### Title

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### Authors

Zhao, Meng
Garland, Theodore
Chappell, Mark A
et al.



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# Metabolic and affective consequences of fatherhood in male California mice

Meng Zhao[a,b], Theodore Garland Jr.[a,b], Mark A. Chappell[a,b], Jacob R. Andrew[a,b], Wendy Saltzman[a,b,*]

[a] Department of Biology, University of California, Riverside, USA
[b] Evolution, Ecology and Organismal Biology Graduate Program, University of California, Riverside, USA

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ABSTRACT

Physiological and affective condition can be modulated by the social environment and parental state in mammals. However, in species in which males assist with rearing offspring, the metabolic and affective effects of pair bonding and fatherhood on males have rarely been explored. In this study we tested the hypothesis that fathers, like mothers, experience energetic costs as well as behavioral and affective changes (*e.g.*, depression, anxiety) associated with parenthood. We tested this hypothesis in the monogamous, biparental California mouse (*Peromyscus californicus*). Food intake, blood glucose and lipid levels, blood insulin and leptin levels, body composition, pain sensitivity, and depression-like behavior were compared in males from three reproductive groups: virgin males (VM, housed with another male), non-breeding males (NB, housed with a tubally ligated female), and breeding males (BM, housed with a female and their first litter). We found statistically significant ($P < 0.007$, when modified for Adaptive False Discovery Rate) or nominally significant ($0.007 < P < 0.05$) differences among reproductive groups in relative testis mass, circulating glucose, triglyceride, and insulin concentrations, pain sensitivity, and anxiety-like behaviors. *A priori* contrasts indicated that VM produced significantly more fecal pellets than BM in the tail-suspension test, had significantly higher glucose levels than NB, and had significantly lower average testis masses than did NB and BM. *A priori* contrasts also indicated that VM had a nominally longer latency to the pain response than NB and that VM had nominally higher insulin levels than did NB. For breeding males, litter size (one to three pups) was a nominally significant positive predictor of body mass, food consumption, fat mass, and plasma leptin concentration. These results indicate that cohabitation with a female and/or fatherhood influences several metabolic, morphological, and affective measures in male California mice. Overall, the changes we observed in breeding males were minor, but stronger effects might occur in long-term breeding males and/or under more challenging environmental conditions.

## 1. Introduction

Parental investment by fathers can greatly increase reproductive success in biparental species by enhancing survival and development of offspring [4,10]. In addition to its influence on offspring, paternal care can potentially influence the physiological, morphological and affective (*e.g.*, depression and anxiety) condition of fathers themselves. Such changes can result in trade-offs between current reproductive success and future survival and reproduction [31]. On the other hand, some of these changes may be beneficial to the father's health, or contribute to better parental care, by helping the parent to meet energetic demands or challenges during parenting.

Many physiological and affective changes in fathers are likely to be mediated by neuroendocrine adjustments during the transition to fatherhood. Depending on the species, fathers may undergo changes in circulating concentrations of gonadal steroids (estrogens, progesterone, androgens), neuropeptides (oxytocin, vasopressin), glucocorticoids (cortisol, corticosterone) and prolactin [68], as well as in expression of their respective receptors [4,62]. Some of these neuroendocrine changes appear to directly influence the expression of parental care and could potentially influence metabolic and affective functions in fathers [68].

Several studies have shown that fatherhood can be energetically costly for fathers in biparental mammals. Prairie vole (*Microtus ochrogaster*) fathers, for example, had lower body mass and less subcutaneous fat than non-fathers [16,48]. At the same time, similar to lactating females [72], fathers spent more time feeding during the postpartum period, possibly leading to recovery in weight. Newly paired male prairie voles also showed increased preference for sucrose solution, suggesting that they needed to increase energy intake [16]. In

* Corresponding author at: Department of Biology, University of California, Riverside CA 92521, USA.
*E-mail address:* Saltzman@ucr.edu (W. Saltzman).

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two biparental primates, common marmosets (*Callithrix jacchus*) and cotton-top tamarins (*Saguinus oedipus*), expectant fathers undergo significant weight increases across their mate's pregnancy, which are thought to prepare males for the energetic demands of fatherhood [83]. This is followed by a drop in body weight during the postpartum period [1,84]. In California mice (*Peromyscus californicus*), males housed with a non-reproductive female were significantly heavier than those housed with a primigravid (first-time pregnant) female [42,67]. In addition, experienced California mouse fathers underwent significant increases in body mass across their mates' pregnancy when housed with pups from their previous litter [67]. Finally, California mouse fathers had smaller fat pads than virgin males [2]. However, other potential metabolic and morphological consequences of being a father, such as changes in glucose regulation and dietary preferences, have rarely been investigated.

A general pattern of reductions in autonomic, neuroendocrine and behavioral reactivity has been reported in mothers of several mammalian species, including humans (*Homo sapiens*), mice (*Mus musculus*), rats (*Rattus norvegicus*) and sheep (*Ovis aries*). This has been attributed in part to hormones associated with parturition and lactation, including prolactin and oxytocin [15,71]. Stress hyporesponsiveness, as well as reduced anxiety and fearfulness [53] in mothers, might reduce the likelihood that maternal care will be disrupted by stress, and could be important for offspring development [71] but potentially at a cost to the mother's self-maintenance and survival [7]. Thus, dampened affect and stress-reactivity might reflect a trade-off between self-maintenance and reproduction [7,81].

Although males do not experience the striking changes in endocrine profiles associated with pregnancy and lactation, paternal experience may elicit neuroendocrine changes capable of modulating stress responses and emotionality in fathers. In one study of prairie voles, fathers displayed higher anxiety-like behavior and/or higher depression-like behavior than virgin males and sexually experienced males without offspring [52]; however, another study of the same species found lower levels of anxiety-related behaviors in fathers than in virgin males [48]. In California mice, two studies found reduced behavioral responses to stress in fathers compared with non-fathers [5,20]. In one of these experiments, fathers engaged in fewer interrupted grooming sequences during a novel-object open-field test compared both to virgins previously exposed to pups and to virgins that had never been exposed to pups [5]. In the second study, virgin males (maintained in unrelated same-sex pairs) and nonbreeding males (vasectomized males housed with intact females) showed behavioral changes in response to predator urine as compared to the 5 min prior to urine exposure, whereas fathers showed no behavioral responses to the same stressor [20]. In contrast to behavioral measures, studies characterizing plasma corticosterone levels in male California mice found minimal differences among reproductive groups after acute stress, after chronic stress, or under baseline conditions [20,23,41,43]. Similarly, no differences were found among male reproductive groups in expression of mRNA for corticotropin-releasing hormone or vasopressin in several brain regions, either under baseline conditions or after exposure to a chronic stressor [23].

In this study we tested the hypothesis that fathers, like mothers [14,72], experience energetic costs as well as behavioral and affective changes associated with parenthood. To do so, we investigated several potential costs of fatherhood in the California mouse, a monogamous, biparental rodent. Fathers engage in all the same parental behaviors as mothers except nursing, and to a similar extent. Fathers can also make important contributions to their pups' survival and development [11,17,26,35,37,82]. We compared several physiological and morphological measures, including blood metabolic markers (glucose, cholesterol and triglycerides), organ masses, body mass, and body composition (fat and lean masses) between new fathers and non-reproductive adult males. We also characterized behaviors potentially associated with differences in metabolic condition, including food intake, preference for high-fat diet, and predatory aggression. 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 [32,61]. Finally, we examined nociceptive responses (an important part of defensive systems influenced by affective state [77]) using the hot-plate test. We predicted that fathers, compared to non-reproductive males, would have changes in body mass and body fat mass, higher food intake, and altered blood glucose and lipid levels. We further predicted that fathers would differ from non-fathers in pain sensitivity, depression-like and anxiety-like behavior, and preferences for highly palatable food and liquid.

## 2. Methods

### 2.1. 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 daily and changed weekly before testing started. During the period of data collection, cages were changed once, on test day 5 (see below).

Mice were weaned at 27–32 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.

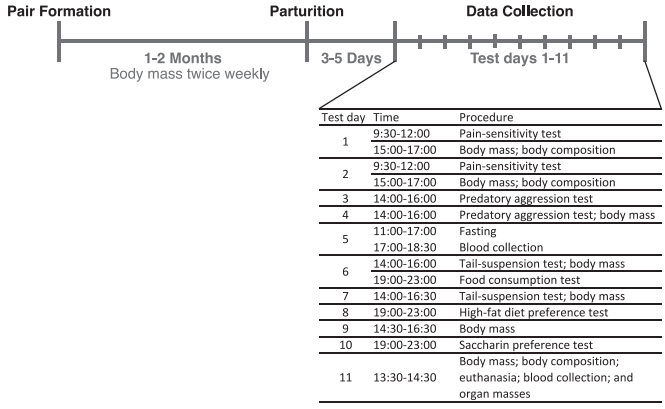
All procedures used were in accordance with the *Guide for the Care and Use of Laboratory Animals* and were approved by the UCR Institutional Animal Care and Use Committee. UCR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

### 2.2. Experimental design

At 158–201 days of age (176.95 ± 1.43 days, mean ± SEM), 44 adult males were randomly assigned to three experimental groups: virgin males (VM, $n = 15$; housed with an unrelated male from their original same-sex group), non-breeding males (NB, $n = 14$; housed with a tubally ligated female and thus able to mate but unable to reproduce; see below) or breeding males (BM, $n = 15$; housed with an intact female). Pairmates in all three groups were no more closely related to each other than first cousins. In the VM group, only one randomly selected male per pair was used for data collection. After the birth of the first litter in each of the breeding pairs (or at a matched time point for VM and NB), animals were left undisturbed for 3–5 days until the beginning of the 11-day testing period (see Fig. 1).

### 2.3. Tubal ligation

Females in non-breeding pairs underwent bilateral ligation of the oviducts as previously described [43]. After surgery, females were housed individually for 14 days until being paired with a male. At the end of the experiment, tubally ligated females were sacrificed by $CO_2$ inhalation and dissected to check for pregnancy. None had visible fetuses.




| Test day | Time | Procedure |
|---|---|---|
| 1 | 9:30-12:00 | Pain-sensitivity test |
| | 15:00-17:00 | Body mass; body composition |
| 2 | 9:30-12:00 | Pain-sensitivity test |
| | 15:00-17:00 | Body mass; body composition |
| 3 | 14:00-16:00 | Predatory aggression test |
| 4 | 14:00-16:00 | Predatory aggression test; body mass |
| 5 | 11:00-17:00 | Fasting |
| | 17:00-18:30 | Blood collection |
| 6 | 14:00-16:00 | Tail-suspension test; body mass |
| | 19:00-23:00 | Food consumption test |
| 7 | 14:00-16:30 | Tail-suspension test; body mass |
| 8 | 19:00-23:00 | High-fat diet preference test |
| 9 | 14:30-16:30 | Body mass |
| 10 | 19:00-23:00 | Saccharin preference test |
| 11 | 13:30-14:30 | Body mass; body composition; euthanasia; blood collection; and organ masses |

**Fig. 1.** Timeline of experimental procedures.

### 2.4. Measurements

#### 2.4.1. Morphology

*2.4.1.1. 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 at 13:00–15:00 h twice weekly, at 3- to 4-day intervals. In addition to providing mass data, this allowed us to assess overall health, habituate the animals to handling, and monitor pregnancies. Subjects were also weighed between 13:30 and 17:20 h on test days 1, 2, 4, 6, 7, 9 and 11 (Fig. 1).

*2.4.1.2. Body composition.* On test days 1, 2, and 11, body composition was assessed using an EchoMRI-100 magnetic resonance whole-body analyzer (Echo Medical Systems, Houston, TX, USA), calibrated in our lab for this species. Mice were weighed and then placed in a plastic tube without anesthesia or sedation, and the masses of lean, fat and water fractions were measured during a scan lasting approximately 90 s. Lean and fat masses were computed as percentages of total body mass. The average of day 1 and day 2 was used in data analysis.

*2.4.1.3. Euthanasia and organ collection.* On test day 11, males were decapitated between 13:30 and 16:30 h, and trunk blood was collected in weigh boats primed with 0.1 ml of heparin (1000 USP units/ml). Immediately after blood collection, organs [heart, liver, leg muscles (left and right triceps surae), testes (left and right) and kidneys (left and right)] were removed rapidly, weighed and stored at − 80 °C. Blood samples were centrifuged for 12 min at 13,300 rpm and 4 °C, and plasma was removed and stored at − 80 °C for leptin assays.

#### 2.4.2. Metabolism/energetics and related behaviors

*2.4.2.1. Food consumption.* Starting at the time of the lights-off (active) period on test day 6, we separated each male from his cage mate(s) in one half of a clean cage for 4 h by inserting a stainless steel mesh partition across the width of the cage. The food hopper was blocked from the cage mate(s) by a small sheet of stainless steel, so that only the subject had access to food in the hopper; a water bottle was also provided. 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 male was returned to his home cage and reunited with his cage mate(s) immediately after the test.

*2.4.2.2. High-fat-diet preference.* Each male was isolated in half of a clean cage for 4 h on test day 8, starting at the time of lights-off (19:00 h), with access to food and water as described above for the food-consumption test. The food hopper was divided by a steel partition into two compartments, each containing ~40 g 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 (right or left) 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 amount of consumed high-fat diet divided by the overall amount of food consumed was calculated as an index of relative preference for high-fat diet.

*2.4.2.3. Predatory aggression.* Tests for predatory aggression on crickets were performed on test days 3 and 4 between 14:00 and 16:00 h. Mice were placed singly in a clean cage containing 22 g of aspen shavings. This amount is enough to cover the cage bottom while preventing crickets from hiding underneath the shavings. No cotton, food, or water was provided. After the mouse was permitted to habituate to the test cage for 15 min, a live cricket (0.2–0.5 g) was weighed and dropped into the cage on the side opposite the mouse, following protocols similar to those of Gammie et al. [30]. The mouse was observed and video-recorded for 7 min, and latency to first appearance of the following behaviors was recorded: *Attack*: pounce on or tear a cricket with the forepaws. Attack, rather than killing, was recorded because the actual time of death of a cricket was difficult to discern. *Eating*: ingest some part of the cricket.

Cricket remains were weighed after the trial period. For each measure, mice that did not attack crickets were assigned a value of 7 min, and values for the two successive trial days were used to assess changes with repeated testing. We did not fast mice prior to testing, in order to minimize disturbance to the animals (especially breeding males and their families). Pilot tests showed that most animals attacked crickets quickly (< 7 min) even without prior fasting.

*2.4.2.4. Blood metabolic profiles.* On test day 5, mice were fasted for 6 h, at approximately 11:00–17:00 h, immediately after which they were anesthetized with isoflurane. Blood was collected from the retro-orbital sinus into heparinized micro-hematocrit capillary tubes. Three samples (70 µl each) were obtained from each mouse. For each of the first two tubes, half of the sample was immediately used to measure glucose level in whole blood with a Contour Next blood glucose monitoring system (Mishawaka, IN, USA). The other half was used to determine total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein (LDL), non-HDL cholesterol (non-HDL), triglyceride and ratio of TC to HDL (TC/HDL) in whole blood with an automated analyzer (model LDX; Cholestech Corporation, Hayward, CA, USA) and Lipid Profile GLU cassettes (item number 10–991). The third sample was centrifuged immediately for 12 min (13,300 rpm, 4 °C), hematocrit was measured, and plasma was removed and stored at − 80 °C for future insulin assays.

*2.4.2.5. Leptin ELISA.* Leptin concentrations in plasma were determined using a mouse leptin enzyme-linked immunosorbent assay (ELISA) kit (Crystal Chem, Downers Grove, IL, USA). 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.

We performed three validation procedures to characterize assay parallelism, accuracy, and precision, following Chauke et al. [20], Good et al. [33], and Harper et al. [40]. In brief, parallelism was determined by comparing the log-logit slope of serially diluted California mouse plasma ($n = 6$) to the log-logit slope of the standard curve (Fig. 2). Slope equality was determined using linear regression in SPSS (IBM Corporation, Somers, NY). The log-logit-transformed slope of the standard curve was statistically indistinguishable from the log-logit-transformed slope of serially diluted California mouse plasma ($P = 0.444$; Fig. 2). To determine accuracy we added 10 µl of California mouse plasma with a known leptin concentration to each point on the standard curve and compared the observed leptin concentration in each

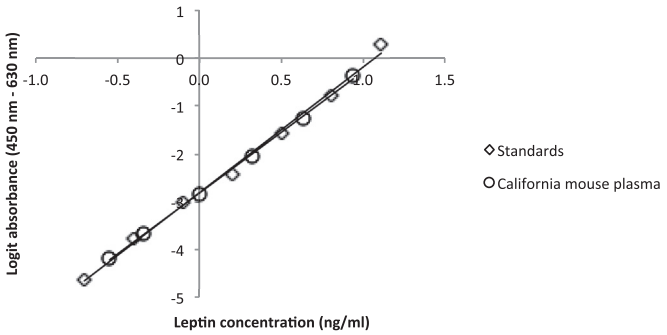



**Fig. 2.** Parallelism of leptin levels from serial dilutions of California mouse plasma (circles) and standards from a commercially available mouse leptin ELISA kit (squares). Data were transformed using the log-logit method. California mouse plasma: y = 2.5443x - 2.8192. Standard curve: y = 2.6274x - 2.8088. Difference between slopes: $P = 0.444$.

sample to the expected concentration. Accuracy was 96.97 ± 2.10% (mean ± SE), and the difference between the observed and expected leptin concentrations per sample was statistically indistinguishable from zero ($P = 0.217$).

Assay precision was determined by calculating intra-assay variation as the coefficient of variation (CV) between triplicate samples in an assay. The intra-assay CV was 5.30%. Inter-assay variation was determined by calculating the CV for the average leptin levels in two plasma pools from laboratory-maintained California mice (one from virgin males and another from breeding males). Inter-assay CVs for the two pools were 5.34% and 2.42%, respectively. Each assay included samples from animals in each of the three reproductive groups.

*2.4.2.6. Insulin ELISA*. Insulin concentrations in plasma were determined using a mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA). All samples were assayed in duplicate 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.

As described above for the leptin ELISA kit, we characterized parallelism, accuracy, and precision of the insulin ELISA. For parallelism, the log-logit-transformed slope of the standard curve was statistically indistinguishable from the log-logit-transformed slope of serially diluted California mouse plasma ($n = 7$; $P = 0.679$; Fig. 3). Average assay accuracy was 105.70 ± 2.70% (mean ± SE), and the difference between the observed and expected insulin concentrations per tube was statistically indistinguishable from zero ($P = 0.071$). The intra-assay CV was 6.01%. Inter-assay CV, using a plasma pool from virgin male California mice, was 1.77%. Each assay included samples from animals in each reproductive group. Samples from two animals (both in the VM group) were above the highest value of the standard curve. We

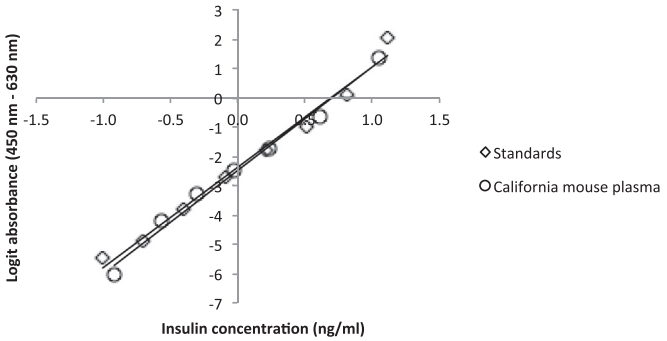



**Fig. 3.** Parallelism of insulin levels from serial dilutions of California mouse plasma (circles) and standards from a commercially available mouse insulin ELISA kit (squares). Data were transformed using the log-logit method. California mouse plasma: y = 3.5358x - 2.4810. Standard curve: y = 3.4276x- 2.3737. Difference between slopes: $P = 0.679$.

therefore used this value (12.8 ng/ml) as a conservative estimate of the insulin concentration for these two samples.

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

*2.4.3.1. Pain sensitivity*. Tests were administered at 09:30–12:00 h on test days 1 and 2, using a protocol modified from one used for lab mice and rats (*e.g.*, [77,78]) (Fig. 1). We used a hot plate (Lab-Line Instruments, Inc., Melrose Park, IL, USA) modified with a custom feedback circuit that permitted precision temperature control. During tests, the hot plate surface was maintained at 44.3 ± 0.2 °C. A pilot study indicated that this temperature was high enough to elicit nociceptive behaviors without causing any tissue damage and was sensitive enough to detect inter-animal differences (unpub. data). Animals were placed in a plexiglass cylinder (6 cm height × 20 cm diameter) on the hot plate. A ventilated plexiglass lid was placed over the cylinder to prevent the mice from standing upright and jumping out. 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. The pilot study revealed that California mice frequently lick their front paws independently of nociceptive behavior, so only hind-paw behaviors were used as measures of nociception.

Animals were removed from the hot plate immediately after showing any of the above behaviors. Animals that did not show any of these behaviors were removed from the hot plate after 120 s. In addition to latency to nociception, we recorded the number of fecal pellets and urine pools deposited on the hot plate, similar to open-field tests, as measures of anxiety [3,13,21,28]. For all three measures, values for the two successive trial days were compared to determine whether pain sensitivity changed with repeated testing.

*2.4.3.2. Tail suspension*. On test days 6 and 7, between 14:00 and 16:00 h, mice were suspended by their tails from a padded plastic clip (at approximately 1/3–2/3 of the distance from the base to the end of the tail, depending on tail length) connected to a ring stand. To block the mice from climbing their tails during the test, a plastic shield was placed under the clip and the tail was passed through a hole in the shield. 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 (www.warthog.ucr.edu). The MAD-1 transduces activity as voltage, with signal intensity correlated to activity intensity. Activity was recorded every 0.004 s for a total of 6 min. LabAnalyst software (www.warthog.ucr.edu) was used for baseline correction and calculation of activity duration [55]. The duration of immobility was measured as the time the force of the mouse's movements was below a threshold, which was determined by comparing manually scored videotapes of the tests with automated scores of individual animals. In addition, we recorded the number of fecal pellets deposited during the 6 min of testing.

*2.4.3.3. Saccharin preference*. Males' preference for saccharin (Sweet'N Low; Cumberland Packing Corp., Brooklyn, NY, USA) solution (0.2% w/v in water) *vs.* water was assessed on test day 10. This concentration was based on a study in *Mus* [50] as well as a pilot study in California mice (unpub. data). Starting at the time of lights-off (19:00 h), each mouse was separated from its cage mate(s) in a clean cage 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. Each syringe was attached to a steel nozzle, to be consistent with the animals' standard daily water bottles. The two syringes were placed on the two sides of the food hopper, with food in between; positions of the two types of liquid were randomly assigned. The syringes were weighed immediately before and after the 4-h test period, and the amount of each liquid consumed was calculated as the difference between initial and final mass. The amount of consumed

**Table 1**
Results of Pearson correlations 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.

| Trait | Unit | Transform | N of paired observations | r of Pearson correlation | P of Pearson correlation | t of paired *t*-test | P of paired *t*-test |
|---|---|---|---|---|---|---|---|
| Pain-sensitivity: latency | Second | none | 38 | 0.598 | **7.30E-05** | 1.216 | 0.232 |
| Pain-sensitivity: number of urine pools | – | one | 43 | 0.599 | **2.20E-05** | 0.496 | 0.623 |
| Pain-sensitivity: number of fecal pellets | – | none | 43 | 0.535 | **2.21E-04** | −2.096 | 0.042 |
| Predatory aggression: latency to attack cricket | Second | $\log_{10}$ | 43 | 0.311 | 0.043 | 4.046 | **2.18E-04** |
| Predatory aggression: latency to eat cricket | Second | $\log_{10}$ | 40 | 0.142 | 0.381 | 1.886 | 0.067 |
| Predatory aggression: mass of cricket consumed | Gram | none | 43 | 0.388 | 0.010 | 2.244 | 0.030 |
| Tail-suspension: duration of mobility | Minute | none | 42 | 0.712 | **1.27E-07** | −1.181 | 0.244 |
| Tail-suspension: number of fecal pellets | – | $\log_{10}$ | 43 | 0.749 | **7.36E-09** | −1.814 | 0.077 |
| Glucose | mg/dl | $\log_{10}$ | 41 | 0.809 | **1.52E-10** | −5.897 | **6.60E-07** |
| Fat mass | Gram | none | 42 | 0.996 | **7.41E-43** | −0.647 | 0.521 |
| Lean mass | Gram | none | 43 | 0.993 | **1.90E-39** | 1.779 | 0.083 |
| Percent fat mass | % | none | 42 | 0.994 | **7.29E-40** | −0.979 | 0.333 |
| Percent lean mass | % | none | 43 | 0.954 | **1.30E-22** | 1.370 | 0.178 |
| Triceps surae mass | Gram | none | 43 | 0.932 | **1.20E-19** | 1.987 | 0.053 |
| Testis mass | Gram | none | 43 | 0.957 | **1.14E-23** | 0.796 | 0.430 |
| Kidney mass | Gram | none | 43 | 0.990 | **1.11E-36** | −2.838 | **0.007** |

Significant *P* values ($P < 0.007$, when modified for Adaptive False Discovery Rate) are both bold and underlined. Nominally significant *P* values ($0.007 < P < 0.05$) are underlined but not bold.

saccharin solution divided by the overall liquid consumption was calculated as an index of relative preference for saccharin solution.

### 2.5. Data analysis

To analyze insulin-glucose dynamics, we calculated two surrogate measures of insulin sensitivity: the homeostatic model assessment of insulin resistance (HOMA-IR = (insulin (mU/l) ∗ glucose (mmol/l)/22.5), and the quantitative insulin check index of insulin sensitivity (QUICKI, 1/(log(insulin (mU/l)) + log(glucose (mg/dl))), both based on fasted glucose and insulin levels [8,9].

All traits were analyzed by analysis of covariance (ANCOVA) using SPSS. Age, time from pairing to measurement, and/or other potentially relevant variables (see Results) were used as covariates. We used *a priori* contrasts in the general linear model (GLM) procedure to compare mean values of each of the three groups (VM, NB, BM). 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 organs, values from the two trials were compared using a paired *t*-test and a Pearson correlation to gauge repeatability, and mean values were used for subsequent analyses. Within the breeding male group, we also performed regression analysis to determine if litter size was a predictor of any trait, while controlling for age, time from pairing to measurement, and/or other variables as covariates.

Excluding nuisance variables such as age, this study generated 180 *P* values, 44 of which were < 0.05 (see Results and online Supplementary Table S1). These tests include a substantial amount of nonindependence because the same individuals were measured for all traits, some traits are correlated, and many tests are interrelated (*e.g.*, the *a priori* contrasts computed for all three groups). 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 24 smallest *P* values would have adjusted *P* values < 0.05 (the highest being 0.007). All *P* values reported in the text and online supplementary material are raw values, not adjusted for multiple comparisons. We refer to *P* values < 0.007 as "significant" and those between 0.007 and 0.05 as "nominally significant." *P* values for the overall ANCOVA tests of a group effect are reported for completeness, but are not emphasized in the text and were not included when applying the Adaptive False Discovery Rate procedure.

## 3. Results

### 3.1. Morphology

#### 3.1.1. 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, and analyzed the slope of this regression as the dependent variable in an ANCOVA with age at first weighing as a covariate. We found no statistically significant differences among VM, NB, and BM (all *P* values for *a priori* contrasts > 0.217). We also analyzed mean body mass during the test days (excluding the one on day 6, taken after fasting on the previous day) and again found no group differences (all $P > 0.459$) (Table 2).

#### 3.1.2. Body composition

Fat and lean masses did not differ among reproductive groups, whether expressed as absolute masses or as percentages of total body mass (Table 2). The data on the two successive testing days for each male were highly correlated and not significantly different between days (Table 1).

#### 3.1.3. Organ masses

Masses of left and right triceps surae, testes, and kidneys all showed high correlations, and right kidneys were significantly heavier than left ones (Table 1), as has been seen in other mammals and with other organs [21,45]. VM had significantly lower average testis masses than did NB ($P = 0.00003$) and BM ($P = 0.00047$) (Fig. 4). No other organ


**Fig. 4.** Estimated marginal means (EMM) and associated standard errors (SE) of testis mass (mean of left and right testes), adjusted for variation in body mass, age, and time since pairing. *A priori* contrasts indicated that virgin males had significantly smaller testis masses than did non-breeding ($P = 0.00003$) and breeding males ($P = 0.00047$).

masses differed between reproductive groups based on *a priori* contrasts (Table 2).

### 3.2. Metabolism/energetics and related behaviors

#### 3.2.1. Blood glucose, lipid and cholesterol profiles

An ANCOVA of hematocrit, with age, time since pairing, and handling time as covariates, indicated no significant difference between groups (Table 1).

$Log_{10}$-transformed fasted glucose concentrations were highly correlated ($r = 0.809$) in the two successive blood samples collected on day 5, but were significantly higher in the second sample (Table 1). ANCOVA of mean values (with age, time since pairing, and handling time as covariates) indicated a positive effect of handling time on $log_{10}$-transformed glucose level ($P = 0.016$), and *a priori* contrasts indicated that VM had significantly higher glucose levels than NB ($P = 0.0066$) (Table 2) (Fig. 5A).

$Log_{10}$-transformed fasted triglyceride levels were significantly lower in BM than in VM ($P = 0.005$) and nominally lower in BM than in NB ($P = 0.011$; Fig. 5B). BM had nominally lower TC ($P = 0.024$) and non-HDL ($P = 0.026$) than VM. No significant differences were found for either HDL or LDL (Table 2).

#### 3.2.2. Circulating leptin and insulin concentrations

An ANCOVA (with age, time since pairing, and percent fat mass as covariates) on $log_{10}$-transformed plasma leptin concentration indicated a positive effect of age ($P = 0.001$) and percent fat mass ($P = 2.38 \times 10^{-11}$), but no significant group differences (Table 2). For $log_{10}$-transformed fasted plasma insulin concentration, ANCOVA (with age, time since pairing, and percent fat mass) revealed a positive effect of body mass ($P = 0.002$), and VM had nominally higher insulin levels than did NB ($P = 0.025$; Table 2). Insulin levels included three outliers with values > 12 ng/ml, all from the VM group. When analyzed without these three outliers, no differences were found between reproductive groups.

#### 3.2.3. Surrogate measures of insulin sensitivity

$Log_{10}$-transformed HOMA-IR was nominally lower in NB than in VM ($P = 0.035$). NB also tended to have higher QUICKI ($P = 0.025$) than VM (Table 2). Both of these measures indicated that insulin sensitivity was higher in NB than in VM.

#### 3.2.4. Food consumption

An ANCOVA (with age, time since pairing and body mass as covariates) indicated that food consumption did not differ between reproductive groups, based on *a priori* contrasts, but water consumption was 51.7% higher in NB than in VM ($P = 0.043$; Table 2).

#### 3.2.5. Predatory aggression

$Log_{10}$-transformed attack latencies on the two successive testing days were weakly correlated ($r = 0.311$, $P = 0.043$) and were significantly shorter on trial 2 than on trial 1 ($P = 0.000218$). An ANCOVA was conducted on mean attack latency raised to the 0.3 power, with age, time since pairing, and cricket mass as covariates. We found no statistically significant differences between groups (Table 2).

$Log_{10}$-transformed latencies to begin eating on the two successive testing days were not correlated and not significantly different (Table 1). An ANCOVA was conducted on $log_{10}$-transformed mean values, with age, time since pairing, and cricket mass as covariates and no group differences were found (Table 2).

The mass of cricket consumed on the two successive testing days was positively correlated ($r = 0.388$, $P = 0.010$) and tended to be lower on day 2 than on day 1 ($P = 0.030$; Table 1). An ANCOVA was conducted on mean values, with age, time since pairing, body mass, and cricket mass as covariates. Again, *a priori* contrasts indicated no significant differences between groups (Table 2).

#### 3.2.6. High-fat-diet preference

The three reproductive groups did not differ significantly in their preference for high-fat diet (Table 2). In general, males did not consistently prefer high-fat diet over standard diet.

### 3.3. Pain sensitivity, depression-like behavior, and anxiety-like behavior

#### 3.3.1. Pain sensitivity

Latencies to pain response on the two successive testing days were significantly correlated ($r = 0.598$) and not significantly different from one another (Table 1). An ANCOVA was conducted on $log_{10}$-transformed mean values from each male, with age, time since pairing, and body mass as covariates. *A priori* contrasts revealed a nominally significant difference between VM and NB ($P = 0.020$), with VM having a longer latency to the pain response (*i.e.*, a higher pain threshold) than NB (Table 2).

The numbers of urine pools excreted on the two successive testing days were significantly correlated ($r = 0.599$) and not significantly different from one another (Table 1). We found no significant differences between groups in an ANCOVA of rank-transformed mean values, with age, time since pairing, and body mass as covariates (Table 2). The numbers of fecal pellets expelled on the two successive testing days were also positively correlated ($r = 0.535$) and nominally lower on day 2 ($P = 0.042$; Table 1). An ANCOVA of rank-transformed mean values with age, time since pairing, and body mass as covariates indicated no differences between groups (Table 2).

#### 3.3.2. Tail-suspension test

The durations of mobility on the two successive testing days were significantly correlated ($r = 0.596$) and not significantly different (Table 1). When one outlier was deleted, the durations of mobility of two trials were more highly correlated ($r = 0.712$) and again not significantly different between days. Mean values were used for comparing groups, after deleting the outlier. An ANCOVA was conducted on squared data, with age, time since pairing, and body mass as covariates. *A priori* contrasts indicated no significant differences between VM, NB and BM (Table 2).

Numbers of fecal pellets on the two successive testing days were highly correlated ($r = 0.702$) and not significantly different from one another (Table 1). When $log_{10}$-transformed to make the distribution closer to bivariate normal, the numbers of fecal pellets were even more highly correlated ($r = 0.749$) and again not significantly different. Mean values were used for comparing groups. An ANCOVA (with age,

**Table 2**
Results of analysis of covariance with *a priori* contrasts comparing virgin males (VM), non-breeding males (NB), and breeding males (BM). Significance levels, estimated marginal means (EMM) and associated standard errors (SE) from ANCOVAs are reported. See text for covariates used in various analyses.

| Trait | Unit | Transform | D.F.[a] | F[a] | Group P[a] | P VM *vs.* NB | P VM *vs.* BM | P NB *vs.* BM | Virgin (VM) | | Non-breeding (NB) | | Breeding (BM) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | EMM | SE | EMM | SE | EMM | SE |
| Pain-sensitivity: latency | Second | $log_{10}$ | 2,37 | 3.063 | 0.059 | 0.020 | 0.120 | 0.445 | 1.527 | 0.052 | 1.344 | 0.055 | 1.404 | 0.053 |
| Pain-sensitivity: number of urine pools | – | rank | 2,37 | 0.319 | 0.729 | 0.644 | 0.736 | 0.433 | 22.091 | 3.350 | 19.846 | 3.509 | 23.775 | 3.412 |
| Pain-sensitivity: number of fecal pellets | – | rank | 2,37 | 0.058 | 0.944 | 0.840 | 0.890 | 0.738 | 21.948 | 3.038 | 22.838 | 3.182 | 21.326 | 3.094 |
| Predatory aggression: latency to attack cricket | Second | **0.3 | 2,37 | 0.761 | 0.475 | 0.960 | 0.307 | 0.271 | 2.172 | 0.116 | 2.180 | 0.113 | 1.997 | 0.114 |
| Predatory aggression: latency to eat cricket | Second | $log_{10}$ | 2,34 | 0.589 | 0.560 | 0.849 | 0.311 | 0.391 | 1.354 | 0.070 | 1.335 | 0.069 | 1.244 | 0.077 |
| Predatory aggression: mass of cricket consumed | Gram | none | 2,36 | 0.220 | 0.804 | 0.513 | 0.775 | 0.727 | 0.203 | 0.027 | 0.178 | 0.026 | 0.192 | 0.026 |
| Tail-suspension: duration of mobility | Minute | **2 | 2,36 | 0.178 | 0.837 | 0.903 | 0.648 | 0.568 | 12.027 | 1.890 | 12.350 | 1.890 | 10.704 | 2.064 |
| Tail-suspension: number of fecal pellets | – | $log_{10}$ | 2,37 | 4.986 | 0.012 | 0.114 | **0.003** | 0.110 | 0.433 | 0.080 | 0.615 | 0.081 | 0.809 | 0.084 |
| Hematocrit | % | none | 2,33 | 0.412 | 0.666 | 0.437 | 0.968 | 0.444 | 47.579 | 0.738 | 48.414 | 0.783 | 47.533 | 0.792 |
| Glucose | mg/dl | $log_{10}$ | 2,34 | 4.309 | 0.021 | **0.007** | 0.059 | 0.386 | 1.910 | 0.018 | 1.839 | 0.017 | 1.861 | 0.016 |
| TC | mg/dl | none | 2,30 | 3.496 | 0.043 | 0.065 | 0.024 | 0.753 | 142.687 | 5.127 | 128.575 | 6.572 | 125.976 | 5.059 |
| HDL | mg/dl | **0.5 | 2,27 | 0.592 | 0.561 | 0.383 | 0.822 | 0.311 | 6.895 | 0.374 | 6.450 | 0.452 | 7.000 | 0.322 |
| TRG | mg/dl | $log_{10}$ | 2,32 | 5.448 | 0.010 | 0.932 | **0.005** | 0.011 | 2.395 | 0.161 | 2.410 | 0.159 | 1.872 | 0.165 |
| LDL | mg/dl | none | 2,14 | 1.065 | 0.375 | 0.177 | 0.472 | 0.574 | 67.437 | 16.526 | 42.831 | 15.739 | 54.351 | 15.665 |
| Non-HDL | mg/dl | none | 2,25 | 2.850 | 0.078 | 0.269 | 0.026 | 0.275 | 94.541 | 8.342 | 84.797 | 7.630 | 74.581 | 7.329 |
| TC/HDL | – | **0.5 | 2,25 | 0.573 | 0.571 | 0.872 | 0.381 | 0.342 | 1.723 | 0.139 | 1.746 | 0.127 | 1.599 | 0.122 |
| Circulating leptin | ng/ml | $log_{10}$ | 2,37 | 0.799 | 0.458 | 0.339 | 0.834 | 0.256 | 0.371 | 0.048 | 0.306 | 0.048 | 0.386 | 0.050 |
| Circulating insulin | ng/ml | $log_{10}$ | 2,33 | 2.765 | 0.078 | 0.025 | 0.380 | 0.267 | 0.174 | 0.128 | − 0.241 | 0.126 | − 0.011 | 0.153 |
| HOMA-IR | – | $log_{10}$ | 2,35 | 2.511 | 0.096 | 0.035 | 0.160 | 0.625 | 0.989 | 0.155 | 0.521 | 0.153 | 0.639 | 0.176 |
| QUICKI | – | $log_{10}$ | 2,35 | 2.760 | 0.077 | 0.025 | 0.262 | 0.365 | − 0.553 | 0.019 | − 0.490 | 0.019 | − 0.518 | 0.023 |
| Food consumption | Gram | $log_{10}$ | 2,36 | 0.106 | 0.899 | 0.708 | 0.803 | 0.912 | 0.345 | 0.039 | 0.319 | 0.041 | 0.333 | 0.041 |
| Water consumption | Gram | $log_{10}$ | 2,35 | 2.555 | 0.092 | 0.043 | 0.783 | 0.090 | 0.498 | 0.046 | 0.634 | 0.047 | 0.517 | 0.047 |
| High-fat diet preference | % | **0.3 | 2,38 | 1.127 | 0.335 | 0.787 | 0.161 | 0.249 | 0.553 | 0.085 | 0.520 | 0.087 | 0.371 | 0.091 |
| Sweet'N Low preference | % | rank | 2,35 | 0.015 | 0.985 | 0.898 | 0.874 | 0.973 | 20.056 | 3.239 | 20.676 | 3.579 | 20.850 | 3.579 |
| Fat mass | Gram | none | 2,37 | 1.195 | 0.314 | 0.308 | 0.143 | 0.621 | 8.275 | 0.787 | 7.104 | 0.835 | 6.501 | 0.840 |
| Lean mass | Gram | none | 2,38 | 0.003 | 0.997 | 0.982 | 0.961 | 0.944 | 34.346 | 1.115 | 34.310 | 1.140 | 34.429 | 1.191 |
| Percent fat mass | % | none | 2,39 | 1.724 | 0.192 | 0.280 | 0.075 | 0.445 | 17.481 | 1.162 | 15.644 | 1.233 | 14.268 | 1.240 |
| Percent lean mass | % | none | 2,39 | 0.982 | 0.384 | 0.438 | 0.171 | 0.522 | 76.131 | 1.132 | 77.385 | 1.157 | 78.464 | 1.164 |
| Heart mass | Gram | rank | 2,37 | 0.134 | 0.875 | 0.806 | 0.607 | 0.776 | 21.274 | 1.980 | 21.966 | 2.019 | 22.811 | 2.099 |
| Liver mass | Gram | $log_{10}$ | 2,36 | 0.353 | 0.705 | 0.439 | 0.907 | 0.530 | 0.278 | 0.018 | 0.258 | 0.018 | 0.275 | 0.019 |
| Triceps surae mass | Gram | none | 2,37 | 1.942 | 0.158 | 0.635 | 0.064 | 0.152 | 0.200 | 0.005 | 0.203 | 0.005 | 0.214 | 0.005 |
| Testis mass | Gram | none | 2,37 | 12.836 | 5.80E-05 | **3.20E-05** | **4.65E-04** | 0.529 | 0.172 | 0.012 | 0.251 | 0.012 | 0.240 | 0.013 |
| Kidney mass | Gram | none | 2,37 | 0.228 | 0.797 | 0.831 | 0.510 | 0.646 | 0.290 | 0.009 | 0.287 | 0.009 | 0.281 | 0.010 |
| Prepartum body weight slopes | – | none | 2,40 | 0.995 | 0.379 | 0.959 | 0.217 | 0.245 | − 0.005 | 0.024 | − 0.007 | 0.025 | − 0.048 | 0.024 |
| Postpartum body weight mean | Gram | none | 2,38 | 0.198 | 0.821 | 0.718 | 0.459 | 0.691 | 45.544 | 1.888 | 44.549 | 1.932 | 43.787 | 2.002 |

TC = total cholesterol, HDL = high-density lipoprotein cholesterol, LDL = low-density lipoprotein cholesterol, non-HDL = non-HDL cholesterol, TRG = triglyceride, TC/HDL = ratio of TC to HDL, 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 ($P < 0.007$, when modified for Adaptive False Discovery Rate) are both bold and underlined. Nominally significant *P* values ($0.007 < P < 0.05$) are underlined but not bold.
[a] *P* values for these overall tests are reported for completeness, but are not emphasized in the text and were not included when applying the Adaptive False Discovery Rate procedure.

time since pairing, and body mass as covariates) revealed a positive effect of body mass on $log_{10}$-transformed fecal pellet number ($P = 0.010$), and *a priori* contrasts indicated that VM produced less pellets than BM ($P = 0.003$; Table 2, Fig. 6).

#### 3.3.3. Saccharine preference

The three reproductive groups did not differ significantly in their preference for saccharin (Table 2). Males in all of the groups tended to prefer the saccharin solution to water.

### 3.4. Effects of litter size

For breeding males, we performed least-squares linear regressions of each trait on relevant covariates (as described above) as well as litter size. Litter size, which ranged from 1 to 3, was a nominally significant positive predictor of body mass ($P = 0.013$), fat mass ($P = 0.020$), percent fat mass ($P = 0.038$), food consumption ($P = 0.018$), and plasma leptin concentration ($P = 0.018$), and a nominally significant negative predictor of percent lean mass ($P = 0.036$; online Supplementary Table S1).

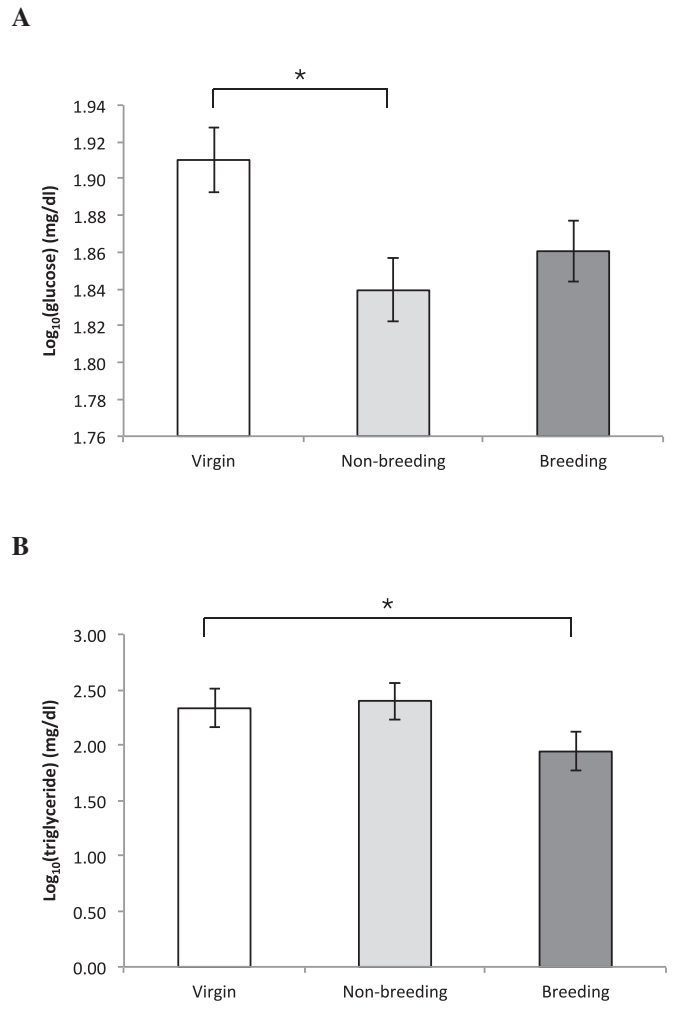



**Fig. 5.** A) EMM ± SE $\log_{10}$-transformed fasted blood glucose levels, adjusted for variation in handling time, age and time since pairing. *A priori* contrasts indicated a significant difference between virgin and non-breeding males ($P = 0.007$). B) EMM ± SE $\log_{10}$-transformed fasted triglyceride levels. Values for breeding males were significantly lower than for virgin males ($P = 0.005$) and nominally lower than for non-breeding males ($P = 0.011$).


**Fig. 6.** EMM ± SE $\log_{10}$-transformed number of fecal pellets expelled in tail-suspension tests, adjusted for variation in body mass, age and time since pairing. Mean values from two trials on each mouse were used for comparing groups. *A priori* contrasts indicated a significant difference between virgin and breeding males ($P = 0.003$).

## 4. Discussion

We hypothesized that in the biparental California mouse, fathers, like mammalian mothers, experience increased metabolic demands [72] and affective changes [71,81], compared to non-reproductive males. We predicted that breeding males, and perhaps males housed with non-breeding females, would demonstrate changes in metabolically important measures of morphology, blood glucose and lipid profiles, hormones, and behavior, as well as in indices of emotionality. Contrary to our expectations, we detected few differences among virgin males, non-breeding males, and breeding males. More specifically, 23 of 167 possible pairwise group comparisons (14%) were significant after correction for multiple comparisons (see Table 2). We did find significant or nominally significant (see Methods) differences among groups in relative testis mass; circulating glucose, triglyceride, and insulin concentrations; insulin sensitivity; pain sensitivity; and anxiety-like behaviors.

### 4.1. Morphology

Unexpectedly, we found no statistically significant differences among reproductive groups in body mass, fat mass or lean mass. In several biparental species (prairie vole, common marmoset, cotton-top tamarin), fathers gain weight during their mate's pregnancy and lose weight during the period of infant care (reviewed in [68]), a pattern suggesting that paternal care is energetically costly, even under laboratory conditions. In contrast, we previously found that California mouse fathers gain body mass across their mates' second pregnancies, corresponding with the period of care of the first litter, but not during their mates' first pregnancies or other pregnancies during which no litter is present [42,67]. Moreover, in our previous study, body mass was lower in expectant fathers housed with a primigravid female than in males housed with a tubally ligated female [67]. Male California mice undergo reductions in testosterone and progesterone, and elevations in prolactin, during the transition to fatherhood, and these hormones can influence body mass and fat/lean mass [6,39,54,59,60,66,84]. Thus, we predicted that breeding males in the present study would have lower body mass and lower fat mass than virgin males and nonbreeding males; however, we found no significant differences among groups. The reason for the disparity between these and previous findings is not clear.

Testis masses (adjusted for body mass) differed among the reproductive groups, with both non-breeding males and breeding males having larger testes than virgin males. Testis mass is known to correlate positively with species variation in sexual function, such as copulatory frequency, sperm production, and sperm per ejaculate, in taxa including rodents [12,47,63]. Our results suggest that cohabitation with a female (and presumably engaging in sexual behavior) is an important cause of increased testis mass. The absence of a difference between non-breeding and breeding males suggests that being a father did not further impair or promote testicular function. No other relative organ masses differed significantly among reproductive groups.

### 4.2. Feeding and predatory behavior

Contrary to our predictions, we found no differences in behaviors potentially associated with metabolic state, including food intake, preference for high-fat diet, and predatory aggression.

We did find that attack latencies in predatory aggression tests were significantly shorter on the second trial than on the first trial, consistent with other studies (*e.g.*, [30,74]). This is likely to reflect an effect of training and/or reduction of fearfulness toward the novel cricket stimulus.

### 4.3. Blood metabolic measures and metabolic hormones

Under fasted conditions, virgin males had higher blood glucose concentrations and nominally higher plasma insulin concentrations than non-breeding males, while neither group differed reliably from breeding males. Fasted insulin levels in virgins were roughly 3 times those in non-breeding males. This pattern suggests that non-breeders are more sensitive to insulin than virgins [79]. This possibility is supported by subsequent analyses of two surrogate measures of insulin sensitivity: HOMA-IR and QUICKI, both based on fasted glucose and insulin levels [8,9]. Both measures indicated that NBs have nominally higher insulin sensitivity than VMs.

The mechanism underlying this difference in insulin sensitivity is not known. In humans and rodent models, insulin resistance is typically associated with high body mass and, in particular, high levels of body fat [18,51]. In our mice, however, neither body mass, body fat, nor circulating levels of the adipocyte hormone leptin differed among reproductive groups. Other hormones might have contributed to the glucose and insulin differences. We measured only insulin and leptin in this study, but other studies in California mice have found differences in circulating concentrations of testosterone, dihydrotestosterone, progesterone, prolactin, and oxytocin among virgin males, males housed with a primigravid female, and fathers, such that each reproductive stage appears to be characterized by a distinct hormonal profile [34,36,75]. Importantly, several of these hormones are known to modulate glucose and lipid metabolism in humans and rodents (reviewed in [69,70,76]). For example, testosterone can increase fat oxidation and lipolysis [69], reduce gluconeogenesis, and increase glycogen synthesis and storage in the liver, thereby lowering circulating glucose levels [70]. Testosterone also improves insulin sensitivity [76]. In our study, NB had significantly higher testis masses than VM. If circulating testosterone levels followed a similar pattern [27], this could potentially account for the observed differences in glucose-insulin dynamics. The biological significance, if any, of the difference in insulin sensitivity in male California mice is unclear.

In addition to insulin and glucose, fasted triglyceride levels differed markedly among reproductive groups: triglycerides in breeding males were significantly lower than those in virgin males and nominally lower than those in non-breeding males, Furthermore, breeding males had nominally lower fasting levels of total cholesterol and non-HDL cholesterol than virgins. These results suggest that lipid metabolism in male California mice is influenced by fatherhood, but not by cohabitation with a female.

Like glucose metabolism, lipid metabolism can be influenced by gonadal steroids (*i.e.*, androgens, estrogens, progestogens [69], and plasma concentrations of both androgens and progesterone differ across reproductive conditions in male California mice [75]. Moreover, in humans, high triglycerides, like high glucose, can be induced by insulin resistance, which is often associated with metabolic syndrome and type II diabetes [64]. However, we saw no evidence of pathology in any of our groups.

The biological significance of the low triglyceride and cholesterol levels in breeding males, or, conversely, of elevated triglyceride and cholesterol levels in virgins, is unknown. Further studies are needed to elucidate the mechanisms by which reproductive status alters glucose and lipid metabolism in male California mice, as well as the consequences of these effects for breeding males.

### 4.4. Behavioral indicators of depression, anxiety, and pain sensitivity

We found no differences among reproductive groups in preference for sucrose solution *vs.* water. Reduced preference for highly palatable liquids is often used as a measure of anhedonia, a common symptom of clinical depression in humans and an index of depression-like behavior in rodents [73,80]. Moreover, breeding, non-breeding, and virgin males did not differ in time spent immobile in the tail-suspension test, another common measure of depression-like behavior in rodents [22]. Thus, we found no evidence that either cohabitation with a female or fatherhood affects depression-like behavior in male California mice.

Interestingly, the number of fecal pellets expelled during tail-suspension tests was significantly higher in breeding males than virgin males. Defecation in response to experimental stressors or pharmacological challenges is often used as a measure of emotionality, particularly fearfulness and anxiety, in rodents [3,13,21,28]. Therefore, our findings suggest that California mouse fathers might be more anxious and/or fearful than virgin males, which might be adaptive under some conditions [68].

This result contrasts with previous findings from our lab that first-time fathers and virgin male California mice showed few differences in responses to a novel object (a measure of neophobia) or behavior in the elevated-plus maze (an index of anxiety) [19]. However, we did not record production of fecal pellets in those tests. In addition, previous studies did not find any differences between fathers and non-fathers in endocrine or neural responses to either acute [19,20,41] or chronic stressors [23]. On the other hand, brief exposure to predator urine elicited acute behavioral changes in both singly housed and paired virgin males but not in fathers [19]. New fathers and expectant fathers also spent more time sniffing and touching a wire mesh ball containing a newborn pup than virgin males [24]. Furthermore, California mouse fathers and virgin males that had been exposed to pups, compared to non-exposed virgin males, had decreased occurrences of incomplete behavioral chains, indicative of reduced stress, during exposure to a novel object [5]. Interestingly, in some mammals, including rats and mice, mothers are less fearful than nulliparous females, and have greatly reduced behavioral, endocrine, and neural responses to stressors [15,71].

Pain sensitivity, as measured in the hot-plate test, was nominally higher in non-breeding males than in virgin males, indicating that pair formation or sexual behavior may induce changes in males' nociceptive system. Pain sensation can be modulated by fear and anxiety, which can themselves be altered by various environmental factors and pharmacological manipulations [77]. For example, manipulations that reduce fear/anxiety (*e.g.*, administration of benzodiazepines) can attenuate the analgesia elicited by stressful situations [44]. In our study, therefore, relatively low anxiety in virgin males (compared to breeding males; as measured by number of fecal pellets in the tail-suspension test) is consistent with their relatively low pain sensitivity in the hot-plate test (compared to non-breeding males).

### 4.5. Effects of litter size

Among breeding males, litter size was a nominally significant, positive predictor of body mass, fat mass (not adjusted for body mass), percent fat mass, food consumption (adjusted for body mass), and plasma leptin levels (adjusted for percent fat mass); litter size was also a nominally negative predictor of percent lean mass. These findings were unexpected and are difficult to interpret, in part because California mice have little variation in litter size; litters in this study ranged from 1 to 3 pups. One possible interpretation is that larger litters represent a greater stimulus to fathers than smaller ones, in terms of increasing food intake to offset energetic demands of fatherhood. In our controlled laboratory environment, in which males have *ad libitum* food and face few energetic challenges, therefore, higher food intake of fathers with larger litters might have led to increased fat storage and, consequently, increased body mass and leptin levels, without corresponding differences in energy utilization, compared to fathers with smaller litters. This possibility is consistent with our previous finding that fathers housed with pups from their first litter gain mass over their mate's subsequent pregnancy, whereas fathers without surviving pups from the first litter do not [67]. If cohabitation with pups does, in fact, stimulate fathers to eat more, we would expect differences in food consumption between fathers and non-breeding males, but this was not

observed. Alternatively, larger litters may stimulate other neuroendocrine pathways that then affect physiological process leading to altered fat mass, *etc.*

## 5. Conclusions

The results of this study reveal few physiological, morphological, or affective changes in first-time California mouse fathers housed under standard lab conditions. Taken at face value, they suggest that fatherhood is minimally stressful for males of this species. However, it is unclear how directly these findings apply to California mice breeding in their natural environment. Our animals were housed in a benign environment, in which food and water were provided *ad lib*, temperature was maintained within or close to the thermoneutral zone [56], and predators were absent. Under these conditions, fathers did not have to invest significant time or energy in foraging, thermoregulating, territorial defense, mate-guarding or taking care of pups. Moreover, males in this study were first-time fathers and were studied during approximately the first half of the lactational period; costs of fatherhood might become apparent only after longer periods of paternal care or multiple reproductive bouts. Future studies should therefore characterize effects of fatherhood on fathers in a more challenging environment, across the entire lactational period, and in long-term breeding males.

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## Conflict of interest

The authors declare that there was no conflict of interests.

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## References

[1] G.G. Achenbach, C.T. Snowdon, Costs of caregiving: weight loss in captive adult male cotton-top tamarins (*Saguinus oedipus*) following the birth of infants, Int. J. Primatol. 23 (2002) 179–189.
[2] J.R. Andrew, W. Saltzman, M.A. Chappell, T. Garland Jr., Consequences of fatherhood in the biparental California mouse (*Peromyscus californicus*): locomotor performance, metabolic rate, and organ masses, Physiol. Biochem. Zool. 89 (2016) 130–140.
[3] J. Archer, Tests for emotionality in rats and mice: a review, Anim. Behav. 21 (1973) 205–235.
[4] K.L. Bales, W. Saltzman, Fathering in rodents: neurobiological substrates and consequences for offspring, Horm. Behav. 77 (2016) 249–259.
[5] M. Bardi, C.L. Franssen, J.E. Hampton, E.A. Shea, A.P. Fanean, K.G. Lambert, Paternal experience and stress responses in California mice (*Peromyscus californicus*), Comp. Med. 61 (2011) 20–30.
[6] K. Blouin, A. Boivin, A. Tchernof, Androgens and body fat distribution, J. Steroid Biochem. Mol. Biol. 108 (2008) 272–280.
[7] V. Bókony, A.Z. Lendvai, A. Liker, F. Angelier, J.C. Wingfield, O. Chastel, Stress response and the value of reproduction: are birds prudent parents? Am. Nat. 173 (2009) 589–598.
[8] A. Borai, C. Livingstone, I. Kaddam, G. Ferns, Selection of the appropriate method for the assessment of insulin resistance, BMC Med. Res. Methodol. 11.1 (2011) 158.
[9] J.E. Bowe, Z.J. Franklin, A.C. Hauge-Evans, A.J. King, S.J. Persaud, P.M. Jones, Metabolic phenotyping guidelines: assessing glucose homeostasis in rodent models, J. Endocrinol. 222 (2014) G13–G25.
[10] K. Braun, F. Champagne, Paternal influences on offspring development: behavioural and epigenetic pathways, J. Neuroendocrinol. 26 (2014) 697–706.
[11] T.W. Bredy, R.E. Brown, M.J. Meaney, Effect of resource availability on biparental care, and offspring neural and behavioral development in the California mouse (*Peromyscus californicus*), Eur. J. Neurosci. 25 (2007) 567–575.
[12] W.G. Breed, J. Taylor, Body mass, testes mass, and sperm size in murine rodents, J. Mammal. 81 (2000) 758–768.
[13] A.M. Bronikowski, P.A. Carter, J.G. Swallow, I.A. Girard, J.S. Rhodes, T. Garland Jr., Open-field behavior of house mice selectively bred for high voluntary wheel-running, Behav. Genet. 31 (2001) 309–316.
[14] F. Bronson, Mammalian reproduction: an ecological perspective, Biol. Reprod. 32 (1985) 1–26.
[15] P. Brunton, J. Russell, A. Douglas, Adaptive responses of the maternal hypothalamic-pituitary-adrenal axis during pregnancy and lactation, J. Neuroendocrinol. 20 (2008) 764–776.
[16] J.C. Campbell, K.D. Laugero, J.A. Van Westerhuyzen, C.M. Hostetler, J.D. Cohen, K.L. Bales, Costs of pair-bonding and paternal care in male prairie voles (*Microtus ochrogaster*), Physiol. Behav. 98 (2009) 367–373.
[17] D. Cantoni, R.E. Brown, Paternal investment and reproductive success in the California mouse, *Peromyscus californicus*, Anim. Behav. 54 (1997) 377–386.
[18] W.T. Cefalu, Z.Q. Wang, S. Werbel, A. Bell-Farrow, J.R. Crouse, W.H. Hinson, J.G. Terry, R. Anderson, Contribution of visceral fat mass to the insulin resistance of aging, Metabolism 44 (1995) 954–959.
[19] M. Chauke, T.R. de Jong, T. Garland Jr., W. Saltzman, Paternal responsiveness is associated with, but not mediated by reduced neophobia in male California mice (*Peromyscus californicus*), Physiol. Behav. 107 (2012) 65–75.
[20] M. Chauke, J.L. Malisch, C. Robinson, T.R. de Jong, W. Saltzman, Effects of reproductive status on behavioral and endocrine responses to acute stress in a biparental rodent, the California mouse (*Peromyscus californicus*), Horm. Behav. 60 (2011) 128–138.
[21] M.A. Coleman, T. Garland Jr., C.A. Marler, S.S. Newton, J.G. Swallow, P.A. Carter, Glucocorticoid response to forced exercise in laboratory house mice (*Mus domesticus*), Physiol. Behav. 63 (1998) 279–285.
[22] J.F. Cryan, C. Mombereau, A. Vassout, The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice, Neurosci. Biobehav. Rev. 29 (2005) 571–625.
[23] T. De Jong, B. Harris, J. Perea-Rodriguez, W. Saltzman, Physiological and neuroendocrine responses to chronic variable stress in male California mice (*Peromyscus californicus*): influence of social environment and paternal state, Psychoneuroendocrinology 38 (2013) 2023–2033.
[24] T.R. De Jong, M. Chauke, B.N. Harris, W. Saltzman, From here to paternity: neural correlates of the onset of paternal behavior in California mice (*Peromyscus californicus*), Horm. Behav. 56 (2009) 220–231.
[26] D. Dudley, Contributions of paternal care to the growth and development of the young in *Peromyscus californicus*, Behav. Biol. 11 (1974) 155–166.
[27] B. Eleftheriou, L. Lucas, Age-related changes in testes, seminal vesicles and plasma testosterone levels in male mice, Gerontology 20 (1974) 231–238.
[28] J. Flint, R. Corley, J.C. DeFries, D.W. Fulker, J.A. Gray, S. Miller, A.C. Collins, A simple genetic basis for a complex psychological trait in laboratory mice, Science 269 (1995).
[30] S.C. Gammie, N.S. Hasen, J.S. Rhodes, I. Girard, T. Garland Jr., Predatory aggression, but not maternal or intermale aggression, is associated with high voluntary wheel-running behavior in mice, Horm. Behav. 44 (2003) 209–221.
[31] T. Garland Jr., Trade-offs, Curr. Biol. 24 (2014) R60–R61.
[32] E.L. Gibson, Emotional influences on food choice: sensory, physiological and psychological pathways, Physiol. Behav. 89 (2006) 53–61.
[33] T. Good, M. Khan, J. Lynch, Biochemical and physiological validation of a corticosteroid radioimmunoassay for plasma and fecal samples in oldfield mice (*Peromyscus polionotus*), Physiol. Behav. 80 (2003) 405–411.
[34] D.J. Gubernick, R.J. Nelson, Prolactin and paternal behavior in the biparental California mouse, *Peromyscus californicus*, Horm. Behav. 23 (1989) 203–210.
[35] D.J. Gubernick, T. Teferi, Adaptive significance of male parental care in a monogamous mammal, Proc. R. Soc. Lond. Ser. B Biol. Sci. 267 (2000) 147–150.
[36] D.J. Gubernick, J.T. Winslow, P. Jensen, L. Jeanotte, J. Bowen, Oxytocin changes in males over the reproductive cycle in the monogamous, biparental California mouse, *Peromyscus californicus*, Horm. Behav. 29 (1995) 59–73.
[37] D.J. Gubernick, S.L. Wright, R.E. Brown, The significance of father's presence for offspring survival in the monogamous California mouse, *Peromyscus californicus*, Anim. Behav. 46 (1993) 539–546.
[39] N. Harada, R. Hanaoka, H. Horiuchi, T. Kitakaze, T. Mitani, H. Inui, R. Yamaji, Castration influences intestinal microflora and induces abdominal obesity in high-fat diet-fed mice, Sci. Rep. (2016) 6.
[40] J.M. Harper, C.W. Leathers, S.N. Austad, Does caloric restriction extend life in wild mice? Aging Cell 5 (2006) 441–449.
[41] B.N. Harris, T.R. de Jong, V. Yang, W. Saltzman, Chronic variable stress in fathers alters paternal and social behavior but not pup development in the biparental California mouse (*Peromyscus californicus*), Horm. Behav. 64 (2013) 799–811.
[42] B.N. Harris, J.P. Perea-Rodriguez, W. Saltzman, Acute effects of corticosterone injection on paternal behavior in California mouse (*Peromyscus californicus*) fathers, Horm. Behav. 60 (2011) 666–675.
[43] B.N. Harris, W. Saltzman, Effect of reproductive status on hypothalamic–pituitary–adrenal (HPA) activity and reactivity in male California mice (*Peromyscus californicus*), Physiol. Behav. 112 (2013) 70–76.
[44] F.J. Helmstetter, Stress-induced hypoalgesia and defensive freezing are attenuated by application of diazepam to the amygdala, Pharmacol. Biochem. Behav. 44 (1993) 433–438.
[45] S. Idelman, The structure of the mammalian adrenal cortex, in: I.C. Jones,

I.W. Henderson (Eds.), General, Comparative and Clinical Endocrinology of the Adrenal Cortex, Academic Press, New York, 1978, pp. 1–180.
[47] G. Kenagy, S.C. Trombulak, Size and function of mammalian testes in relation to body size, J. Mammal. 67 (1986) 1–22.
[48] W.M. Kenkel, G. Suboc, C. Sue Carter, Autonomic, behavioral and neuroendocrine correlates of paternal behavior in male prairie voles, Physiol. Behav. 128 (2014) 252–259.
[50] E.M. Kolb, Neurobiological and Physiological Underpinnings of High Voluntary Wheel Running, University of California, Riverside, 2010 (PhD Dissertation (Unpublished)).
[51] G.F. Lewis, A. Carpentier, K. Adeli, A. Giacca, Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes, Endocr. Rev. 23 (2002) 201–229.
[52] C. Lieberwirth, Y. Wang, X. Jia, Y. Liu, Z. Wang, Fatherhood reduces the survival of adult-generated cells and affects various types of behavior in the prairie vole (*Microtus ochrogaster*), Eur. J. Neurosci. 38 (2013) 3345–3355.
[53] J.S. Lonstein, J. Maguire, G. Meinlschmidt, I.D. Neumann, Emotion and mood adaptations in the peripartum female: complementary contributions of GABA and oxytocin, J. Neuroendocrinol. 26 (2014) 649–664.
[54] J. Lovejoy, G. Bray, C. Greeson, M. Klemperer, J. Morris, C. Partington, R. Tulley, Oral anabolic steroid treatment, but not parenteral androgen treatment, decreases abdominal fat in obese, older men, Int. J. Obes. Relat. Metabol. Disord. 19 (1995) 614–624.
[55] J.L. Malisch, C.W. Breuner, E.M. Kolb, H. Wada, R.M. Hannon, M.A. Chappell, K.M. Middleton, T. Garland Jr., Behavioral despair and home-cage activity in mice with chronically elevated baseline corticosterone concentrations, Behav. Genet. 39 (2009) 192–201.
[56] B.K. McNab, P. Morrison, Body temperature and metabolism in subspecies of *Peromyscus* from arid and mesic environments, Ecol. Monogr. (1963) 63–82.
[59] J.E. Nettleship, T.H. Jones, K.S. Channer, R.D. Jones, Physiological testosterone replacement therapy attenuates fatty streak formation and improves high-density lipoprotein cholesterol in the Tfm mouse an effect that is independent of the classic androgen receptor, Circulation 116 (2007) 2427–2434.
[60] S.T. Page, J.K. Amory, F.D. Bowman, B.D. Anawalt, A.M. Matsumoto, W.J. Bremner, J.L. Tenover, Exogenous testosterone (T) alone or with finasteride increases physical performance, grip strength, and lean body mass in older men with low serum T, J. Clin. Endocrinol. Metab. 90 (2005) 1502–1510.
[61] N. Pecoraro, F. Reyes, F. Gomez, A. Bhargava, M.F. Dallman, Chronic stress promotes palatable feeding, which reduces signs of stress: feedforward and feedback effects of chronic stress, Endocrinology 145 (2004) 3754–3762.
[62] J.P. Perea-Rodriguez, E.Y. Takahashi, T.M. Amador, R.C. Hao, W. Saltzman, B.C. Trainor, Effects of reproductive experience on central expression of progesterone, oestrogen α, oxytocin and vasopressin receptor mRNA in male California mice (*Peromyscus californicus*), J. Neuroendocrinol. 27 (2015) 245–252.
[63] B. Preston, I. Stevenson, J. Pemberton, D. Coltman, K. Wilson, Overt and covert competition in a promiscuous mammal: the importance of weaponry and testes size to male reproductive success, Proc. R. Soc. Lond. B Biol. Sci. 270 (2003) 633–640.
[64] C.K. Roberts, A.L. Hevener, R.J. Barnard, Metabolic syndrome and insulin resistance: underlying causes and modification by exercise training, Compr. Physiol. 3 (2013) 1–58.
[66] A. Sainsbury, L. Zhang, Role of the hypothalamus in the neuroendocrine regulation of body weight and composition during energy deficit, Obes. Rev. 13 (2012) 234–257.
[67] W. Saltzman, B. Harris, T. Jong, P. Nguyen, J. Cho, M. Hernandez, J. Perea-Rodriguez, Effects of parental status on male body mass in the monogamous, biparental California mouse, J. Zool. 296 (2015) 23–29.
[68] W. Saltzman, T.E. Ziegler, Functional significance of hormonal changes in mammalian fathers, J. Neuroendocrinol. 26 (2014) 685–696.
[69] S. Santosa, M.D. Jensen, The sexual dimorphism of lipid kinetics in humans, Front. Endocrinol. 6 (2015).
[70] M. Shen, H. Shi, Sex hormones and their receptors regulate liver energy homeostasis, Int. J. Endocrinol. 2015 (2015).
[71] D.A. Slattery, I.D. Neumann, No stress please! Mechanisms of stress hyporesponsiveness of the maternal brain, J. Physiol. 586 (2008) 377–385.
[72] J.R. Speakman, The physiological costs of reproduction in small mammals, Philos. Trans. R. Soc. B Biol. Sci. 363 (2008) 375–398.
[73] T. Strekalova, R. Spanagel, D. Bartsch, F.A. Henn, P. Gass, Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration, Neuropsychopharmacology 29 (2004) 2007–2017.
[74] R.S. Surwit, C.M. Kuhn, C. Cochrane, J.A. McCubbin, M.N. Feinglos, Diet-induced type II diabetes in C57BL/6J mice, Diabetes 37 (1988) 1163–1167.
[75] B.C. Trainor, I.M. Bird, N.A. Alday, B.A. Schlinger, C.A. Marler, Variation in aromatase activity in the medial preoptic area and plasma progesterone is associated with the onset of paternal behavior, Neuroendocrinology 78 (2003) 36–44.
[76] O. Varlamov, C.L. Bethea, C.T. Roberts, Sex-specific differences in lipid and glucose metabolism, Front. Endocrinol. 5 (2015) 241.
[77] L.F. Vendruscolo, F.A. Pamplona, R.N. Takahashi, Strain and sex differences in the expression of nociceptive behavior and stress-induced analgesia in rats, Brain Res. 1030 (2004) 277–283.
[78] S.A. Weaver, J. Diorio, M.J. Meaney, Maternal separation leads to persistent reductions in pain sensitivity in female rats, J. Pain 8 (2007) 962–969.
[79] G. Wilcox, Insulin and insulin resistance, Clin. Biochem. Rev. 26 (2005) 19–39.
[80] P. Willner, R. Muscat, M. Papp, Chronic mild stress-induced anhedonia: a realistic animal model of depression, Neurosci. Biobehav. Rev. 16 (1992) 525–534.
[81] J. Wingfield, R. Sapolsky, Reproduction and resistance to stress: when and how, J. Neuroendocrinol. 15 (2003) 711–724.
[82] S.L. Wright, R.E. Brown, The importance of paternal care on pup survival and pup growth in *Peromyscus californicus* when required to work for food, Behav. Process. 60 (2002) 41–52.
[83] T.E. Ziegler, S.L. Prudom, N.J. Schultz-Darken, A.V. Kurian, C.T. Snowdon, Pregnancy weight gain: marmoset and tamarin dads show it too, Biol. Lett. 2 (2006) 181–183.
[84] T.E. Ziegler, S.L. Prudom, S.R. Zahed, A. Parlow, F. Wegner, Prolactin's mediative role in male parenting in parentally experienced marmosets (*Callithrix jacchus*), Horm. Behav. 56 (2009) 436–443.