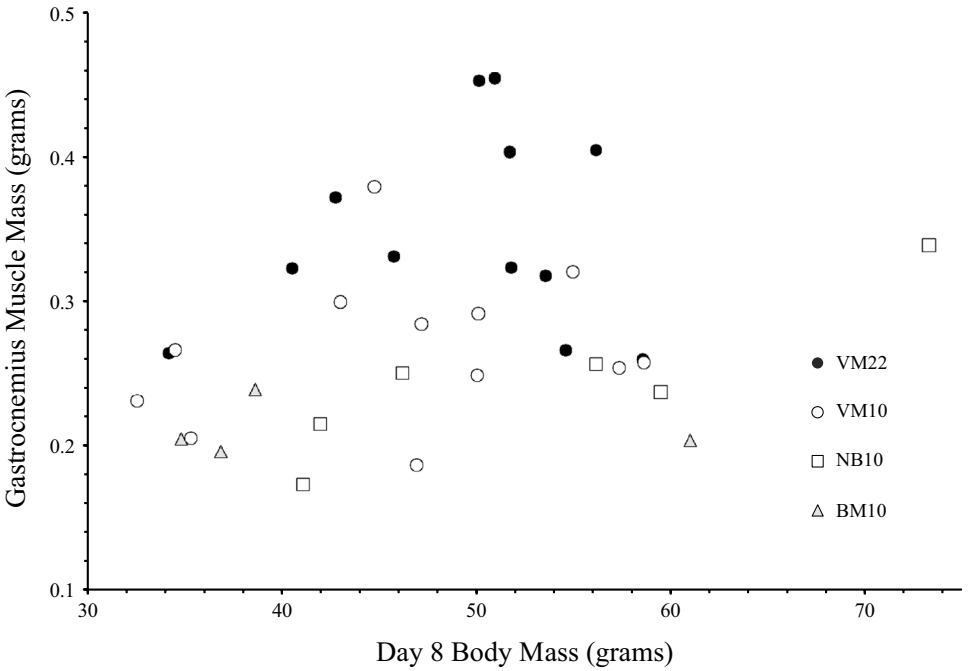

Fig. 6 Gastrocnemius muscle mass versus body mass of mice in Experiment 2. Closed circles—virgin males (VM22) housed at 22 °C ($n=12$), open circles—VM housed at 10 °C (VM10, $n=12$), open squares—non-breeding males (NB10) housed at 10 °C ($n=6$), grey triangles—breeding males (BM10) housed at 10 °C BM ($n=4$). Gastrocnemius muscle mass was lower in both NB10 ($P=0.004$) and VM10 ($P=3.63\times10^{-4}$) compared to VM22 and VM10 had larger gastrocnemius muscles than NB10 ($P=3.65\times10^{-4}$)

($P=0.007$) and VM10 ($P=0.012$) had higher fat mass than VM22 (day 6).

BMR was substantially higher (21–34%) in BM10 ($P=3.00\times10^{-6}$) and NB10 ($P=1.00\times10^{-6}$) than in VM22 (Table 3). $\dot{V}O_2$max was slightly lower (by 7%) in BM10 compared to VM22 ($P=0.018$; Fig. 3), whereas $\dot{V}O_2$sum was 12% higher in VM10 than VM22 ($P=0.011$; Fig. 4).

At the end of Experiment 2, several morphometric variables differed among groups (Table 3). Log_{10}-transformed head width was lower in BM10 ($P=0.004$) than in VM22. When body length was used as a covariate, log_{10}-transformed head width was relatively higher in BM10 ($P=0.002$) compared to VM22. Foot length followed a similar pattern, regardless of whether body length was used as a covariate: both BM10 (both $P\leq0.003$) and NB10 (both $P\leq0.007$) had longer feet than VM22 (Fig. 5). Finally, baculum length (without body length as a covariate) was significantly higher in BM10 than in VM22 ($P=0.015$).

Organ masses (log-transformed in some analyses) were compared among groups by ANCOVA with log-transformed body mass as a covariate (Table 3). Ventricles were heavier in NB10 ($P=0.002$) and VM10 ($P=2.40\times10^{-5}$) than in VM22, and kidneys were heavier in VM10 than VM22 ($P=1.15\times10^{-4}$). Both stomach and intestine masses were higher in NB10 ($P=0.014$, $P=0.002$, respectively) and VM10 ($P=0.011$, $P=1.50\times10^{-5}$) compared to VM22. Baculum mass was higher in BM10 ($P=0.001$) compared to VM22. Finally, gastrocnemius muscle mass was lower in both NB10 ($P=0.004$) and VM10 ($P=3.63\times10^{-4}$) compared to VM22 (Fig. 6). No other organ masses were affected by long-term cold acclimation.

Effects of reproductive condition at 10 °C

We compared breeding, non-breeding, and virgin males, all housed at 10 °C, to examine possible interactions between reproductive condition and long-term cold acclimation. Breeding males showed several differences from non-breeding and/or virgin males. BMR (with body mass as a covariate) was higher (by 10–34%) in BM10 than in both NB10 ($P=0.003$) and VM10 ($P=1.34\times10^{-7}$), but $\dot{V}O_2$max (with body mass as a covariate) was 15% lower in BM10 than in VM10 ($P=0.014$; Fig. 3). Foot length (with or without body length as a covariate) was higher in BM10 than in VM10 (both $P\leq0.005$; Fig. 5), as was baculum length BM10 (without body length as a covariate: $P=0.013$; with body length as a covariate: $P=0.014$). Ventricle mass (body mass as a covariate) was lower in BM10 than in VM10 ($P=0.007$).

Several traits differed between non-breeding males and virgin males. BMR (with body mass as a covariate) was 13% higher in NB10 than in VM10 ($P=9.40\times10^{-8}$). Body length ($P=0.001$; Fig. 2; Table 3), foot length (with or without body length as a covariate; all $P\leq0.015$; Fig. 5), baculum length (with body mass as a covariate; $P=0.005$), adrenal mass (with body mass as a covariate; $P=0.014$), intestine mass (with body mass as a covariate; $P=0.003$), and baculum length (with body mass as a covariate; $P=0.001$) were all higher in NB10 than in VM10. However, VM10 had larger thigh muscles (with body mass as a covariate; $P=0.005$), and gastrocnemius muscles than NB10 ($P=3.65\times10^{-4}$; Fig. 6). There were no differences among BM10, NB10, and VM10 in head length or in brain, fat, lung, liver, spleen, pancreas, adrenal, caecum, or testis masses.

Discussion

Previous work on captive California mice indicated that the intensive and sustained paternal care provided by fathers had quite minor impacts on their energy metabolism, performance, and morphology (Saltzman et al. 2015; Andrew et al. 2016; Zhao et al. 2017, 2018). However, these small effects may have been a function of a benign laboratory environment that did not reflect the more demanding conditions faced by wild California mice, particularly because the species routinely reproduces in winter, when (presumably) thermoregulatory costs may be substantial (Ribble 1992). Accordingly, a primary goal in the present study was to examine how more ecologically realistic thermal environments affect the physiology of breeding and non-breeding males. We expected that cold acclimation would alter energy metabolism in California mice, as occurs in numerous other small mammals (specifically, enhanced thermogenic capacity, elevated thermoneutral RMR and BMR, and changes in body composition). We also predicted that a male's reproductive status would affect his acclimatory plasticity, such that cold acclimation would impact fathers—with the burden of large investments in offspring care—differently from non-fathers in terms of morphology, behavior, and energy metabolism. Although our results supported some of these predictions, the effects of fatherhood were inconsistent, and we found substantial quantitative differences in several aspects of cold acclimation between California mice and other small mammals, including two closely related *Peromyscus* species. Additionally, the low sample size for breeding males (see "Methods" and "Results") reduced our statistical power to detect differences among groups. Nevertheless, the lack of large or numerous differences in the many variables measured suggests that parenthood does not have substantial impact on male physiology, morphology, or energy metabolism.

In most temperate or high-latitude small rodents, winter acclimatization or long-term laboratory acclimation to cold induces large increases in thermogenic capacity and cold tolerance (Hart 1971; Lynch 1973; Heldmaier et al. 1989;

Nespolo et al. 1999). For example, in freshly captured white-footed mice (*Peromyscus leucopus)* from Michigan (USA), winter animals acclimatized to sub-zero temperatures had 70% higher thermogenic capacity ($\dot{V}O_2$sum) than mice captured in summer (Wickler 1980). In free-living deer mice (*P. maniculatus)* from California, acclimatization to 6–9 °C winter temperatures increased $\dot{V}O_2$sum by 39% compared to summer values from the same wild population (Hayes 1989a), and laboratory acclimation to 3–5 °C increased $\dot{V}O_2$sum by 30–64% (Hayes and Chappell 1986, 1990; Rezende et al. 2004b; Chappell et al. 2007). Notably, both *P. maniculatus* and *P. leucopus* exhibited acclimatory increases in $\dot{V}O_2$sum that were much larger (by 2.5- to 5.8-fold) than the ca. 11–12% augmentation we found in male *P. californicus*. In addition to being unusually weak, the increase of $\dot{V}O_2$sum after prolonged cold exposure in *P. californicus* was independent of reproductive status (Table 3), contrary to our expectations that breeding and non-breeding males would differ in acclimatory responsiveness.

The striking contrast in cold acclimation among *Peromyscus* species might be explained in several ways. First, the 10 °C acclimation temperature we used for *P. californicus* is somewhat higher than temperatures used in other studies (typically ~ 3–5 °C; e.g., Rezende et al. 2004b, 2009). However, it was only slightly warmer than the 6–9 °C environmental temperatures that elicited a threefold-larger summer-to-winter $\dot{V}O_2$sum change in wild deer mice from California (Hayes 1989a). Second, California mice are large (~ 50 g) compared to deer mice and white-footed mice (20–25 g), and their size may make them more resistant to heat loss than their smaller congeners (due to a lower surface/volume ratio and possibly an increased ability to support thicker and more effective insulation). This may reduce selection for high thermogenic capacity. Third, unlike previous *Peromyscus* studies, we housed California mice in groups (pairs or families), and huddling may have reduced the requirement for thermogenesis as it does in other small mammals (Gilbert et al. 2010).

Finally, it is possible that winter temperatures in the natural range of *P. californicus* are not sufficiently cold to have selected for the evolution of strong acclimatory responses (see Hayes and O'Connor 1999). California mice inhabit fairly low altitudes (generally below 1600 m) in central and southern California and northwestern Baja California Norte (Hall 1981; Brylski and Harris 1990); the animals in our study descended from a wild population in the coastal Santa Monica mountains of southern California. Much of the range of *P. californicus*, especially near coastal areas, has a Mediterranean climate with mild winters (mean monthly minima of 5–10 °C, with temperatures rarely falling to slightly below 0 °C). These conditions are much less thermally demanding than the severe subzero winter temperatures routinely experienced by most populations of *P. maniculatus* and *P. leucopus,* which is consistent with these species' much stronger acclimatory response to cold. Similarly, the small acclimatory changes in thermogenic capacity (ca. 10%) found in the South American fossorial rodent *Spalacopus cyanus* were also attributed to life in an environment that does not expose the species to selection to withstand severe cold (Nespolo et al. 2001).

In addition to enhanced $\dot{V}O_2$sum, several other responses to long-term cold exposure are frequently observed in small mammals. Cold acclimation or acclimatization is accompanied by elevated resting or basal metabolic rate (RMR or BMR) in many rodents (Hart 1971; Derting and Austin 1998; Zub 2014), including *Peromyscus* species (Hayward 1965; Hill 1983). However, a study of wild *P. maniculatus* found no statistically significant difference in BMR between summer- and winter-acclimatized animals, despite large seasonal changes in $\dot{V}O_2$sum (Hayes 1989b), and a laboratory study of that species found little evidence that BMR was affected by cold acclimation (Russell and Chappell 2007). When it occurs, increased RMR or BMR following cold acclimation is usually interpreted as an energy cost necessitated by alterations to the suite of sub-organismal traits required to support the aerobic demands of higher thermogenic capacity (sensu Bennett and Ruben 1979). These include shifts in body and fat mass, especially hypertrophy of brown adipose tissue (Smith and Horwitz 1969; Konarzewski and Diamond 1994; Vaanholt et al. 2009), changes in sizes of visceral organs (heart, liver, kidneys, or digestive tract, individually or summed; Hammond and Kristan 2000; Russell and Chappell 2007; Rezende et al. 2009; Vaanholt et al. 2009; Zub 2014), altered musculoskeletal mass (Russell and Chappell 2007), and elevated hematocrit (Heldmaier et al. 1982; Deveci et al. 2001; Rezende et al. 2009). However, little consistency has been found in correlations between sub-organismal traits and either minimal or maximal aerobic metabolism (RMR or BMR, and $\dot{V}O_2$max or $\dot{V}O_2$sum, respectively), both interspecifically and intraspecifically (e.g., Russell and Chappell 2007; Dlugosz et al. 2012; Andrew et al. 2016). This suggests that no single organ or organ system is the primary 'driver' of (or limiting factor for) acclimatory shifts in aerobic metabolism.

Our findings for a large set of morphometric and physiological traits in male California mice parallel the interspecific findings: we found little consistency in responses to cold acclimation (Table 3) or among sub-organismal correlates of aerobic metabolism (Table S2). Contrary to our initial hypothesis, reproductive status (virgin, non-breeding, or breeding) was not a reliable predictor of the acclimatory responsiveness of aerobic physiology. For example, exercise capacity ($\dot{V}O_2$max) increased after cold acclimation in breeding males but not in virgin or nonbreeding males, whereas thermogenic capacity ($\dot{V}O_2$sum) increased after cold acclimation in virgin males, but not in breeding or

nonbreeding males (Table 3). BMR, often viewed as reflecting the overall metabolic demand on the organism or the minimal cost of living (e.g., McNab and Morrison 1963; Bennett and Ruben 1979; Ricklefs et al. 1996; Hulbert and Else 2004; Biro et al. 2018), did conform to predictions: following cold acclimation, it differed significantly among the three reproductive categories, being highest in breeding males and lowest in virgin males (Table 3). Moreover, BMR in breeding and non-breeding males responded to cold acclimation, but that of virgin males did not (Table 3). The findings for BMR are consistent with the expectation that the 'workload' of offspring care, particularly in a cold environment, places higher demands on fathers than on nonbreeding or virgin males.

Another expectation derived from the 'workload' concept is that the sizes of organs associated with supporting aerobic metabolism and energy processing (e.g., heart, liver, kidneys, digestive system) should correlate with energy use. Therefore, these organs should be larger in fathers than in non-breeding or virgin males, and they should be larger in males acclimated to 10 °C than in those acclimated to 22 °C (e.g., Hammond and Kristan 2000; Hammond and Wunder 1995). Neither of these expectations was strongly supported in our California mice (Tables 3, S2). We speculate that, at least in part, the absence of consistent organ-mass effects may be attributable to the small change in thermogenic capacity in *P. californicus* compared to most rodents.

In *P. maniculatus*, the large increase in $\dot{V}O_2$sum after cold acclimation is accompanied by a smaller but significant increase in exercise-induced $\dot{V}O_2$ max, possibly as a result of enhanced oxygen uptake in skeletal muscles as a side effect of the increased oxygen delivery to brown adipose tissue required for intense nonshivering thermogenesis (e.g., Hayes and Chappell 1986; Hayes and Chappell 1990). In *P. californicus*, $\dot{V}O_2$ max also changed following cold acclimation in some experimental groups, but results were inconsistent: $\dot{V}O_2$ max increased in the short-term experiment (Table 2), but in the long-term experiment it decreased in breeding males and was not affected in non-breeding and virgin males (Table 3). If, as previously suggested (Hayes and Chappell 1986, 1990), elevated $\dot{V}O_2$max following cold acclimation results from enhancements to oxygen delivery in support of increased thermogenic capacity, then, as speculated for organ sizes, the inconsistent findings for California mice may be a reflection of this species' relatively small acclimatory changes in $\dot{V}O_2$sum.

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