Effects of a Physical and Energetic Challenge on Male California Mice (*Peromyscus californicus*): Modulation by Reproductive Condition

Meng Zhao¹, Theodore Garland, Jr.¹, Mark A. Chappell¹, Jacob R. Andrew¹, Breanna N. Harris², Wendy Saltzman¹

¹ Department of Evolution, Ecology, and Organismal Biology, University of California, Riverside

² Department of Biological Sciences, Texas Tech University

Corresponding author:

Wendy Saltzman

Department of Evolution, Ecology, and Organismal Biology

University of California, Riverside

Riverside, CA 92521

Saltzman@ucr.edu

Phone: 951-827-6356

Fax: 951-827-4286
Abstract

Reproduction strongly influences metabolism, morphology and behavior in female mammals. In species in which males provide parental care, reproduction might have similar effects on fathers. We examined effects of an environmental challenge on metabolically important physiological, morphological, and behavioral measures, and determined whether these effects differed between reproductive and non-reproductive males, in the biparental California mouse (*Peromyscus californicus*). Males were paired with an ovary-intact female, an ovariectomized female treated with estrogen and progesterone to induce estrus, or an untreated ovariectomized female. Within each group, half of the animals were housed under standard laboratory conditions and half in cages requiring them to climb wire towers to obtain food and water; these latter animals were also fasted for 24 hours every third day. We predicted that few differences would be observed between fathers and non-reproductive males under standard conditions, but that fathers would be in poorer condition than non-reproductive males under challenging conditions. Body and fat mass showed a housing condition x reproductive group interaction: the challenge condition increased body and fat mass in both groups of non-reproductive males, but breeding males were unaffected. Males housed under the physical and energetic challenge had higher blood lipid content, lower maximal aerobic capacity and related traits (hematocrit and relative triceps surae mass), increased pain sensitivity and increased number of fecal boli excreted during tail-suspension tests (a measure of anxiety), compared to controls. Thus, our physical and energetic challenge paradigm altered metabolism, morphology and behavior, but these effects were largely unaffected by reproductive condition.

Keywords: anxiety, body composition, fatherhood, metabolism, paternal care, reproduction
**Introduction**

Female mammals undergo changes in physiology, morphology and emotionality as a result of becoming mothers (Slattery and Neumann, 2008; Speakman, 2008; Woodside et al., 2012). Although males do not experience pregnancy and lactation, they too might be expected to undergo physiological, morphological, and emotional changes across different reproductive states, especially in species in which males provide significant care for their offspring. Few studies have addressed this possibility; however, fathers in many biparental mammalian species (i.e., in which both parents contribute to offspring care) undergo systematic changes in levels of numerous hormones and neuropeptides, including several that are known to have pronounced effects on energetics, metabolism, and behavior (Saltzman and Ziegler, 2014).

Fatherhood can be energetically costly in biparental mammals, corresponding to changes in energetically relevant physiological, morphological and behavioral measures. Prairie vole (*Microtus ochrogaster*) fathers, for example, had lower body mass and less subcutaneous fat than non-fathers (Campbell et al., 2009; Kenkel et al., 2014). Similar to lactating females in many species, prairie vole fathers spent more time feeding during the postpartum period, possibly leading to recovery in body mass (Campbell et al., 2009; Speakman, 2008). Newly paired male prairie voles also showed increased preference for sucrose solution, suggesting that they needed to increase energy intake (Campbell et al., 2009) or possibly that they experienced alterations in taste or in the brain's reward system. In two biparental primates, common marmosets (*Callithrix jacchus*) and cotton-top tamarins (*Saguinus oedipus*), expectant fathers underwent significant increases in body mass across their mate’s pregnancy, which are thought to prepare males for the energetic demands of fatherhood (Ziegler et al., 2006). This was followed by a drop in body mass during the postpartum period (Achenbach and Snowdon, 2002; Ziegler et al., 2009).
Fatherhood may also be associated with changes in anxiety-like and depression-like behavior in some biparental mammals, although very few studies have tested this possibility. One study of prairie voles found that fathers displayed more anxiety-like behavior and/or more depression-like behavior than virgin males and sexually experienced males without offspring (Lieberwirth et al., 2013). Another study, however, found lower levels of anxiety-related behaviors in prairie vole fathers than in virgin males (Kenkel et al., 2014).

The California mouse (*Peromyscus californicus*) is a genetically monogamous and biparental rodent in which males may undergo affective and metabolic changes when they become fathers. Fathers engage in all the same parental behaviors as mothers except nursing, and to a similar extent (Bredy et al., 2007; Cantoni and Brown, 1997; Dudley, 1974; Gubernick and Teferi, 2000; Gubernick et al., 1993; Wright and Brown, 2002). Previous studies in our lab found that males housed with a non-reproductive female were significantly heavier than those housed with a first-time pregnant female (Saltzman et al., 2015), and fathers showed a significant rise in body mass across their mate’s pregnancy, but only if housed with pups from the previous litter (Harris et al., 2011; Saltzman et al., 2015). California mouse fathers also had smaller subcutaneous fat pads than virgin males (Andrew et al., 2016). Two studies found that California mouse fathers have reduced behavioral responses to stress, as compared to males with no previous exposure to pups (Bardi et al., 2011; Chauke et al., 2011); however, other studies reported no differences between fathers and non-fathers in neuroendocrine responses to either acute (Chauke et al., 2011; Harris and Saltzman, 2013) or chronic stressors (De Jong et al., 2013). We also found that virgin males produced significantly more fecal boli than breeding males in the tail-suspension test (a measure of anxiety), had significantly higher blood glucose levels than non-breeding males housed with a tubally ligated female, and significantly lower average testis
masses than non-breeding and breeding males (Zhao et al., 2017). However, we did not find any significant effects of reproductive status on males’ resting metabolic rate (RMR), maximal oxygen consumption (VO$_2$max), food intake (all mass-adjusted), blood leptin levels, pain sensitivity, or depression-like behavior (Andrew et al., 2016; Zhao et al., 2017).

In sum, few affective, metabolic or physiological effects of fatherhood have been found in California mice. All of these studies, however, were carried out on animals housed under standard laboratory conditions; we have speculated that more, and more pronounced, effects might occur under more challenging environmental conditions (Zhao et al., 2017). In natural environments, animals face many energetic and physical challenges, such as predation, limited food availability, and extreme temperatures (McPhee, 2004), and therefore might be more affected by the additional demands of parenthood, compared to captive animals housed under standard conditions.

In the present study, we subjected adult male California mice to two simultaneous environmental challenges: climbing (required for obtaining food and water) and fasting for 24 hours every third day. We aimed to identify the physiological, morphological, and behavioral effects of these challenges and to determine if they differed between fathers and non-fathers. We compared morphological (body mass, body composition, organ masses) and physiological measures (blood metabolic markers; plasma insulin, leptin and corticosterone levels; maximal oxygen consumption during forced running [VO$_2$max]) between new fathers and non-reproductive adult males housed under both standard and challenging conditions. We also characterized behaviors potentially associated with metabolic condition (food intake, preference for high-fat diet). A tail-suspension test was performed as an index of depression-like and anxiety-like behaviors, and preference for artificial sweetener was assessed to investigate
anhedonia, (i.e., a reduced ability to experience pleasure from rewarding activities), a common symptom of depression (Gibson, 2006; Pecoraro et al., 2004). Finally, we examined nociceptive responses, an important part of defensive systems influenced by affective state (Vendruscolo et al., 2004). We predicted that few differences would be observed between fathers and non-reproductive males housed under standard laboratory conditions, as in previous studies, but that under challenging conditions, fathers would have decreased body mass and body fat mass, higher food intake, and altered blood glucose and lipid levels, compared to non-reproductive males. We further predicted that the environmental challenges would reveal differences between fathers and non-fathers in pain sensitivity, depression-like and anxiety-like behavior, and preferences for highly palatable food and liquid.

Materials and methods

Animals

California mice were bred in our colony at the University of California, Riverside (UCR) and were descended from mice from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Animals were housed in polycarbonate cages (44 × 24 × 20 cm) with aspen shavings as bedding and cotton wool as nesting material. Food (Purina 5001 Rodent Chow, LabDiet, Richmond, IN, USA) and tap water were provided ad lib. The colony was on a 14:10 light:dark cycle, with lights on at 05:00 h and lights off at 19:00 h. Room temperature was approximately 21 °C and humidity was about 55%. Cages were checked twice daily and changed weekly.

Mice were weaned at 27-30 days of age, prior to the birth of younger siblings. At weaning, animals were ear-punched for individual identification and housed in same-sex groups
of 3-4 related and/or unrelated, age-matched individuals.

Sample sizes were based on prospective power analysis (G*Power 3; Faul et al. 2009), using the magnitude of group differences and the within-group standard errors observed in our previous studies on California mice. All procedures used were in accordance with the *Guide for the Care and Use of Laboratory Animals* and were 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 (AAALAC).

**Experimental design**

At 130-175 days of age (mean ± SE = 154.94 ± 1.29 days), 64 adult males were randomly assigned to three categories: virgin males (VM, n=21; housed with an ovariectomized female and thus without opportunities to copulate), non-breeding males (NB, n=22; housed with an ovariectomized female treated with estradiol benzoate and progesterone and thus able to copulate but not conceive; see below) and breeding males (BM, n=21; housed with a sham-ovariectomized female and thus able to copulate and reproduce). Pairmates in all three groups were no more closely related to each other than first cousins. NB were used to control for mating, and VM were used to control for cohabitation with an adult female. Half of the pairs (n=31; 10-11 per reproductive condition) were housed under standard lab conditions from birth (CTRL) and the other half (n=33; 10-12 per reproductive condition) were housed under standard lab conditions until pairing and moved to challenging conditions immediately after pairing (CHAL), in which they were 1) required to climb towers (see below) in order to obtain food and water and 2) fasted for 24 h every third day. After the birth of the first litter in each breeding pair (or at a matched time point for VM and NB), animals were left undisturbed for 2-3 days until the
beginning of the 14-day testing period (see Fig. 1). At postpartum day 27 or 28, female mates and pups of BM were weighed and their body composition was assessed (see below). Pups were also checked daily for eye opening.

Climbing towers and food restriction

A climbing-exercise paradigm modified from previous studies of lab mice (*Mus musculus*) and rats (*Rattus norvegicus*) (Lionikas and Blizard, 2008; Mori et al., 2003; Notomi et al., 2001) was used to subject half of the animals (CHAL condition) to a physical and energetic challenge. The challenged mice were housed in cages connected to 2 wire mesh (hardware cloth of ~5 mm) towers measuring ~8 cm in diameter and ~50 cm in height. Food was located at the top of one tower, and a water bottle was at the top of the other tower. In addition, all food was removed for 24 h at 10:00-11:00 h every third day. Water was available *ad libitum* every day.
**Ovariectomy, sham-ovariectomy, and hormone treatment**

Female mice were ovariectomized using antiseptic techniques and standard surgical procedure. Briefly, mice were anesthetized with isoflurane gas using a vaporizer. The incision region (1 cm above genital area) was shaved and sanitized, and a ventral midline incision (approximately 1 cm) was made. The left and right oviducts were clamped tightly with a hemostat at the fallopian tubes, and the ovaries were removed. All reproductive structures were repositioned back in the abdominal cavity, the abdominal incision was closed with absorbable sutures (Monocryl Suture 4-0 FS-2, Ethicon, San Angelo, TX) and the skin was sealed using tissue glue (Vetbond Tissue Adhesive 1469SB, St. Paul MN, USA). After surgery, females were housed individually for 7 days until being paired with a male. At the end of the experiment, ovariectomized females were sacrificed by CO$_2$ inhalation and dissected to check for pregnancy. None had visible fetuses. For sham-ovariectomies, all procedures were identical to those for ovariectomies except that the oviducts were not clamped and the ovaries were not removed.

California mice mate after pairing and again on the day of parturition (Gubernick, 1988). To mimic this pattern, the ovariectomized females from NB were treated with estradiol benzoate (Sigma-Aldrich, St. Louis, MO, USA) (0.072 mg, s.c.) two days before the breeding males were paired and also on the day when the breeding pairs gave birth; each estrogen treatment was followed 48 h later by treatment with progesterone (Sigma-Aldrich, St. Louis, MO, USA) (0.48 mg, s.c.). Pilot data showed that this treatment paradigm, with estrogen followed by progesterone 48 h later, usually induced mating behaviors in ovariectomized females within 13 h, while untreated ovariectomized females were never observed copulating (unpublished data).
Measurements

Morphology

Body mass

From the time of pair formation until the birth of the first litter in breeding pairs (or a matched time point for VM and NB), all males, as well as the females in breeding pairs, were weighed to the nearest 0.01 g at 14:00-16:00 h twice weekly, at 3- to 4-day intervals. In addition to providing body-mass data, this allowed us to assess overall health, habituate the animals to handling, and monitor pregnancies through patterns of mass gain in females. Subjects were also weighed between 13:30 and 16:00 h on test days 1, 2, 8, 9, and 13, as well as between 9:00 and 11:00 h on test days 11 and 12 (Fig. 1).

Body composition (days 1, 2, and 13)

Body composition was assessed with an EchoMRI-100 magnetic resonance whole-body analyzer (Echo Medical Systems, Houston, TX, USA) as previously described (Zhao et al., 2017). Lean and fat masses were computed both as absolute values and as percentages of total body mass (analyses using body mass as a covariate yielded similar results). For each measure, the average of days 1, 2, and 13 was used in analysis.

Euthanasia and organ collection (day 14)

Males were decapitated between 13:30 and 16:00 h. Trunk blood was collected in a heparinized weighing boat, and organs [heart, liver, leg muscles (left and right triceps surae), testes (left and right), adrenal glands (left and right) and kidneys (left and right)] were removed and weighed as previously described (Zhao et al., 2017). Blood samples were centrifuged for 12 min at 13,300
rpm and 4 °C, and plasma was removed and stored at -80 °C for corticosterone assays.

**Metabolism, energetics, and related behaviors**

**Food consumption (day 5)**

Beginning at the start of the active period (lights-off; 19:00 h) on test day 5, each male’s mate (and pups, for BM) was moved to a clean cage next to the home cage for 4 h. The males remained in their home cages (which included climbing towers for CHAL males). The food and water bottle were weighed immediately before and after the 4-h test period, and the amounts consumed were determined as the difference between initial and final mass. The bedding was checked before and after the test to confirm that no shredded food was present. Males were reunited with their cagemates in their home cages immediately after the test.

**High-fat-diet preference (day 11)**

Each male was isolated in its home cage for 4 h, starting at the time of lights-off (19:00 h), as described above for the food-consumption test. The food hopper was divided by a steel partition into two compartments, each containing ~100g of standard diet (13.5% Kcal fat, Purina 5001 Rodent Chow, LabDiet, Richmond, IN, USA) or high-fat diet (43.6% Kcal fat, Modified Diet 5001, TestDiet, Richmond, IN, USA). The positions of the two diets were assigned randomly. The food in each part of the hopper was weighed immediately before and after the 4-h test period, and the amount of each diet consumed was determined as the difference between initial and final mass. The bedding was checked before and after the test to make sure food wasting did not bias results (Koteja et al., 2003). The amount of high-fat diet consumed divided by the overall amount of food consumed was calculated as an index of relative preference for high-fat diet.
Maximum aerobic metabolic rate (days 11 and 12)

Maximal oxygen consumption (VO_{2}max) was measured as described previously (Andrew et al., 2016; Chappell and Dlugosz, 2009), immediately after weighing on days 11 and 12. Briefly, mice were forced to run in an enclosed running-wheel respirometry chamber. The wheel was rotated from low to high speed until either the O_{2} concentration did not increase with increasing exercise intensity or the mouse could not keep up. Flow rates (2,400 mL/min) and gas concentrations were measured every s using Warthog LabHelper software (http://www.warthog.ucr.edu). Excurrent air was subsampled (~150 mL/min), scrubbed of CO_{2} and dried with soda lime and Drierite, and sent through an oxygen analyzer. Reference air was taken at the beginning and end of every trial, and a baseline was fitted by linear regression. Oxygen consumption was then calculated with Warthog LabAnalyst (http://www.warthog.ucr.edu). Instantaneous corrections were used to account for the mixing and washout characteristics of the chamber. VO_{2}max was determined as highest VO_{2} averaged over 1 min. We tested VO_{2}max for repeatability across the two days of tests, and the higher of the two values for each animal was used for analysis.

Blood metabolic profiles

Glucose and lipid profiles (day 6)

Mice were fasted for 6 h, at approximately 11:00-17:00 h, immediately after which they were anesthetized with isoflurane and blood was collected from the retro-orbital sinus as previously described (Zhao et al., 2017). Four samples were obtained from each mouse using heparinized capillary tubes. Each of the first two tubes (40 μl each) was immediately used to determine total cholesterol (TC), high-density lipoprotein cholesterol (HDL), triglyceride, and ratio of TC to
HDL (TC/HDL) in whole blood using an automated analyzer (model LDX; Cholestech Corporation, Hayward, CA, USA) and Lipid Profile GLU cassettes (item number 10-991). We did not formally validate the Cholestech LDX analyzer for *Peromyscus californicus*; however, it has been validated extensively for human samples (details in package insert), within the following ranges: TC – 100-500 mg/dL, HDL – 15-100 mg/dL, triglyceride – 45-650 mg/dL. This analyzer has also been used in several other rodent studies (Hammad et al., 2003; Meek et al., 2014; Stedman et al., 2006). The third sample (< 20 μl) was used to measure glucose concentration in whole blood twice with a Contour Next blood glucose monitoring system (Mishawaka, IN, USA). We did not formally validate this system for *Peromyscus californicus*; however, it has been validated for humans (details in user guide), can measure glucose in the range of 20-600 mg/dL and has been used in other studies in rodents (Carvas et al., 2012; Croce et al., 2013; Fonken et al., 2010). The fourth tube (70 μl) was centrifuged immediately for 12 min (13,300 rpm, 4 °C), hematocrit was measured, and plasma was removed and stored at -80 °C for leptin and insulin assays.

**Leptin ELISA**

Leptin concentrations in plasma were determined using a laboratory mouse (*Mus*) leptin enzyme-linked immunosorbent assay (ELISA) kit (cat. no. 90030; Crystal Chem, Downers Grove, IL, USA) that we previously validated for *P. californicus* (Zhao et al., 2017). All samples were assayed in triplicate at the recommended volume (5 μl). The kit standards generate a curve adequate to measure leptin concentrations between 0.2 and 12.8 ng/ml. Intra-assay coefficient of variation (CV) was 5.23%, and inter-assay CV was 4.08% for a plasma pool from adult male.
California mice. Each assay included samples from animals in each of the six experimental groups (2 housing conditions x 3 reproductive groups).

**Insulin ELISA**

Insulin concentrations in plasma were determined using a mouse (Mus) insulin ELISA kit (cat. no. 90080; Crystal Chem, Downers Grove, IL, USA) previously validated for *P. californicus* (Zhao et al., 2017). All samples were assayed in triplicate at the recommended volume (5 µl). The kit standards generate a curve adequate to measure insulin concentrations between 0.1 and 12.8 ng/ml, and intra- and inter-assay CVs were 5.07% and 7.84%, respectively. Each assay included samples from animals in each of the six groups.

To analyze insulin-glucose dynamics, we calculated two surrogate measures of insulin sensitivity: the homeostatic model assessment of insulin resistance (HOMA-IR, \((\text{insulin (mU/l)} \times \text{glucose (mmol/l)})/22.5\)), and the quantitative insulin check index of insulin sensitivity (QUICKI, \(1/(\log(\text{insulin (mU/l)})+\log(\text{glucose (mg/dl)}))\)), both based on fasted glucose and insulin levels (Borai et al., 2011; Bowe et al., 2014).

**Corticosterone radioimmunoassay**

Plasma corticosterone concentration for each male was determined using a radioimmunoassay kit (cat. no. 07120103; MP Biomedicals, Solon, OH) previously validated for *P. californicus* (Chauke et al., 2011). The assay was run according to manufacturer’s instructions, but the assay standard curve was extended down from 25 to 12.5 ng/ml (90-91% bound) and went to 1000 ng/ml (20-21% bound). Samples were diluted anywhere from 1:200 to 1:800 in order to ensure that values were contained within the curve. All samples were diluted and run in duplicate. Intra-
and inter-assay CVs were calculated using a kit-provided control and were 1.97% and 3.24%, respectively.

**Behavioral indicators of pain sensitivity, depression, and anxiety**

**Pain sensitivity (days 2 and 3)**

Tests were administered between 09:00 h and 11:00 h using a protocol previously described (Zhao et al., 2017). Briefly, animals were placed in a ventilated plexiglass cylinder (6 cm height x 20 cm diameter) on a hot plate (Lab-Line Instruments, Inc., Melrose Park, IL, USA) maintained at 44.3 ± 0.2°C. The time from placement on the hot plate until shaking, licking or sustained lift of either of the hind paws, whichever occurred first, was recorded as an index of latency to pain response. Animals were removed from the hot plate immediately after showing any of the above behaviors, or after 120 s if they showed no responses. In addition to latency to nociceptive behavior, we recorded the number of fecal boli and urine pools deposited on the hot plate, as commonly performed for the open-field test, a standard test of anxiety-like behavior (Archer, 1973; Bronikowski et al., 2001; Colman et al., 2007; Flint et al., 1995).

**Tail suspension (days 8 and 9)**

Between 14:00 and 16:00 h, mice were suspended by their tails from a ring stand for 6 min, and the duration of immobility was measured as previously described (Zhao et al., 2017). Briefly, the ring stand was placed on an activity detector unit (MAD-1: Sable Systems International, Henderson, NV, USA) interfaced to a Macintosh computer equipped with an A-D converter and LabHelper software. Activity was recorded every 0.004 s. LabAnalyst software was used for baseline correction and calculation of activity duration (Malisch et al., 2009). Duration of time
spent immobile is interpreted as a positive indicator of depression-like behavior (Cryan et al., 2005). In addition, we recorded the number of fecal boli produced during the 6 min of testing.

**Saccharin preference (day 2)**

Males’ preference for saccharin (Sweet’N Low; Cumberland Packing Corp., Brooklyn, NY, USA) solution (0.2% w/v in water) vs. water was assessed as a measure of anhedonia, as previously described (Zhao et al., 2017). Briefly, starting at the time of lights-off (19:00 h), each mouse was separated from its cage mate(s) for 4 h, as described above, with access to standard chow and two plastic syringes, one containing ~35 ml of water and the other containing ~35 ml of 0.2% saccharin solution. Positions of the two types of liquid were randomly assigned. The syringes were weighed immediately before and after the 4-h test period. The amount of consumed saccharin solution divided by the overall liquid consumption was calculated as an index of relative preference for saccharin solution. We have previously found that male California mice in all reproductive conditions tend to prefer the saccharin solution over water (Zhao et al., 2017).

**Data Analysis**

All traits were analyzed by analysis of covariance (ANCOVA) using SPSS. Age and/or other potentially relevant variables (see Results) were used as covariates. All tests were two-tailed. For each analysis, residuals were checked for (1) skewness and (2) homogeneity of variance using Levene’s test, and dependent variables were transformed as needed. For traits measured twice and for tests conducted on two successive days, as well as for paired (right and left) organs, values from the two trials or two organs were compared using a paired t-test and a Pearson correlation to gauge repeatability (Table 1), and mean values were used for subsequent analyses (except for VO_{2max}). For the breeding pairs, we also performed ANCOVAs to
determine if litter size, pup development and mothers’ body composition differed between animals in the CTRL and CHAL conditions.

Excluding such nuisance variables as age, this study generated 145 P values, 48 of which were < 0.05. These tests include a substantial amount of non-independence because the same individuals were measured for all traits, some traits were correlated, and many tests were interrelated. To compensate for non-independence in multiple related tests, we used the Adaptive False Discovery Rate procedure as implemented in PROC MULTTEST in SAS 9.4 (SAS Inc., Cary, NC, USA). Based on this procedure, the 43 smallest P values would have adjusted P values < 0.05 (the highest being 0.019). For simplicity, all P values reported in the text and tables are raw values, not adjusted for multiple comparisons; however, we refer to P values ≤ 0.019 as “significant” and those between 0.019 and 0.05 as “nominally significant.”
Results

Morphology

Body mass

To examine changes in body mass within individuals, we analyzed all masses recorded between the time of pairing and parturition (for breeding males) or a comparable time point (for virgin males and non-breeding males). For each male, we computed a least-squares linear regression of body mass on measurement day. We analyzed the slope of this regression as the dependent variable in an ANCOVA with age at first weighing as a covariate. We found a nominally significant difference between CHAL and CTRL, with prepartum body mass increasing more rapidly in CHAL males than in CTRL males (P = 0.043). Neither the main effect of reproductive group nor the housing condition x reproductive group interaction was significant (Table 2).

We also analyzed mean body mass during the test days and again found no main effect of reproductive group; however, we found a significant main effect of housing condition, with CHAL heavier than CTRL (P < 0.0001). The interaction between housing and reproductive conditions was nominally significant (P = 0.037): both VM and NB had higher body mass in CHAL than in CTRL, whereas BM did not (Table 2, Fig. 2).

Body composition

Squared fat mass was significantly higher in CHAL compared to CTRL (P < 0.0001) when using age as a covariate, with an interaction between housing and reproductive conditions (P = 0.019) (Table 2, Fig. 3). Similar to body mass, both VM and NB had higher fat mass in CHAL than in CTRL, whereas BM did not (interaction P = 0.019). When expressed as percentage of total body mass, squared percent fat mass was significantly higher in CHAL compared to CTRL (P = 0.0001); however, the interaction effect was no longer significant.
No significant effects were found for absolute lean mass. When expressed as a percentage of total body mass, however, percent lean mass was significantly lower in CHAL compared to CTRL \((P = 4.90 \times 10^{-7})\). The interaction between housing and reproductive condition was significant for percent lean mass \((P = 0.045)\), with both VM and NB, but not BM, showing lower percent lean mass in CHAL as compared to CTRL. Neither lean mass nor percent lean mass showed a significant main effect of reproductive group.

Note that fat mass and percent fat mass had positively skewed residuals, and transforms did not eliminate the skewness, but the main results are clear (Fig. 4).

**Organ masses**

Body mass was used as a covariate in all organ-mass analyses. Masses of left and right triceps surae, testes, adrenals and kidneys all showed high correlations within individual animals, and similar to Zhao et al., 2017, right adrenals and kidneys were significantly heavier than left ones (Table 1), as has been seen in other mammals and with other organs (e.g., Coleman et al., 1998; Idelman, 1978). One BM from the CTRL condition had much higher \((-2x)\) triceps surae mass than the other CTRL males and was excluded from analysis as an outlier. Males from CHAL had higher square-root-transformed liver mass \((P = 0.001)\), higher rank-transformed mean triceps surae mass \((P = 3.9 \times 10^{-5})\), and lower average testis mass \((P = 3.33 \times 10^{-7})\) than did CTRL. No other organ masses differed among reproductive groups or between housing conditions, and no interactions occurred (Table 2). Note that mean triceps surae mass had a skewed distribution and significant heterogeneity of variance, and transforms did not eliminate the heterogeneity or skewness (Fig. 4).
Metabolism/energetics and related behaviors

Blood glucose, lipid, and cholesterol profiles

An ANCOVA of hematocrit, with age as a covariate, indicated a significant difference between housing conditions ($P = 0.003$), with CTRL higher than CHAL (Table 1). Hematocrit was not influenced by a main effect of reproductive group or a housing condition x reproductive group interaction.

Fasted plasma glucose concentrations were highly correlated in the two successive blood samples ($r = 0.970$), but did not differ significantly among groups (Table 1). CHAL had higher fasted TC ($P = 1.01 \times 10^{-8}$), HDL ($P = 0.017$) and rank-transformed triglyceride levels ($P = 0.0001$) than CTRL (Table 2), but none of these measures was affected by a significant main effect of reproductive group or a housing condition x reproductive group interaction. Note that TC had a skewed distribution and significant heterogeneity of variance, and transforms did not eliminate these patterns.

Circulating leptin, insulin, and corticosterone concentrations

An ANCOVA (with age and percent fat mass as covariates) on log$_{10}$-transformed plasma leptin concentration found a positive effect of percent fat mass ($P = 4.72 \times 10^{-8}$) and a significant difference between housing conditions ($P = 0.006$), with CHAL having higher leptin levels than CTRL (Table 2); however, no main effect of reproductive group or housing condition x reproductive group interaction was found.

For log$_{10}$-transformed fasted plasma insulin concentration, ANCOVA (with age and body mass as covariates) revealed a positive effect of body mass ($P = 0.001$) but no main effects of housing conditions or reproductive groups, and no housing condition x reproductive group interaction.
interaction (Table 2). When analyzed without body mass as a covariate, \(\log_{10}\)-transformed fasted plasma insulin concentration was significantly higher in CHAL compared to CTRL (\(P = 0.0005\)).

For \(\log_{10}\)-transformed baseline plasma corticosterone concentration, ANCOVA (with age, time of day and time elapsed from disturbance to decapitation as covariates) revealed a positive effect of time of day (\(P = 0.001\)), but no main effects of housing conditions or reproductive groups, and no housing condition x reproductive group interaction (Table 2).

**Surrogate measures of insulin sensitivity**

\(\log_{10}\)-transformed HOMA-IR was higher in CHAL than in CTRL (\(P = 0.000824\)). CHAL also had lower QUICKI (\(P = 0.00131\)) than CTRL (Table 2). Both of these measures indicated that insulin sensitivity was lower in CHAL than in CTRL. Neither HOMA-IR nor QUICKI was significantly affected by reproductive group or by a housing condition x reproductive group interaction.

**Food consumption**

An ANCOVA (with age and body mass as covariates) indicated that food consumption did not differ between housing conditions or among reproductive groups and was not affected by a housing condition x reproductive group interaction (Table 2).

**High-fat-diet preference**

Preference for high-fat diet did not differ between housing conditions or among reproductive groups and was not affected by a housing condition x reproductive group interaction (Table 2). In general, males did not consistently prefer high-fat diet over standard diet (grand mean ± SE = 0.660 ± 0.042).
Maximum aerobic metabolic rate

Measurements of VO$_2$max were highly repeatable within individuals and did not differ between the two days. ANCOVA (with age and body mass as covariates) indicated a positive effect of body mass (P < 3×10$^{-5}$) and a significant difference between housing conditions (P < 0.0015), with CHAL lower than CTRL (Table 2). VO$_2$max was not affected by reproductive group or by an interaction between housing condition and reproductive group.

Pain sensitivity, depression-like behavior, and anxiety-like behavior

Pain sensitivity

Individual animals’ latencies to nociceptive behavior on the two successive testing days were significantly correlated (r = 0.313) and not significantly different from one another (Table 1). An ANCOVA was conducted on log$_{10}$-transformed mean values from each male, with age and body mass as covariates, and revealed a significant difference between housing conditions (P = 0.005), with CHAL having a shorter latency to the pain response (i.e., a lower pain threshold) than CTRL (Table 2). The main effect of reproductive group and the housing condition x reproductive group interaction were not significant.

The numbers of urine pools excreted on the two successive testing days were significantly correlated (r = 0.454) and not significantly different from one another (Table 1). An ANCOVA of rank-transformed mean values with age and body mass as covariates found nominally fewer urine pools in CHAL than in CTRL (Table 2). The numbers of fecal boli expelled on the two successive testing days were also positively correlated (r = 0.340; Table 1). We found no significant difference between reproductive groups and no significant housing condition x reproductive group interaction (Table 2).
**Tail-suspension test**

The durations of immobility on the two successive testing days were significantly correlated ($r = 0.722$) within individual animals and not significantly different (Table 1). An ANCOVA conducted on rank-transformed mean values (with age and body mass as covariates) indicated no significant differences between housing conditions or among reproductive groups, and the housing condition x reproductive group interaction was not significant (Table 2).

Numbers of fecal boli expelled on the two successive testing days were correlated ($r = 0.399$) and not significantly different from one another (Table 1). Mean values were used for comparing groups. An ANCOVA (with age and body mass as covariates) revealed a positive effect of body mass on rank-transformed fecal boli number ($P = 0.026$) and a significant difference between housing conditions, with CHAL producing more boli than CTRL ($P = 0.016$; Table 2: the effect disappeared without body mass as a covariate). Number of fecal boli was not influenced by a significant main effect of reproductive group or a housing condition x reproductive group interaction.

Note that both duration of immobility and number of fecal boli had skewed distributions and significant heterogeneity of variance, and transforms did not eliminate these statistical complications.

**Saccharin preference**

Preference for saccharin solution over water did not differ between housing conditions or among reproductive groups and was not affected by a housing condition x reproductive group interaction (Table 2). In general, males did not consistently prefer the saccharin solution over water (grand mean ± SE = 0.650 ± 0.037).
Maternal morphology and pup development

Neither litter size at weaning (range: 1 - 3, 2.0 ± 0.1) nor day of eye opening of first pup (15.6 ± 0.7) differed between offspring of breeding males in the CTRL and CHAL conditions. In both mothers and pups, however, several measures of body composition differed at the time of weaning (postpartum day 27). CHAL mothers had nominally lower body mass (P = 0.030; CTRL = 49.57 ± 1.19 g, CHAL = 44.81 ± 0.93 g) and significantly lower lean mass (P = 0.009; CTRL = 39.86 ± 0.71 g, CHAL = 34.82 ± 0.75 g) than CTRL mothers. Similarly, CHAL pups had lower mean mass (P = 0.016; CTRL = 19.15 ± 0.46 g, CHAL = 13.39 ± 1.07 g) but not total litter mass, as well as lower percent lean mass, than CTRL pups (P = 0.017; CTRL = 15.48 ± 0.29 g, CHAL = 11.28 ± 0.78 g) when using mother’s age and body mass as covariates. CHAL pups also had lower fat mass than CTRL pups with only mother’s age as a covariate (P = 0.001), but the effects disappeared when including mother’s body mass as a covariate, which had a nominally significantly effect on pups’ fat mass (P = 0.034).

Discussion

Fatherhood in biparental mammals is thought to be energetically and metabolically expensive, at least in some species (Achenbach and Snowdon, 2002; Campbell et al., 2009; Ziegler et al., 2009). Correspondingly, fathers in these species, including the California mouse, typically undergo changes in circulating levels of several metabolically important hormones and/or their receptors, including androgens, progesterone, and prolactin, across their mate’s pregnancy and postpartum period (Gubernick and Nelson, 1989; Perea-Rodriguez et al., 2015; Saltzman and Ziegler, 2014; Trainor et al., 2003). In the present study, we characterized energetically and metabolically relevant physiological, morphological, and behavioral measures in breeding, non-
breeding, and virgin male California mice housed under both standard laboratory conditions and energetically and physically challenging conditions, in which 1) mice had to climb wire-mesh towers to obtain food and water, and 2) food was removed for 24 h every third day. Overall, we found that the climbing + fasting paradigm had numerous morphological, physiological, and behavioral effects on male mice but, contrary to our predictions, very few of these effects were modulated by the males’ reproductive condition.

**Body composition**

After being paired with a female, males in the challenge condition underwent more rapid increases in body mass than those housed in standard cages. Subsequently, following the birth of the breeding males’ first litters, fatherhood apparently limited the ability of males to increase their fat mass under energetically challenging conditions.

In several biparental species, fathers gain body mass during their mate's pregnancy and lose mass during the period of infant care (reviewed in Saltzman and Ziegler, 2014), suggesting that paternal care is energetically costly, even under laboratory conditions. In California mice, on the other hand, we previously found that fathers gain body mass across their mates' second or subsequent pregnancies, corresponding to the period of care of the previous litter, but not during their mates' first pregnancies or other pregnancies during which no litter is present (Harris et al., 2011; Saltzman et al., 2015). In a recent study, moreover, body mass, fat mass, and food consumption in California mouse fathers were positively correlated with litter size (Zhao et al., 2017). These findings, together with the results of the present study, suggest that male California mice gain body mass and fat, presumably due to changes in food consumption and/or energy expenditure, in response to (potential) energetic challenges (i.e, presence of offspring, repeated
fasting, climbing exercise). This pattern likely reflects an adaptive response to cues signaling actual or potential increases in energetic demand in natural environments. In a laboratory setting, in contrast, the energetic demands of parental care or climbing exercise alone may be easily offset by the available food, supply (even under our food-restriction paradigm), allowing animals to not only maintain but build their fat reserves during periods of increased demand. Our findings further suggest that this ability was constrained by the energetic demands of fatherhood.

Food restriction can decrease body mass, body fat content, and resting oxygen consumption, and alter mass of several organs, in lab rats and mice, depending on the restriction paradigm and strain or species of animals (Colman et al., 2007; Faulks et al., 2006; Rothwell and Stock, 1982; Santos-Pinto, 2001; Sohal et al., 2009). In a study of Mus, however, Li et al. (2010) found that food-restricted animals that were provided with a limited amount of food two hours before lights-off each day ate significantly more than ad lib-fed controls immediately following daily food provisioning. Food-restricted animals in that study, as in ours, had higher body mass and fat mass than controls. In another study, rats under food restriction ate fewer meals but consumed more food during each meal and spent more time eating per meal than did rats fed ad lib (Duffy et al., 1989). In contrast to the mouse and rat studies, however, we did not find any significant effects of housing or reproductive condition on food consumption. This might reflect a limitation of our experimental design in that that we measured food consumption over only a single 4-h period (to avoid more prolonged separation of males from their mates and pups), which might be too short to detect any difference. In addition, if animals housed under challenging conditions increased their food intake, relative to control animals, when food was returned after their 24-h fasting periods every third day, this pattern would not have been detected by our one-time food-intake measurement on test day 5, when food was available ad lib.
Blood metabolic profile and liver mass

In our previous study (Zhao et al., 2017), virgin males had higher blood glucose concentrations, nominally higher plasma insulin concentrations, and nominally higher insulin sensitivity than non-breeding males, while neither group differed from breeding males. In contrast, none of these measures differed significantly among reproductive conditions in the current study. This difference might be accounted for by differences between the two experiments in the composition of the virgin and non-breeding groups. In the earlier study, virgin males were housed with another male and non-breeding males were housed with a tubally ligated female. In the current experiment, to better control for cohabitation with a female, males in the virgin group were housed with an ovariectomized female and non-breeding males were housed with an ovariectomized female treated with estrogen and progesterone. Testosterone can improve insulin sensitivity (Varlamov et al., 2015), increase fat oxidation and lipolysis (Santosa and Jensen, 2015), reduce gluconeogenesis, and increase glycogen synthesis and storage in the liver, thereby lowering circulating glucose levels (Shen and Shi, 2015). Cohabitation with a female (current study) might increase testosterone levels, compared to cohabitation with a male (previous study) (e.g., Mongolian gerbil [Brown et al., 1995], Djungarian hamster [Reburn and Wynne-Edwards, 1999], cotton-top tamarin [Ziegler and Snowdon, 2000]), potentially leading to increased insulin sensitivity. Although we did not measure testosterone levels in either the present experiment or the previous one, we did find that virgin males in the previous study (housed with another virgin male), but not in the current one (housed with an ovariectomized female), had significantly lower testis masses than males housed with females, consistent with a possible effect of testosterone on insulin sensitivity.
In contrast to reproductive condition, housing condition influenced insulin sensitivity in the present study: both HOMA-IR and QUICKI (see Methods) indicated that males housed under standard lab conditions had higher insulin sensitivity than those housed under challenging conditions, although neither blood glucose nor plasma insulin levels differed significantly. Males housed under standard conditions also had higher testis masses than those in the challenge condition, again raising the possibility that group differences in testosterone levels might have contributed to differences in insulin sensitivity. The high leptin levels in the challenged mice also might have contributed to their lower insulin sensitivity, as insulin resistance can be exacerbated by hyperleptinemia (Chen et al., 2017). The combination of high leptin levels with high fat mass and high blood lipid levels suggests that the challenged animals might also have had lower leptin sensitivity than the control mice (Lin et al., 2000). Similar patterns have been observed in other animals under conditions of low food availability (e.g., hibernating brown bears, *Ursus arctos horribilis*: Rigano et al., 2017; starving humans: Soeters and Soeters, 2012), suggesting that this might be a beneficial response to food restriction by maintaining energy availability.

Basal plasma corticosterone levels were not influenced by either housing condition or reproductive status of the males. The glucocorticoid hormones corticosterone and cortisol have pronounced effects on glucose, fat, and protein metabolism and can be influenced by social and reproductive conditions, such as pair bond formation and parenthood (Carter, 1998; Uchoa et al., 2014). In humans, for example, both circulating and salivary cortisol levels were lower in men after their infants were born than during their partners’ late pregnancies (Berg and Wynne-Edwards, 2002; Storey et al., 2000). Male marmosets had an increase in urinary cortisol levels after the birth of their first litter but a decrease after the birth of their second litter (Nunes et al., 2001). Prairie vole fathers had significantly lower serum corticosterone levels compared to
virgins and paired males without offspring (Campbell et al., 2009). In previous studies from our lab, however, neither basal nor stress-induced plasma corticosterone levels differed between California mouse fathers, virgin males, and males that were either vasectomized or housed with a tubally ligated female, indicating that, as in the present study, corticosterone concentrations were not influenced by reproductive status (Chauke et al., 2011; de Jong et al., 2013; Harris and Saltzman, 2013).

In many species, corticosterone levels can also be influenced by environmental challenges, such as food restriction (Heiderstadt et al., 2000; Stamp et al., 2008). In a previous study, a chronic variable stress paradigm increased basal plasma corticosterone concentrations in male California mice, and this effect did not differ between fathers, virgins, and males housed with tubally ligated females (De Jong et al., 2013). In the present study, however, corticosterone levels, measured >26 h after food was returned to the challenged animals from the previous 24-h fast, did not differ between males housed under standard lab conditions and energetically challenging conditions. This finding was unexpected. It is possible that our challenge paradigm was not sufficiently stressful to maintain higher corticosterone levels after the food was returned. This could be related to our previous findings that California mice are relatively resistant to stress. For example, several acute stressors elicit only very transient corticosterone elevations (Harris et al., 2012), and in our chronic variable stress paradigm, we used three acute stressors per day because, in contrast to lab mice and rats (e.g., Cox et al., 2011; Onaolapo et al., 2017), 1-2 stressors per day did not consistently elevate basal plasma corticosterone levels (De Jong et al., 2013; Harris et al., 2013; unpub. data). Another possibility is that the challenge paradigm in the present study did alter circulating corticosterone levels, but only during some parts of the diel rhythm. A limitation of our study is that we measured corticosterone at only a single time point,
which might have obscured effects of the challenge on diel rhythms.

**Behavior**

Both time spent immobile in the tail-suspension test and anhedonia (reduced preference for sweet solution in the saccharin preference test) are considered markers of depression-like behavior (Cryan et al., 2005; Schrader, 1997). Neither measure differed between reproductive groups or housing conditions, consistent with our previous findings (Zhao et al., 2017). In addition, virgin, non-breeding, and breeding males in the present study did not differ significantly in an indicator of anxiety-like behavior (number of fecal boli produced) during tail-suspension tests or pain-sensitivity tests. Housing conditions, on the other hand, affected measures of both anxiety-like behavior and pain sensitivity. Males housed under challenging conditions had a shorter latency to nociceptive behavior, indicative of higher pain sensitivity, and produced more fecal boli during the tail-suspension test, possibly indicating greater anxiety (Archer, 1973; Bronikowski et al., 2001; Coleman et al., 1998; Flint et al., 1995; Pometlová et al., 2016).

Activation of analgesic mechanisms can be modulated by fear and anxiety: manipulations that induce fear/anxiety can provoke analgesia (i.e., reduce pain sensitivity) (Vendruscolo et al., 2004). Thus, we expected that animals in the challenge condition would show lower pain sensitivity and more anxiety-like behavior during tail-suspension tests. We found, however, that while animals in the challenge condition showed higher pain sensitivity, they also showed more anxiety-like behavior (fecal boli) during tail-suspension tests. The mechanism and functional significance of this difference between mice in the two housing conditions are not known. In humans, fasting is frequently accompanied by analgesia, mood enhancement and heightened
alertness (Michalsen et al., 2006; Michalsen 2010); however, effects of food restriction on anxiety-like behaviors vary in rodents (Jahng et al., 2007; Levay et al., 2007).

**VO$_2$max and correlates**

VO$_2$max is a measure of maximal aerobic metabolic rate, with higher VO$_2$max indicating a greater capacity for sustained, aerobically supported exercise. Endurance exercise training can increase VO$_2$max, and decrease fat and body mass, in humans and rodents (e.g., Huang et al., 2005; Joyner and Coyle, 2008; Swallow et al., 1998). While VO$_2$max typically responds positively to aerobic exercise conditioning, it does not necessarily show the same response to strength training. Strength training can, however, positively influence bone mineral density and muscle mass in humans and lab rodents (Coll-Risco et al., 2016; Hentschke et al., 2017; Layne and Nelson, 1999; Tipton and Ferrando, 2008). Towers similar to the ones used in the present study have been shown to increase bone mineral density in two strains of lab mice during a 4-week period (Lionikas and Blizard, 2008; Mori et al., 2003). In the same study, a strain × exercise interaction in the forelimb biceps brachii muscle was also observed, but no effect of climbing exercise was found on food intake, body mass or mass of hindlimb muscles (Lionikas and Blizard, 2008; Mori et al., 2003). A similar study found that hindlimb muscles of rats housed in towered cages were heavier than those of control rats after 4 weeks of exercise, but not after 8 weeks of exercise (Notomi et al., 2001).

In the present experiment, we found that mass-adjusted VO$_2$max was lower in animals housed in cages with climbing towers than in those housed under standard conditions. In addition, challenged mice had lower hematocrit and triceps surae mass, both of which typically are positively related to VO$_2$max within species of mammals and birds (Chappell et al., 1999;
Chappell et al., 2007; Kanstrup and Ekblom, 1984). The opposite effects that we observed for animals housed with towers, compared to studies of rats and lab mice, might be accounted for by the different type of exercise training that occurred and/or by our food-restriction paradigm. Previous studies found that food restriction decreased hindlimb muscle mass (Sohal et al., 2009: 6 or 23 months) in lab mice as well as resting oxygen consumption in rats and mice (Rothwell and Stock, 1982: 17 days; Santos-Pinto and Griggio, 2011: 3 months; Sohal et al., 2009), but to our knowledge, effects of food restriction on VO$_2$max have not been studied. In addition, challenged mice may have compensated for the additional exercise required by the climbing towers by decreasing other activities, similar to what has been shown in lab rodents given access to running wheels (e.g., Copes et al., 2015).

**Mothers and pups**

At the time of weaning, mothers and pups in the breeding pairs had lower body mass under challenging conditions than under standard conditions, indicating that the climbing + food-restriction paradigm was costly for the females and their offspring. The effects of the challenge condition on pup lean mass and fat mass were apparently mediated by the mother’s body mass, because the differences were reduced when mother’s mass was included as a covariate. On the other hand, neither the number of pups weaned per litter nor the age at eye opening differed between conditions. Reproductive female rodents under food restriction or elevated energy demands can have reduced body mass compared to reproductive females housed under standard conditions (Sabau and Ferkin, 2013) and have been reported to spend less time (Marsteller and Lynch, 1987; Sabau and Ferkin, 2013; Smart, 1976; Smart and Preece, 1973), more time (Masoro et al., 1982; Wiener et al., 1977), or similar amounts of time (Crnic, 1976; König, 1989)
engaging in maternal behavior. The effects of food restriction on females’ milk yield or milk composition are not well studied. Because pup survival did not decline in the present study but maternal condition did, females in the challenge condition seemed to favor investment in their offspring rather than in themselves. It is also possible that the low body mass of pups in the challenge condition resulted, at least in part, from direct exposure of the pups to food restriction: California mouse pups begin eating solid food by 20 days of age (unpub. obs.), at least one week before the age of weaning. Unfortunately, we did not collect longitudinal data on mothers and pups, which would have allowed us to better evaluate this possibility.

Conclusions

Overall, our findings suggest that our physical and energetic challenge paradigm led to decreased peripheral sensitivity to both insulin and leptin, associated with increased body fat and body mass, in male California mice. Additionally, males in the challenge condition showed decrements in measures related to exercise performance. For the most part, these effects of the challenge paradigm were not modulated by reproductive status. Thus, we found little support for our hypothesis that subjecting males to an energetic challenge would reveal costs of fatherhood. It is certainly possible that other types or combinations of stressors or energetic challenges, such as low ambient temperature or exposure to predators, would reveal more pronounced effects of fatherhood; however, our findings, in combination with results of our previous studies (Andrew et al., 2016; Zhao et al., 2017), suggest that being a father is not necessarily particularly expensive, at least in this biparental rodent. Alternatively, fathers in this species and perhaps other biparental mammals might experience increased energetic demands, compared to non-
fathers, but these demands might be counteracted by the hormonal changes that males undergo as they transition into fatherhood (Saltzman and Ziegler, 2014).
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Conflict of interest

The authors declare that there was no conflict of interests.

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References


**Fig. 1. Timeline of experimental procedures.** PPD = postpartum day. For logistical reasons, test procedures shown as occurring on PPD 2-16 were performed one day later (i.e., on PPD 3-17) for approximately half of the animals in each experimental condition. Removal and return of food was performed only for animals in the Challenge condition.
Fig. 2. Estimated marginal means (EMM) and standard errors (SE) of male postpartum body mass, adjusted for age. ANCOVA revealed an interaction between housing and reproductive conditions \( (P = 0.037) \) and a main effect of housing condition \((P < 0.001)\). Sample sizes are shown in the bars.
Fig. 3. EMM ± SE of squared fat mass, adjusted for variation in age. ANCOVA revealed an interaction between housing and reproductive conditions (P = 0.019) and a main effect of housing condition (P < 0.0001). Sample sizes are shown in the bars.
ANCOVA revealed that fat mass, triceps surae mass, testis mass and liver mass all differed significantly between Control (open shapes, n=34) and Challenge (closed shapes, n=30) conditions, but lean mass and heart mass did not. Fat mass and triceps surae mass also showed higher variance among Challenge individuals than among Control animals. Liver mass is shown on a log₁₀-transformed y-axis. Circles – VM
(n=21), triangles – NB (n=22), squares – BM (n=21). (One BM in the Control condition was an outlier with very high triceps surae mass, and was excluded from the graph and statistical analyses of this trait.)
Table 1. Results of Pearson correlations (Spearman for urine and feces production) and paired t-tests comparing values from the two trials for tests conducted on two successive days, and for paired organ masses. Positive t values indicate that trial 1 > trial 2 or, for paired organs, left > right. Significant P values (P ≤ 0.019, when modified for Adaptive False Discovery Rate) are both bold and underlined. Nominally significant P values (0.019 < P < 0.05) are underlined but not bold.

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Table 2. Results of ANCOVAs comparing the three reproductive groups (breeding male, non-breeding male, virgin male) in the two housing conditions (control, challenge). Significance levels, estimated marginal means (EMM) and associated standard errors (SE) from ANCOVAs are reported. See text for covariates used in various analyses. TC - total cholesterol, HDL - high-density lipoprotein cholesterol, TRG - triglyceride, HOMA-IR - insulin sensitivity calculated from homeostatic model assessment of insulin resistance, QUICKI - insulin sensitivity calculated from quantitative insulin check index of insulin sensitivity. Significant P values (P ≤ 0.019, when modified for Adaptive False Discovery Rate) are both bold and underlined. Nominally significant P values (0.019 < P < 0.05) are underlined but not bold.

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