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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.

22 **Keywords:** Biparental care, cold acclimation, costs of reproduction, energetics, exercise
23 performance, fatherhood, morphology, paternal care, physiology, rodent

24 **1. Introduction**

25 The rodent genus *Peromyscus* (often called 'deer mice') includes ~56 species in North
26 and Central America (King, 1968; Hill, 1983; Bedford and Hoekstra, 2015). Although most
27 follow the standard mammalian reproductive pattern of exclusively maternal parental care, the
28 California mouse (*P. californicus)* is genetically monogamous and biparental, with pairs forming
29 lifelong bonds and males contributing extensively to offspring care (Gubernick and Alberts,
30 1987b; Ribble and Salvioni, 1990; Ribble, 1991). California mice produce multiple litters per
31 year, and fathers assist with parturition and exhibit all of the parental behaviors (huddling,
32 grooming, protection, and transportation of offspring) characteristic of mothers except lactation
33 (Dudley, 1974; Gubernick and Alberts, 1987a; Lee and Brown, 2002). Care by fathers increases
34 offspring survival in both field conditions (Gubernick and Teferi, 2000) and laboratory
35 environments involving stress (e.g., cold exposure or exercise; Gubernick et al., 1993; Cantoni
36 and Brown, 1997). Paternal care also has lasting impacts on offspring development, including
37 social, aggressive, and mating behaviors, neural and endocrine function, and cognitive ability
38 (Braun and Champagne, 2014; Bales and Saltzman, 2016).

39 How the intense and sustained investment in offspring affects male California mice is an
40 intriguing question. Although the physiological and endocrine correlates of reproduction in
41 female mammals have been studied intensively (e.g. Gittleman and Thompson, 1988; Hammond,
42 1997; Speakman, 2008), much less is known about the physiological impacts of parenthood on
43 fathers. In some biparental mammals, including California mice, becoming a father alters neural
44 circuitry and endocrinology (Saltzman and Ziegler, 2014; Bales and Saltzman, 2016), but effects
45 on energetics, performance, and morphology are less clear. In the biparental common marmoset
46 (*Callithrix jacchus*), cotton-top tamarin (*Saguinus oedipus*), and California mouse, expectant

47 fathers gain body mass during their mate's pregnancy, with subsequent mass loss after
48 parturition (Achenbach and Snowdon, 2002; Ziegler et al., 2006; Harris et al., 2011; Saltzman et
49 al., 2015). However, other studies of California mice found no differences in body mass
50 between breeding and non-breeding males, or any consistent effects of fatherhood on fat or lean
51 mass (Andrew et al., 2016; Zhao et al., 2017; Zhao et al., 2018).

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

64 For many small mammals, cold and its associated thermoregulatory costs are pervasive
65 challenges, as the environmental temperatures they experience - particularly at night, when most
66 small mammals are active - are routinely below thermoneutrality (Hill, 1983; Feist and White,
67 1989). This is especially pertinent during winter in temperate and high-latitude habitats. Small
68 body size goes hand-in-hand with high surface/volume ratios and also constrains the ability of
69 small mammals to add insulation (fur or subcutaneous fat), so acclimatization to winter

70 conditions is usually based primarily on increased thermogenic capacity (e.g., Hart, 1971;
71 Dawson and Olson, 1988), mainly via brown adipose tissue (Heldmaier et al., 1982; Heldmaier
72 et al., 1989; Cannon and Nedergaard, 2004). In rodents, including *Peromyscus* species, cold
73 acclimatization (or acclimation) typically increases thermogenic capacity by 30-50% (Hart,
74 1971; Lynch, 1973; Heimer and Morrison, 1978; Wickler, 1980; Heldmaier et al., 1982; Hayes
75 and Chappell, 1986; Nespolo et al., 1999; Rezende et al., 2004a). This enhanced thermogenic
76 capacity is often accompanied by elevated resting or basal metabolic rates (RMR or BMR; e.g.,
77 Hart, 1957; Hayward, 1965; Russell and Chappell, 2007, Zub, 2014). Winter acclimatization or
78 acclimation may also induce substantial alterations in body composition and organ morphology
79 (e.g., Smith and Horwitz, 1969; Heldmaier et al., 1982; Konarzewski and Diamond, 1994;
80 Hammond and Kristan, 2000; Deveci et al., 2001; Brzęk et al., 2007; Rezende et al., 2009;
81 Vaanholt et al., 2009; Zub, 2014). These changes may impact both energy budgets (Hayes,
82 1989b) and aspects of performance in addition to cold tolerance (e.g., exercise capacity; Hayes
83 and Chappell, 1986).

84 In its natural range in California and Baja California, the California mouse breeds
85 throughout the year, except for the dry summer (Gubernick, 1988), so parents must care for some
86 litters during the cold winter months. Given the apparent impact of an experimental energy
87 stress on fathers in laboratory conditions (Zhao et al., 2018), an understanding of the effects of
88 ecologically relevant thermal conditions - including winter temperatures - may be important for
89 understanding the evolution of the species' monogamous mating system. Accordingly, we
90 performed two experiments to evaluate the effects of cold acclimation on male morphology and
91 physiology. First, we acclimated mice for short periods (~1 month) to temperatures typical for
92 winter in the species' natural habitat (5°C or 10°C) and measured body composition, hematocrit,

93 energy metabolism (thermoneutral RMR, maximal oxygen consumption in exercise [$\dot{V}O_2$ max]),
94 exercise performance (grip strength, sprint speed) and predatory aggression. Since many small
95 mammals spend much of their lives at environmental temperatures below their thermal neutral
96 zones, we also performed a longer-term (~6 months) cold acclimation at 10°C. For this
97 experiment we measured body composition, hematocrit, and energy metabolism (BMR, $\dot{V}O_2$
98 max, and thermogenic capacity). To ascertain the effects of pair bonding and fatherhood, we
99 compared virgin males (housed with another adult male), breeding males (housed with an adult
100 female and their first litter) and non-breeding males (housed with an ovariectomized, estrogen-
101 and progesterone-treated female).

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

111

112

113 **2. Methods**

114 *2.1 Animals*

115 California mice were born and raised in our colony at the University of California,

116 Riverside (UCR) and were descended from animals purchased from the Peromyscus Genetic
117 Stock Center (University of South Carolina, Columbia, SC, USA; ancestors captured in the Santa
118 Monica Mountains, CA, USA). Animals were housed in polycarbonate cages (44 x 24 x 20 cm)
119 with aspen shavings for bedding but no additional nesting material. Food (Purina 5001 Rodent
120 Chow, LabDiet, Richmond, IN, USA; caloric content – 28.5% protein, 13.5% fat, 58.0%
121 carbohydrate) and water were available *ad libitum*. Lighting was on a 14:10 cycle (lights on at
122 05:00 h, off at 19:00 h), with humidity maintained at approximately 55% and ambient
123 temperature at 22.1 ± 0.9°C (mean ± SE) except where stated otherwise. Mice were checked
124 twice daily, and cages were cleaned once per week. At weaning age (27-31 days; 28.0 ± 0.3,
125 mean ± SE), animals were ear-punched for identification and placed in same-sex groups of 3-4
126 related and/or unrelated, age-matched individuals.

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

131

132 *2.2 Experiment 1– Short-Term Cold Acclimation*

133 When males reached 60-114 (88.4 ± 1.1, mean ± SE) days of age, they were moved in
134 their virgin groups either to an environmental chamber set at 5°C (VM5, initial $N = 42$, final $N =$
135 29; see Results for explanation of decreases is sample sizes) or 10°C (VM10, initial $N = 70$, final
136 $N = 50$) or to a new room with ambient temperature set at 22°C (VM22, initial $N = 72$, final $N =$
137 61) (Fig. 1: Experiment 1). Beginning at 14-38 (24.8 ± 1.5) days in their respective temperature
138 conditions, males from each group underwent a series of test procedures over a 7-day period

139 (Table 1). All males remained housed with their cage mates throughout the period of data
140 collection, except where indicated below.
141
142 *2.3 Experiment 2– Long-Term Cold Acclimation*
143 Virgin males from the 10°C (VM10, initial $N = 14$, final $N = 12$; see Results) and 22°C
144 (VM22, initial $N = 14$, final $N = 12$) conditions [age: 126-198 days (147.0 ± 5.1)] in experiment
145 1 were pair-housed with a virgin male cage mate from their original same-sex group and
146 maintained at their respective housing temperatures until 346-421 (381.5 ± 4.8) days of age (Fig.
147 1: Experiment 2). Additional virgin males that had been housed at 10°C in experiment 1 [age:
148 123-173 days (142.7 ± 4.4)] were randomly paired with an age-matched virgin female in one of
149 two conditions (Fig. 1). Breeding males (BM10, initial $N = 14$, final $N = 4$) were paired with a
150 sham-ovariectomized female, and non-breeding males (NB10, initial $N = 14$, final $N = 6$) were
151 paired with an ovariectomized female treated periodically with estradiol benzoate and
152 progesterone (see below) to induce estrous behavior. After 207-295 days (243.0 ± 7.4), all virgin
153 males, breeding males, and non-breeding males underwent an 8-day testing period (Table 1),
154 after which they were euthanized and dissected (see below). BM10 [age: 342-380 days (357.3 ±
155 6.8)] and NB10 [age: 340-363 days (346.5 ± 5.5)] were compared with the age-matched VM10
156 [age: 381-392 days (387.8 ± 1.2)] and VM22 [age: 374-401 days (389.5 ± 2.9)].
157
158 *2.4 Ovariectomies and Estrogen/Progesterone Treatment*
159 Females in experiment 2 underwent bilateral ovariectomies prior to being paired with
160 NB10 (to allow mating but prevent pregnancy) or sham-ovariectomies prior to being paired with
161 BM10. Females were anesthetized with isoflurane, and surgeries were performed under aseptic

162 conditions using standard procedures as previously described (Zhao et al., 2018). Females were
163 then housed individually for two weeks to recover before being paired with males. Forty-eight
164 hours prior to pairing, NB10 females were injected with estradiol benzoate (0.072 mg, s.c.;
165 suspended in sesame oil, Sigma-Aldrich, St. Louis, MO, USA). At the time of pairing, they were
166 injected with progesterone (0.48 mg, s.c.; suspended in sesame oil, Sigma-Aldrich, St. Louis,
167 MO, USA) (Zhao et al., 2018). A pilot study (unpub.) found that this treatment usually led to
168 mating behaviors in ovariectomized females ~13 h after progesterone injection, whereas
169 untreated ovariectomized females were never observed to copulate. At the end of the
170 experiment, ovariectomized females were euthanized by CO_2 inhalation and dissected to check
171 for the presence of fetuses in the uterine canal; no females had visible fetuses.
172
173 *2.5 Body Mass*
174 Males in experiments 1 and 2, as well as breeding females in experiment 2, were weighed
175 to ± 0.1 g twice per week (13:00-15:00 h) at approximately 3- to 4-day intervals from pairing
176 until the beginning of their test period. This procedure was used to monitor pregnancies in
177 breeding females (experiment 2) and animal health, and to habituate animals to handling.
178
179 *2.6 Body Composition*
180 Body composition was measured in experiment 1 on test days 1 (13:00-14:30 h) and 7
181 (9:00-10:30 h) and in experiment 2 on test day 6 (12:00-14:00) (Table 1). Males were weighed
182 and then scanned with a magnetic resonance whole-body analyzer (EchoMRI-100; Echo Medical
183 Systems, Houston, TX, USA) to assess fat mass, lean mass, free water mass, and total water
184 mass that was calibrated in our lab for this species (Zhao et al., 2017; Zhao et al., 2018). Scans

185 lasted ~90 s and did not require anesthesia or sedation. Here we report fat and lean masses only
186 (unaltered and as percentages of total body mass).
187
188 *2.7 Hematocrit*
189 Blood samples (~200 µl) were collected in experiment 1 on test days 1 (9:00-10:30 h) and
190 7 (13:00-14:30 h) and in experiment 2 on test day 8 (12:30-14:30 h) (Table 1). Mice were
191 anesthetized with isoflurane and blood was collected from the retro-orbital sinus into heparinized
192 microhematocrit capillary tubes (Chauke et al., 2011; Harris et al., 2011; Andrew et al., 2016).
193 Blood was centrifuged at 4°C and 1300 RPM (~1,900 g) for 12 min (Sorvall Legend Micro 21R;
194 Thermo Scientific), and hematocrit was recorded.
195
196 *2.8 Predatory Aggression*
197 Mice were tested for predatory aggression (Gammie et al., 2003; Zhao et al., 2017),
198 without prior fasting, in experiment 1 (test days 2 and 3, 13:30-15:00 h). Briefly, the mouse was
199 placed singly in a clean cage with a thin layer of aspen shavings covering the floor and no food
200 or water. After a 15-min habituation period, a live adult cricket (0.2-0.5 g) was dropped into the
201 cage on the side opposite the mouse. Behavior was video recorded until either the cricket was
202 killed or 7 min had elapsed. Videos were scored for latency to attack and latency to kill the
203 cricket. If the mouse did not kill the cricket, it was assigned a latency of 7 min. Predatory
204 aggression was tested on two successive days to determine repeatability. The shorter latency of
205 each animal's two tests was used for comparisons among temperature conditions. California
206 mice are omnivorous, with a diet that includes arthropods (Merritt, 1974; Reid et al., 2013).
207

208 *2.9 Resting Metabolic Rate (RMR)*

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

219

220 *2.10 Basal Metabolic Rate (BMR)*

221 BMR was measured in experiment 2 on test day 1 (8:30-16:30 h). The procedure for
222 measuring BMR was identical to the method for obtaining RMR, except that food was removed
223 8h before testing began.

224

225 *2.11 Forced-Exercise Maximal Oxygen Consumption ($\dot{V}O_2$ max)*

226 $\dot{V}O_2$ max was measured in experiment 1 (test days 5 and 6; 10:00-11:30 h) and in
227 experiment 2 (test days 4 and 5; 11:30-13:30 h), using a running-wheel respirometer
228 (circumference: 51.8 cm; effective volume: 900 mL) as previously described (Dlugosz et al.,
229 2012; Andrew et al., 2016). Air flow through the wheel was 2400 mL/min, and ambient
230 temperature was 22.0 ± 0.2°C (mean ± SE). Excurrent air was subsampled (~150 mL/min) and

231 dried with soda lime and Drierite prior to oxygen measurements (Applied Electrochmistry S-3A;
232 Sunnyvale, CA, USA). Mice were weighed, placed in the wheel, and given ~2 min to acclimate.
233 We then started rotation and gradually increased rotation speed approximately every 30s until
234 either oxygen concentration did not change or mice could no longer maintain position. Flow rate
235 and O_2 concentration were measured every second using LabHelper. Reference air was taken at
236 the beginning and end of trials, and a baseline was computed by linear regression. We calculated
237 $\dot{V}O_2$ in LabAnalyst using the Mode 1 equation, and computed $\dot{V}O_2$max as the highest $\dot{V}O_2$
238 averaged over 1 min (ml O_2/h). We measured $\dot{V}O_2$max on each of the two test days to assess
239 repeatability, and the higher of the two values for each animal was used for further statistical
240 analyses.
241
242 *2.12 Maximal Thermogenic Capacity*
243 The maximal $\dot{V}O_2$ during thermogenesis (summit metabolism; $\dot{V}O_2$sum) was only
244 measured in experiment 2 on test day 7 (11:00-13:00 h), using acute cold exposure in heliox
245 (21% O_2: 79% He by volume) to quickly elicit maximal thermogenic capacity without use of
246 dangerously low temperatures and attendant risk of frostbite injury (Rosenmann and Morrison,
247 1974; Chappell et al., 2003). Males were separated from their cage mates, weighed, and placed
248 in a Plexiglas metabolic chamber (volume 850 mL) with a small amount bedding, inside an
249 environmental cabinet. Excurrent air was subsampled (~150 mL/min) and dried with soda lime
250 and Drierite prior to oxygen analysis (Applied Electrochemistry S-3A). Reference air was taken
251 at the beginning and end of trials, and a baseline was computed by linear regression. Flow rates
252 (1700 mL/min), temperature, and O_2 concentrations were measured every second using
253 LabHelper. Mice were placed in the chamber at a moderately low temperature (0 to -5°C), after

254 which temperature was reduced by ~1°C/min until it stabilized or declined despite decreasing

255 ambient temperature. At this point, mice were removed from the chamber and a temperature

256 probe was inserted into the anus to determine the final body temperature. We computed $\dot{V}O_2$ in

257 LabAnalyst using the Mode 1 equation, and $\dot{V}O_2$ sum was determined as the highest $\dot{V}O_2$

258 averaged over 1 min (ml O_2/h). We did not test for $\dot{V}O_2$ sum in experiment 1 because the

259 duration of cold exposure was variable and insufficient to achieve stable cold acclimation

260 responses (Rezende et al., 2004b).

261

262 *2.13 Grip Strength*

263 Grip strength was tested in experiment 1 on test days 2 and 3 (9:30-11:00 h). Mice were

264 suspended by their tail over a horizontal wire-mesh surface (0.25" grid) attached to a force gauge

265 (HF-10N, M&A Instruments Inc., Arcadia, CA, USA). The mouse was lowered until both the

266 forelimbs and hindlimbs were touching the mesh without pulling on the force gauge. Once the

267 mouse had relaxed on the mesh, the end of its tail was gently pulled horizontally until it released

268 its grip (Meyer et al., 1979; Maurissen et al., 2003). Peak force value was recorded and the test

269 was repeated once. The higher value from each day was used to assess repeatability, and the

270 higher of the two values was used for analysis comparing groups.

271

272 *2.14 Sprint Speed*

273 Maximum sprint speed (Djawdan and Garland 1988) was measured in experiment 1 on

274 test days 5 and 6 (14:00-15:30 h) using a ‘racetrack’ (8 m long by 10 cm wide, with 30 cm high

275 walls) equipped with 12 sets of aligned photocells at 50-cm intervals (Andrew et al., 2016). A

276 mouse was placed near the start of the track and encouraged to walk or run down the track 2 - 4

277 times for familiarization at the start of each test. The male was then returned to the starting area,
278 the photocells were activated, and the mouse was chased down the track with a padded strip of
279 plastic (~10 cm wide by ~30 cm long), tripping successive photocells as it ran. Sprint speed was
280 measured 5 times on each of the two days, yielding a total of 10 trials per individual; the fastest
281 1.0-m interval on each day was recorded. Trials were scored subjectively as 'poor', 'fair',
282 'okay', 'good' or 'excellent' depending on mouse cooperation. Trials with scores of poor or fair
283 were excluded from analysis. The highest values from each day were used to determine
284 repeatability, and the single highest value for each individual was used as its maximum sprint
285 speed.

286

287 *2.15 Euthanasia and Organ Collection*

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

296

297 *2.16 Statistical Analysis*

298 In both experiments, we examined repeatability for all measures taken on two trials or
299 two paired organs, using Pearson's correlations and paired *t*-tests. We used single values (e.g.,

300 mean or maximum) for comparisons of group means for these measures. Repeatability analysis
301 was conducted in the same manner as in previous studies (Andrew et al., 2016; Table S1). We
302 also computed multiple linear regressions of the performance measures (BMR, $\dot{V}O_2$ max, and
303 $\dot{V}O_2$ sum) for experiment 2 on relevant organ masses and hematocrit (Table S2). For all
304 measures, we used analysis of covariance (ANCOVA) in SPSS 24.0 (see below for covariates
305 used) to compare traits among groups (experiment 1: VM5, VM10, VM22; experiment 2: VM22,
306 VM10, NB10, BM10). Data were $\log_{10}$- or rank-transformed prior to analysis where appropriate
307 (noted on Tables 2 and 3); results are presented in untransformed units (as estimated marginal
308 mean ± standard error unless otherwise noted).
309 For experiment 1, male age and testing cohort differed among the three groups (both $P <$
310 0.001), whereas the number of days between relocation to new housing and testing did not ($P =$
311 0.318). All three variables were used as covariates in analysis. For experiment 2, age, testing
312 cohort, and days between relocation to new housing and testing differed significantly among the
313 four groups (all $P < 0.006$), but for reasons explained below, were not used as covariates in
314 analysis. We also used body mass, body length, and cricket mass as covariates where
315 appropriate (denoted in Tables 2 and 3). We performed the overall F-test for group differences
316 (Tables S3 and S4) and *a priori* contrasts among all of the groups for experiments 1 and 2. We
317 discuss only the *a priori* contrasts between groups.
318 Excluding nuisance variables such as age, cohort, and duration of acclimation,
319 experiment 1 generated 60 P values, 15 of which were < 0.05 (underlined values in Table 2), and
320 experiment 2 generated 258 P values, 61 of which were < 0.05 (underlined values in Table 3).
321 These tests include a substantial amount of non-independence because the same individuals were
322 measured for all traits, some traits were correlated, and many were interrelated. Numerous

323 methods to compensate for non-independence in multiple related tests, and hence control the
324 number of false positives, are available. No single procedure performs best in all situations, and
325 indeed multiple types of error rates can be defined, where "each of them might be appropriate
326 and useful for some inferential situation" (Benjamini, 2010). Moreover, some workers argue that
327 such corrections are often unnecessary and undesirable. Given such controversies, we computed
328 both the False Discovery Rate (FDR) and Adaptive False Discovery Rate procedures in PROC
329 MULTTEST in SAS 9.4 (SAS Inc., Cary, NC, USA). For experiment 1, the smallest four values
330 would have adjusted P values < 0.05 (0.034 being the highest) using the Adaptive FDR
331 procedure, while no values would have adjusted P values < 0.05 using the FDR procedure. We
332 used the Adaptive FDR procedure for experiment 1. For simplicity, all P values are reported in
333 the text and tables as raw values, not adjusted for multiple comparisons; however, we refer to P
334 values ≤ 0.034 as “significant” (bold and underlined in Table 2) and those between 0.034 and
335 0.05 as “nominally significant” (underlined but not bold in Table 2). For experiment 2, the
336 smallest 34 values would have adjusted P values < 0.05 (0.007 as the highest) using the Adaptive
337 FDR procedure, whereas the smallest 49 values would have adjusted P values < 0.05 (0.018 as
338 the highest) using the FDR procedure. We used the FDR procedure for experiment 2. Similar to
339 experiment 1, we refer to P values ≤ 0.018 as “significant” (underlined in Table 3) and those
340 between 0.018 and 0.05 as “nominally significant” (bolded and underlined in Table 3).
341

342 **3. Results**

343 *3.1 Experiment 1-Short-Term Cold Acclimation*

344 In experiment 1, mortality did not differ significantly among groups. Twenty-nine of 42
345 (69.0%) virgin males acclimating to 5°C survived to the last day of testing compared to 50 of 70

346 (71.4%) virgin males acclimating to 10°C and 61 of 72 (84.7%) virgin males housed at 22°C (χ^2
347 = 4.9, P = 0.085). Deaths occurred either during testing procedures (N = 3 VM5, 6 VM10, 5
348 VM22) or of unknown causes under undisturbed conditions in the home cage (10 VM5, 14
349 VM10, 6 VM22).

350 With body mass as a covariate, RMR was significantly higher in 5°C males than in males
351 housed at higher temperatures (VM10: P = 0.002, VM22: P = 3.00x10^{-6}; Table 2). Forced-
352 exercise $\dot{V}O_2$max (with body mass as a covariate) was slightly higher (by 6-9%) for VM5 (P =
353 0.001) and VM10 (P = 0.004) than for VM22. No other measures (i.e., body mass, fat mass,
354 lean muscle mass, organ masses, hematocrit, predatory aggression, sprint speed) differed
355 significantly between virgin males housed at the three temperatures (Table 2).
356
357 *3.2 Experiment 2-Long-Term Cold Acclimation*

358 Mortality differed among groups in experiment 2 (χ^2 = 8.8, P = 0.031). Survival rates of
359 males to the final day of testing were 85.7% (12 of 14) for VM22, 85.7% (12 of 14) for VM10,
360 42.9% (6 of 14) for NB10, and 57.1% (8 of 14) for BM10. Again, deaths occurred either during
361 testing (N = 2 VM22, 1 VM10, 2 NB, 1 BM) or in the home cage for unknown reasons (N = 0
362 VM22, 1 VM10, 6 NB, 5 BM). Of the 8 surviving breeding pairs, two did not produce offspring;
363 thus, the survival rate for pairs that bred was 42.9% (6 of 14). When these latter two pairs were
364 removed from analysis, the difference in survival rate among groups was even more highly
365 significant (χ^2 = 11.2, P = 0.011).
366
367 *3.2.1 Long-Term Acclimation to 10°C*

368 To determine possible interactions of reproductive/mating status and cold acclimation,

369 we compared each group of males housed at 10°C (BM10, NB10, VM10) with the control group
370 of VM22. Body length, measured at euthanasia, was lower in NB10 ($P = 0.001$) and VM10 ($P =$
371 $5x10^{-6}$) than in VM22 (Fig. 2; Table 3). Body mass did not differ significantly between VM22
372 controls and any of the three groups housed at 10°C on test days 1, 4 or 8, but when body length
373 was used as a covariate, NB10 were heavier than VM22 on all three test days ($P = 0.015$, $P \leq$
374 0.001, and $P \leq 0.001$, respectively) and had higher lean mass ($P = 0.002$). With body length as a
375 covariate, both NB10 ($P = 0.007$) and VM10 ($P = 0.012$) had higher fat mass than VM22 (day
376 6).

377 BMR was substantially higher (21-34%) in BM10 ($P = 3.00x10^{-6}$) and NB10 ($P =$
378 $1.00x10^{-6}$) than in VM22 (Table 3). $\dot{V}O_2$ max was slightly lower (by 7%) in BM10 compared to
379 VM22 ($P = 0.018$; Fig. 3), whereas $\dot{V}O_2$ sum was 12% higher in VM10 than VM22 ($P = 0.011$;
380 Fig. 4).

381 At the end of experiment 2, several morphometric variables differed among groups
382 (Table 3). Log_{10}-transformed head width was lower in BM10 ($P = 0.004$) than in VM22. When
383 body length was used as a covariate, log_{10}-transformed head width was relatively higher in
384 BM10 ($P = 0.002$) compared to VM22. Foot length followed a similar pattern, regardless of
385 whether body length was used as a covariate: both BM10 (both $P \leq 0.003$) and NB10 (both $P \leq$
386 0.007) had longer feet than VM22 (Fig. 5). Finally, baculum length (without body length as a
387 covariate) was significantly higher in BM10 than in VM22 ($P = 0.015$).

388 Organ masses (log-transformed in some analyses) were compared among groups by
389 ANCOVA with log-transformed body mass as a covariate (Table 3). Ventricles were heavier in
390 NB10 ($P = 0.002$) and VM10 ($P = 2.40x10^{-5}$) than in VM22, and kidneys were heavier in VM10
391 than VM22 ($P = 1.15x10^{-4}$). Both stomach and intestine masses were higher in NB10 ($P =$

392 0.014, P = 0.002, respectively) and VM10 (P = 0.011, $P = 1.50 \times 10^{-5}$) compared to VM22.

393 Baculum mass was higher in BM10 (P = 0.001) compared to VM22. Finally, gastrocnemius

394 muscle mass was lower in both NB10 (P = 0.004) and VM10 ($P = 3.63 \times 10^{-4}$) compared to VM22

395 (Fig. 6). No other organ masses were affected by long-term cold acclimation.

396

397 *3.2.2 Effects of Reproductive Condition at 10°C*

398 We compared breeding, non-breeding, and virgin males, all housed at 10°C, to examine

399 possible interactions between reproductive condition and long-term cold acclimation. Breeding

400 males showed several differences from non-breeding and/or virgin males. BMR (with body

401 mass as a covariate) was higher (by 10-34%) in BM10 than in both NB10 (P = 0.003) and VM10

402 ($P = 1.34 \times 10^{-7}$), but $\dot{V}O_2$ max (with body mass as a covariate) was 15% lower in BM10 than in

403 VM10 (P = 0.014; Fig. 3). Foot length (with or without body length as a covariate) was higher

404 in BM10 than in VM10 (both $P \leq 0.005$; Fig. 5), as was baculum length BM10 (without body

405 length as a covariate: P = 0.013; with body length as a covariate: P = 0.014). Ventricle mass

406 (body mass as a covariate) was lower in BM10 than in VM10 (P = 0.007).

407 Several traits differed between non-breeding males and virgin males. BMR (with body

408 mass as a covariate) was 13% higher in NB10 than in VM10 ($P = 9.40 \times 10^{-8}$). Body length (P =

409 0.001; Fig. 2; Table 3), foot length (with or without body length as a covariate; all $P \leq 0.015$;

410 Fig. 5), baculum length (with body mass as a covariate; P = 0.005), adrenal mass (with body

411 mass as a covariate; P = 0.014), intestine mass (with body mass as a covariate; P = 0.003), and

412 baculum length (with body mass as a covariate; P = 0.001) were all higher in NB10 than in

413 VM10. However, VM10 had higher thigh muscles (with body mass as a covariate; P = 0.005),

414 and gastrocnemius muscles than NB10 ($P = 3.65 \times 10^{-4}$; Fig. 6). There were no differences among

415 BM10, NB10, and VM10 in head length or in brain, fat, lung, liver, spleen, pancreas, adrenal,
416 caecum, or testis masses.
417
418 **4. Discussion**

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

438 Nevertheless, the lack of large or numerous differences in the many variables measured suggests
439 that parenthood does not have substantial impact on male physiology, morphology, or energy
440 metabolism.

441 In most temperate or high-latitude small rodents, winter acclimatization or long-term
442 laboratory acclimation to cold induces large increases in thermogenic capacity and cold tolerance
443 (Hart, 1971; Lynch, 1973; Heldmaier et al., 1989; Nespolo et al., 1999). For example, in freshly
444 captured white-footed mice (*Peromyscus leucopus)* from Michigan (USA), winter animals
445 acclimatized to sub-zero temperatures had 70% higher thermogenic capacity ($\dot{V}O_2$ sum) than
446 mice captured in summer (Wickler, 1980). In free-living deer mice (*P. maniculatus)* from
447 California, acclimatization to 6-9°C winter temperatures increased $\dot{V}O_2$ sum by 39% compared
448 to summer values from the same wild population (Hayes, 1989a), and laboratory acclimation to
449 3-5 °C increased $\dot{V}O_2$ sum by 30%-64% (Hayes and Chappell, 1986; Hayes and Chappell, 1990;
450 Rezende et al., 2004b; Chappell et al., 2007). Notably, both *P. maniculatus* and *P. leucopus*
451 exhibited acclimatory increases in $\dot{V}O_2$ sum that were much larger (by 2.5-5.8-fold) than the ca.
452 11-12% augmentation we found in male *P. californicus*. In addition to being unusually weak,
453 the increase of $\dot{V}O_2$ sum after prolonged cold exposure in *P. californicus* was independent of
454 reproductive status (Table 3), contrary to our expectations that breeding and non-breeding males
455 would differ in acclimatory responsiveness.

456 The striking contrast in cold acclimation among *Peromyscus* species might be explained
457 in several ways. First, the 10°C acclimation temperature we used for *P. californicus* is
458 somewhat higher than temperatures used in other studies (typically ~3-5°C; e.g., Rezende et al.,
459 2004b; Rezende et al., 2009). However, it was only slightly warmer than the 6-9°C

460 environmental temperatures that elicited a 3-fold-larger summer-to-winter $\dot{V}O_2$ sum change in
461 wild deer mice from California (Hayes, 1989a). Second, California mice are large (~50 g)
462 compared to deer mice and white-footed mice (20-25 g), and their size may make them more
463 resistant to heat loss than their smaller congeners (due to a lower surface/volume ratio and
464 possibly an increased ability to support thicker and more effective insulation). This may reduce
465 selection for high thermogenic capacity. Third, unlike previous *Peromyscus* studies, we housed
466 California mice in groups (pairs or families), and huddling may have reduced the requirement for
467 thermogenesis as it does in other small mammals (Gilbert et al., 2010).

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

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

504 Our findings for a large set of morphometric and physiological traits in male California
505 mice parallel the interspecific findings: we found little consistency in responses to cold
506 acclimation (Table 3) or among sub-organismal correlates of aerobic metabolism (Table 4).
507 Contrary to our initial hypothesis, reproductive status (virgin, non-breeding, or breeding) was not
508 a reliable predictor of the acclimatory responsiveness of aerobic physiology. For example,
509 exercise capacity ($\dot{V}O_2$ max) increased after cold acclimation in breeding males but not in virgin
510 or nonbreeding males, whereas thermogenic capacity ($\dot{V}O_2$ sum) increased after cold acclimation
511 in virgin males, but not in breeding or nonbreeding males (Table 3). BMR, often viewed as
512 reflecting the overall metabolic demand on the organism or the minimal cost of living (e.g.
513 McNab and Morrison, 1963; Bennett and Ruben, 1979; Ricklefs et al., 1996; Hulbert and Else,
514 2004; Bior et al., 2018), did conform to predictions: following cold acclimation, it differed
515 significantly among the three reproductive categories, being highest in breeding males and
516 lowest in virgin males (Table 3). Moreover, BMR in breeding and non-breeding males
517 responded to cold acclimation, but that of virgin males did not (Table 3). The findings for BMR
518 are consistent with the expectation that the 'workload' of offspring care, particularly in a cold
519 environment, places higher demands on fathers than on non-breeding or virgin males.
520 Another expectation derived from the 'workload' concept is that the sizes of organs
521 associated with supporting aerobic metabolism and energy processing (e.g., heart, liver, kidneys,
522 digestive system) should correlate with energy use. Therefore, these organs should be larger in
523 fathers than in non-breeding or virgin males, and they should be larger in males acclimated to
524 10°C than in those acclimated to 22°C (e.g. Hammond and Kristan, 2000; Hammond and
525 Wunder, 1995). Neither of these expectations was strongly supported in our California mice
526 (Tables 3, 4). We speculate that, at least in part, the absence of consistent organ-mass effects

527 may be attributable to the small change in thermogenic capacity in *P. californicus* compared to

528 most rodents.

529 In *P. maniculatus,* the large increase in $\dot{V}O_2$ sum after cold acclimation is accompanied

530 by a smaller but significant increase in exercise-induced $\dot{V}O_2$ max, possibly as a result of

531 enhanced oxygen uptake in skeletal muscles as a side effect of the increased oxygen delivery to

532 brown adipose tissue required for intense nonshivering thermogenesis (e.g., Hayes and Chappell,

533 1986; Hayes and Chappell, 1990). In *P. californicus,* $\dot{V}O_2$ max also changed following cold

534 acclimation in some experimental groups, but results were inconsistent: $\dot{V}O_2$ max increased in

535 the short-term experiment (Table 2), but in the long-term experiment it decreased in breeding

536 males and was not affected in non-breeding and virgin males (Table 3). If, as previously

537 suggested (Hayes and Chappell, 1986; Hayes and Chappell, 1990), elevated $\dot{V}O_2$ max following

538 cold acclimation results from enhancements to oxygen delivery in support of increased

539 thermogenic capacity, then, as speculated for organ sizes, the inconsistent findings for California

540 mice may be a reflection of this species' relatively small acclimatory changes in $\dot{V}O_2$ sum.

541

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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

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

				***P* of *a priori* Contrasts**			**5°C VM**			**10°C VM**			**22°C VM**		
Traits	**Units**	**Trans.**	**Covar.**	**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**	**3.00x10^{-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	sec	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	sec	Rank	C	0.791	0.380	0.142	25	63.88	8.59	40	69.96	5.66	49	55.92	4.48

NC = No Covariate, B = Body Mass, C = Cricket Mass

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.

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). 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.

				***P* of *a priori* Contrasts**						**10°C BM**			**10°C NB**			**10°C VM**			**22°C VM**		
Traits	**Units**	**Trans.**	**Covar.**	**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	**$3.00x10^{-6}$**	**$1.00x10^{-6}$**	0.764	**0.003**	**$1.34x10^{-7}$**	**$9.40x10^{-8}$**	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**	**$5.00x10^{-6}$**	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

Head Length	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
Head Length	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 Width	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 Width	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
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**	**2.40×10^{-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	**1.15×10^{-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**	**1.50×10^{-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	**3.63×10^{-4}**	**0.004**	0.616	0.067	**3.65×10^{-4}**	4	0.220	0.028	6	0.236	0.023	12	0.272	0.016	12	0.346	0.016

NC = No Covariate, B = Body Mass, L = Body Length

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.

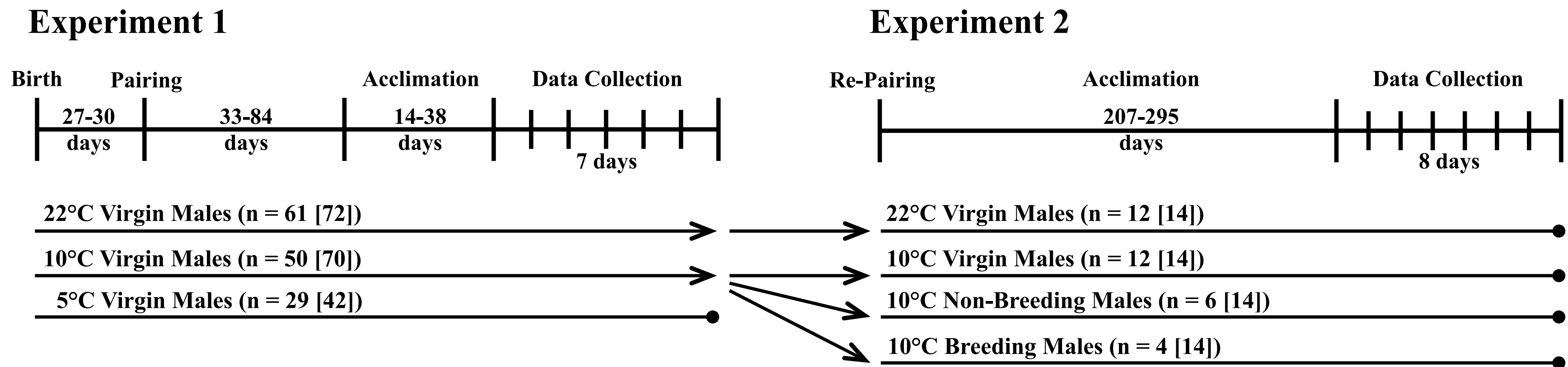

Figure 1. Timeline and use of male California mice in experiments 1 and 2. An arrow at the end of a line indicates that males were re-paired and used in experiment 2. A closed circle at the end of a line indicates that males were euthanized and used for morphological measurements, and were not used in 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.

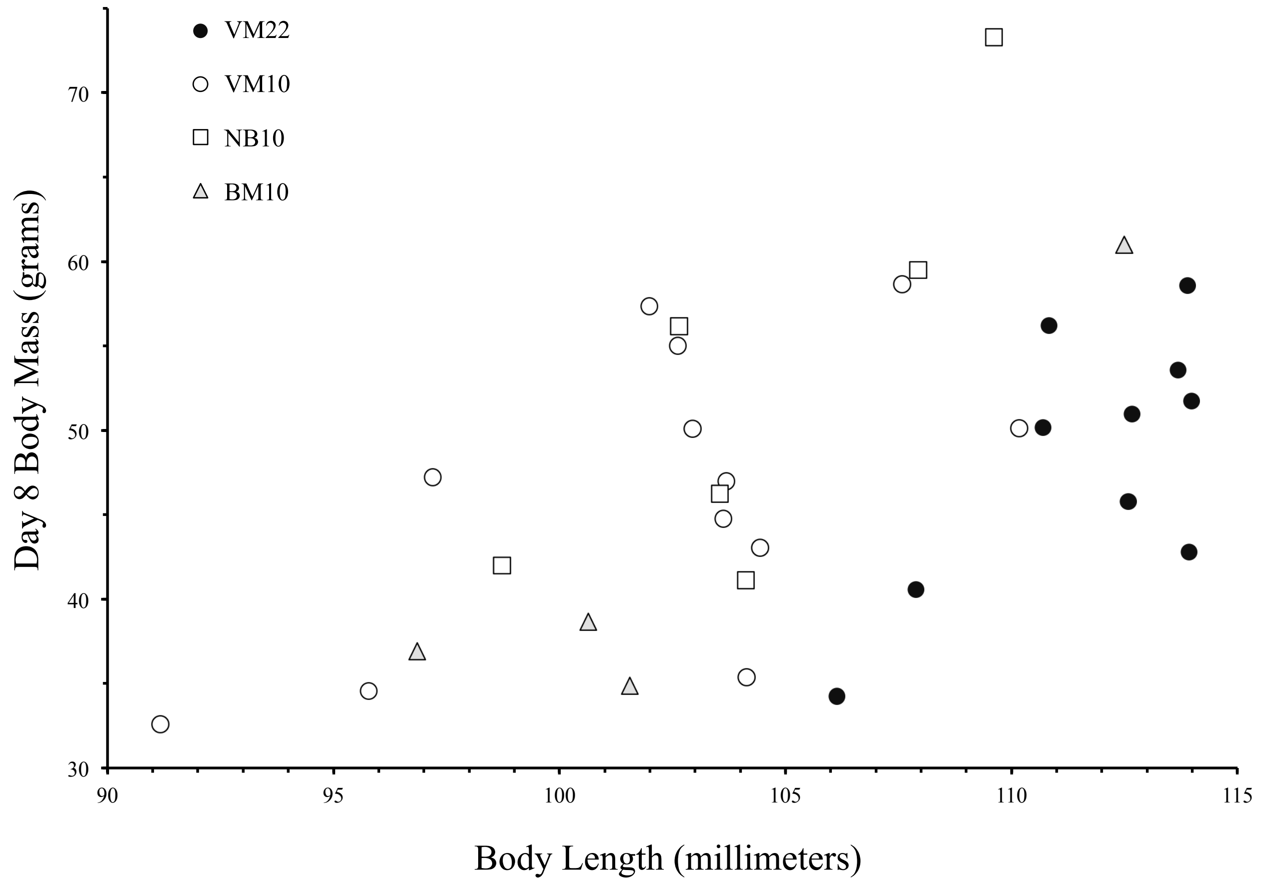

Figure 2. Body length versus day 8 body mass of mice in experiment 2 on the y-axis. Closed circles-virgin males (VM22) housed at 22°C (n=12), open circles-VM housed at 10°C (VM10, n=12), open squares-non-breeding males (NB10) housed at 10°C (n=6), grey triangles-breeding males (BM10) housed at 10°C BM (n=4). Body length was lower in NB10 ($P = 0.001$) and VM10 ($P = 5x10^{-6}$) than in VM22 and higher in NB10 than in VM10 ($P = 0.001$).

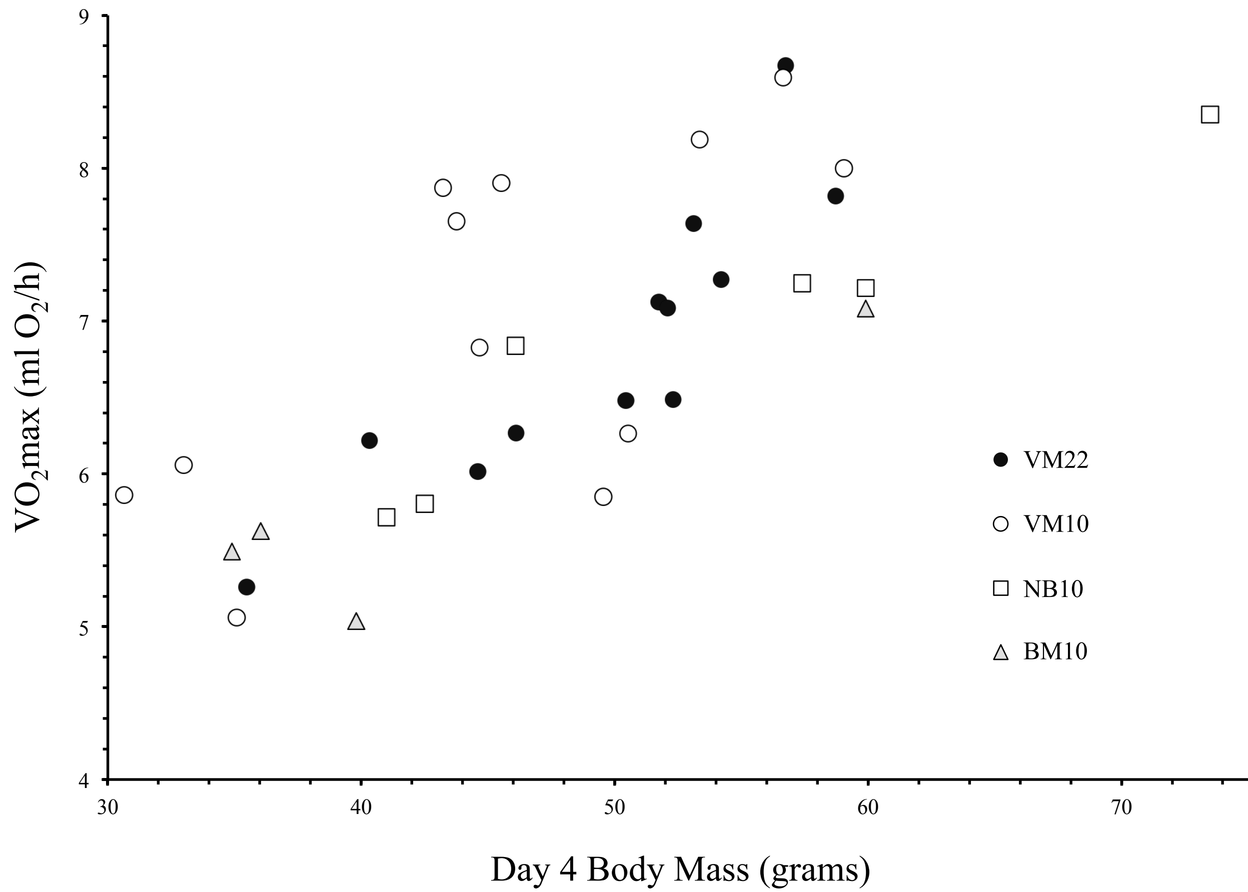

Figure 3. $\dot{V}O_2$ max versus day 4 body mass of mice in experiment 2. Closed circles-virgin males (VM22) housed at 22°C (n=12), open circles-VM housed at 10°C (VM10, n=12), open squares-non-breeding males (NB10) housed at 10°C (n=6), grey triangles-breeding males (BM10) housed at 10°C BM (n=4).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

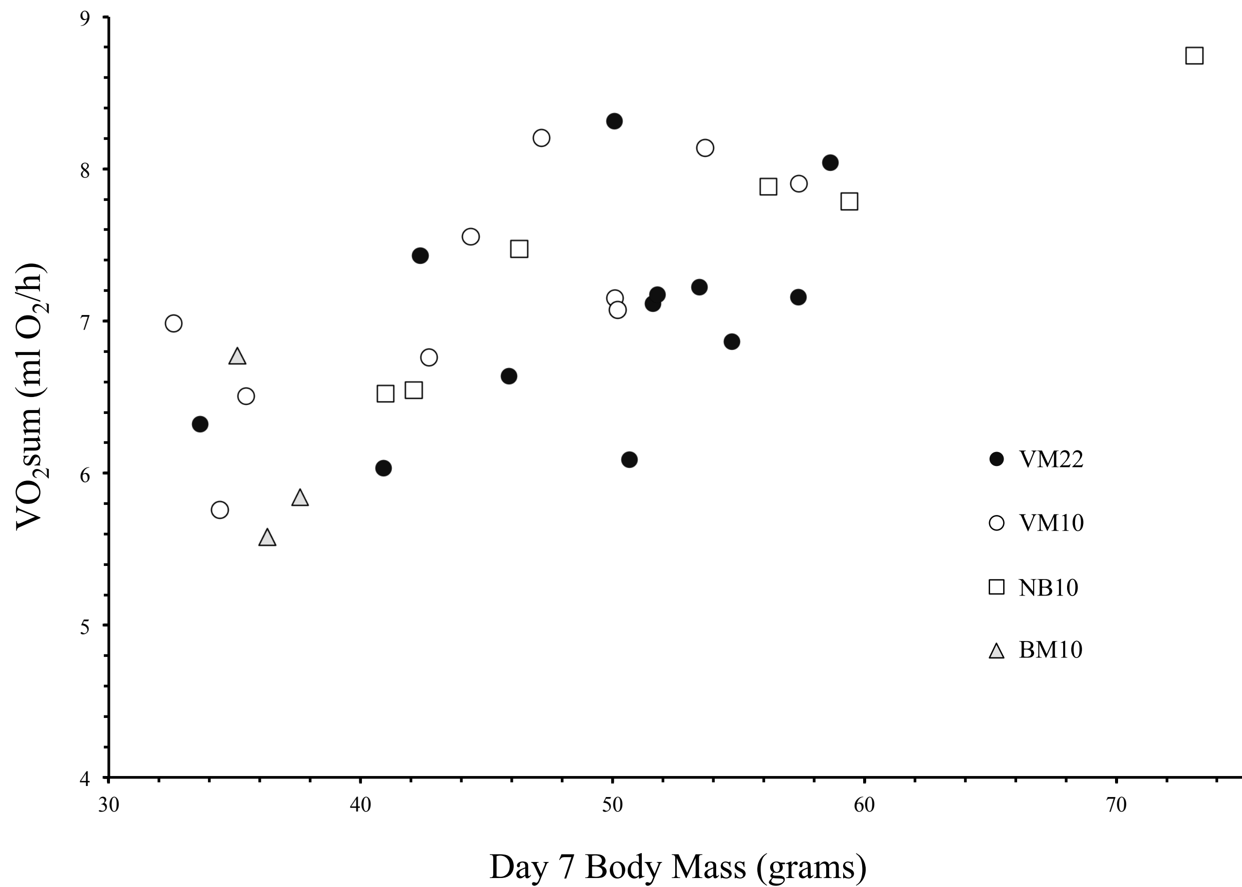

Figure 4. $\dot{V}O_2$ sum versus day 7 body mass for mice in experiment 2. Closed circles-virgin males (VM22) housed at 22°C (n=12), open circles-VM housed at 10°C (VM10, n=12), open squares-non-breeding males (NB10) housed at 10°C (n=6), grey triangles-breeding males (BM10) housed at 10°C BM (n=4).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

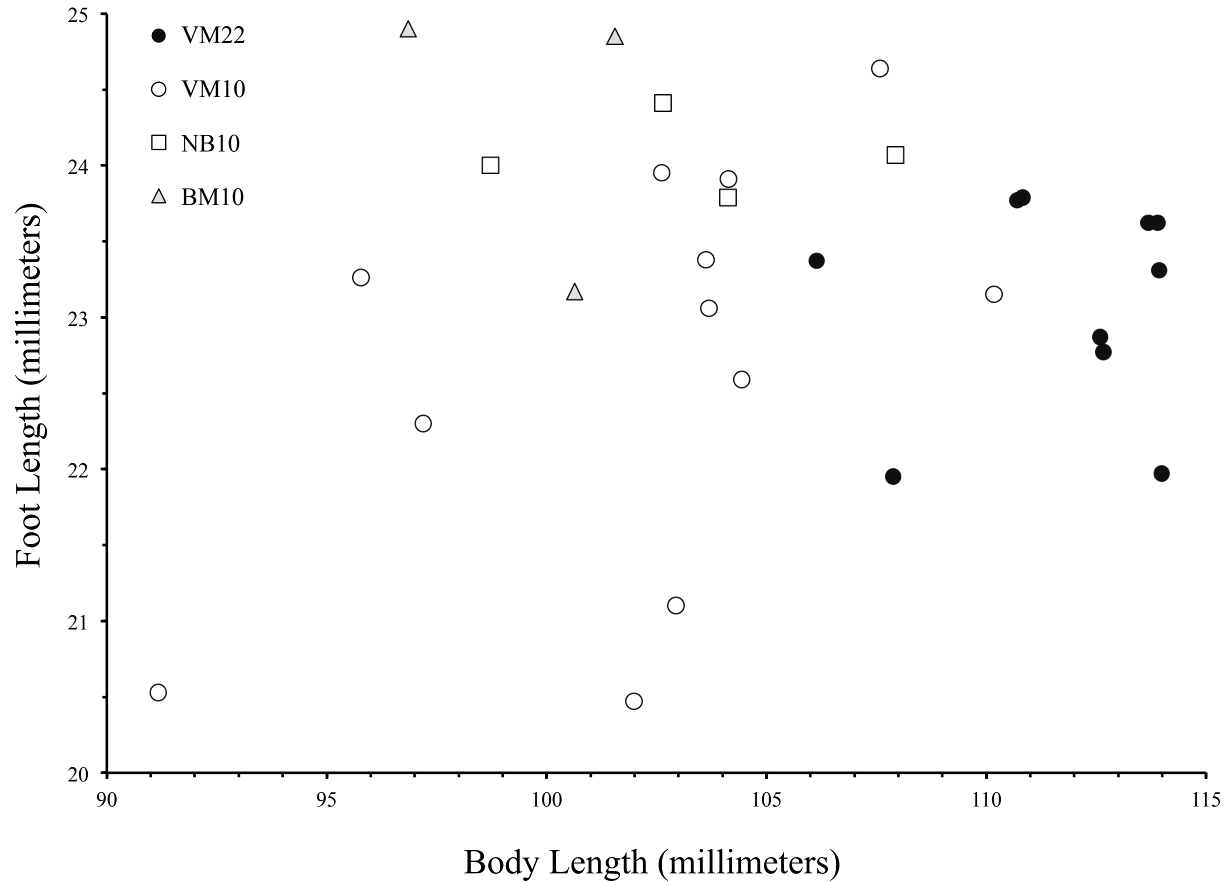

Figure 5. Foot length versus body length of mice in experiment 2. Closed circles-virgin males (VM22) housed at 22°C (n=12), open circles-VM housed at 10°C (VM10, n=12), open squares-non-breeding males (NB10) housed at 10°C (n=6), grey triangles-breeding males (BM10) housed at 10°C BM (n=4). Both BM10 (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.

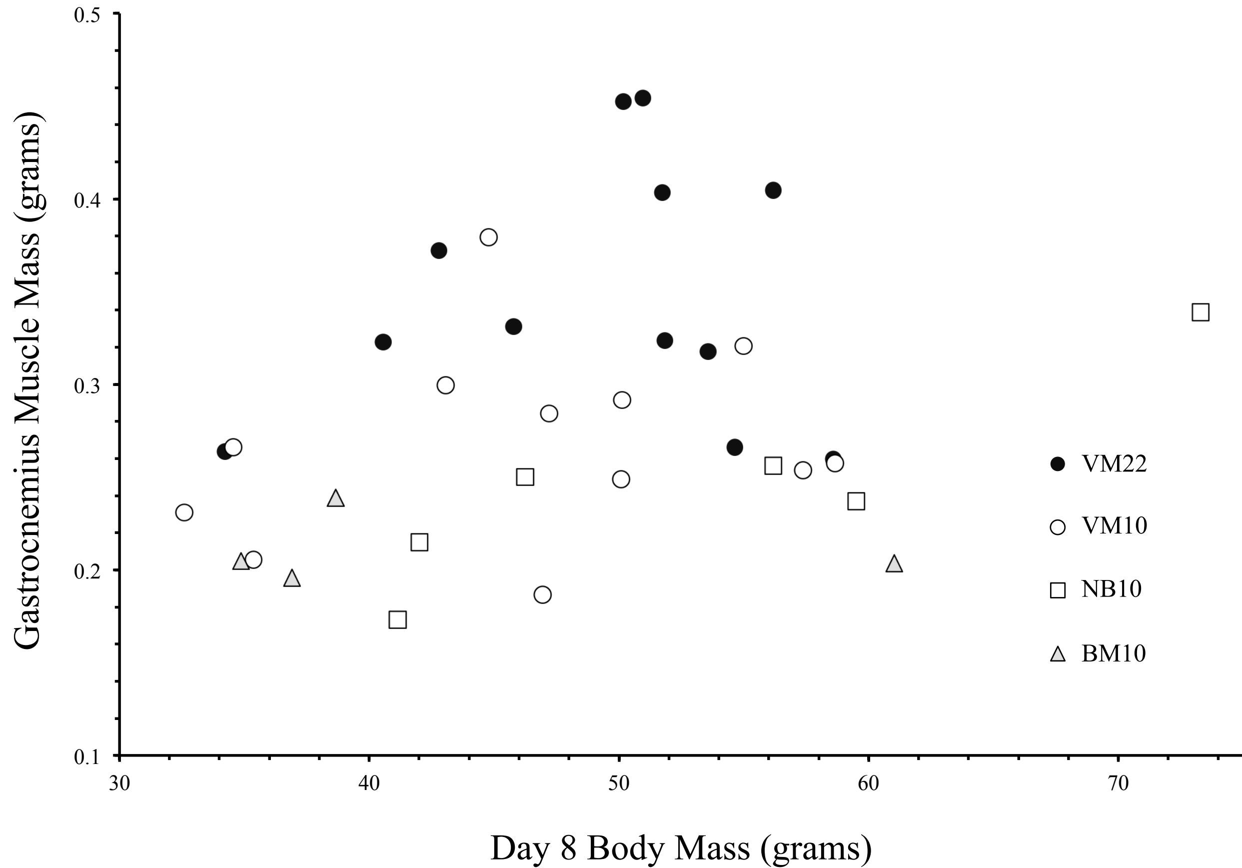

Figure 6. Gastrocnemius muscle mass versus body mass of mice in experiment 2. Closed circles-virgin males (VM22) housed at 22°C (n=12), open circles-VM housed at 10°C (VM10, n=12), open squares-non-breeding males (NB10) housed at 10°C (n=6), grey triangles-breeding males (BM10) housed at 10°C BM (n=4).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