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Affective, Behavioral, and Physiological Effects of Reproduction in California Mice

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Evolution, Ecology, and Organismal Biology

by

Meng Zhao

June 2018

Dissertation Committee:
Dr. Wendy Saltzman, Chairperson
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The Dissertation of Meng Zhao is approved:

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Chapter 2: **Effects of a physical and energetic challenge on male California mice (*Peromyscus californicus*): modulation by reproductive condition.**

Meng Zhao, Theodore Garland Jr., Mark A. Chappell, Jacob R. Andrew, Breanna N. Harris, and Wendy Saltzman

Published in Journal of Experimental Biology 221 (2018), jeb168559.

The co-authors listed in these publications directed, supervised or provided technical expertise in the research which forms the basis for this dissertation.

DEDICATION

I dedicate this dissertation to my family

ABSTRACT OF THE DISSERTATION

Affective, Behavioral, and Physiological Effects of Reproduction in California Mice

by

Meng Zhao

Doctor of Philosophy, Graduate Program in Evolution, Ecology, and Organismal Biology
University of California, Riverside, June 2018
Dr. Wendy Saltzman, Chairperson

Being a mother is energetically costly and can entail trade-offs between reproduction and self-maintenance. In biparental species, fathers, as well as mothers, care for offspring and their contributions improve offspring survival. It is unknown if fathers in biparental species, like mothers, experience costs of parenting. It is also not clear whether the beneficial consequences of paternal care for offspring are mediated exclusively through direct effects on offspring or whether they can be mediated indirectly through beneficial effects on mothers. This dissertation investigates energetic costs as well as behavioral and affective changes associated with fatherhood, and morphological, physiological and affective conditions in single mothers rearing offspring without assistance from their mates in the monogamous, biparental California mouse (*Peromyscus californicus*). Results demonstrate that cohabitation with a female and/or fatherhood decreases blood

glucose levels, alters lipid profiles, as well as protects body mass and fat mass from being affected by energetic challenge. Fathers also buffer their mates from potentially adverse consequences of motherhood. Mothers rearing pups without assistance from a mate can maintain pup survival and development as normal, but experience morphological and endocrine changes that might be harmful in the long term. These experiments provide important implications for the understanding of trade-offs between reproduction and self-maintenance in a biparental breeding system.

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Introduction

Among the four levels of analyses in animal behavior proposed by Tinbergen (1963) – mechanism, ontogeny, evolution and adaptive value - the adaptive value of a behavioral trait explains how the behavior contributes to survival and reproductive success. Trivers (1972) first defined parental investment as “any investment by the parent in an individual offspring that increases the offspring's chance of surviving.” Essentially, parental care evolves because it increases the reproductive output (i.e., inclusive fitness) of the parent (Alexander, 1974). However, parenthood can entail trade-offs between reproduction and self-maintenance (Garland, 2014, Hamilton, 1964), and sexual and parental experience can influence physiological and psychological condition in both females and males (Saltzman & Ziegler, 2014, Speakman, 2008). Thus, an understanding of the effects of parenthood on parents could have implications for understanding and improving parents' health and, consequently, the health of their offspring.

In comparison with the ubiquitous involvement of females in parental care in mammals, only 5-10% of mammals are biparental, meaning that both parents invest some effort in taking care of their young (Kleiman, 1977). Paternal care occurs if the male can achieve higher reproductive success through the care of offspring than if he provides no care (West & Capellini, 2016, Wittenberger & Tilson, 1980). Indeed, paternal care greatly enhances offspring survival rate and development in at least some biparental species (Cantoni & Brown, 1997, Gecas & Schwalbe, 1986, Macintyre, 1992), as well as influences aspects of the offspring's future adult behavior, such as aggression (Rosenfeld et al., 2013). Although males do not go through pregnancy and lactation as females do,

they appear to undergo a series of hormonal changes during the transition to fatherhood that may directly promote paternal behavior. Firstly, changes in the levels of gonadal steroids (estrogens, progesterone and testosterone) can influence the display of paternal behavior (Ogawa et al., 1998, Schum & Wynne-Edwards, 2005, Timonin et al., 2008). Suppression of testosterone level has been proposed to reduce aggression toward the offspring and increase paternal response to infant stimuli in human fathers (Fleming et al., 2002, Storey et al., 2000). Progesterone appears to have an inhibitory effect on paternal behavior, so a decrease in progesterone has been hypothesized to occur in fathers (Kentner et al., 2010). Secondly, both oxytocin and vasopressin (AVP) have been widely recognized as neuropeptides mediating affiliation and are considered integral to reproduction and parental behavior (Insel, 1992). In addition, elevated circulating prolactin levels have been found in fathers compared to virgins and/or expectant fathers of many biparental species (Brown et al., 1995, Gettler et al., 2012, Gubernick & Nelson, 1989, Reburn & Wynne-Edwards, 1999, Ziegler et al., 1996). Lastly, the glucocorticoids (cortisol or corticosterone) in males are also implicated in social affiliation and pair bond formation (Carter, 1998, Wynne-Edwards, 2001) in monogamous species. In addition to promoting paternal care, the changes in some hormones may play a role in indirectly facilitating paternal care or paternal survival through metabolic, neural and emotional effects that help fathers meet the demands of parenting. However, the physiological and affective (e.g., stress-responsiveness, depression, anxiety and fearfulness) changes caused by fatherhood in these biparental animals are rarely investigated.

In the biparental mammals, the presence of the father can enhance pup survival and development (Cantoni & Brown, 1997, Gubernick & Teferi, 2000, Gubernick et al., 1993, Wright & Brown, 2002). It is unknown, however, whether these beneficial consequences of paternal care are mediated exclusively through direct effects on offspring or whether they can also be mediated indirectly through beneficial effects on mothers. In humans, mothers raising children alone have poorer physical and mental health than those with partners (Beatson-Hird et al., 1989, Burstrom et al., 2010, Cairney et al., 1999, Young et al., 2005), and their children have higher mortality and morbidity (Daryanani et al., 2016, East et al., 2006). Studies in other monogamous animals have also shown that separation from the mate can induce changes in behavior, physiology, and even depression and anxiety (Bosch et al., 2009, Castro & Matt, 1997, French et al., 2007). These studies have typically been performed on nonbreeding animals, however; it is unknown if there are any effect of fathers on their mate's health condition or how these effects might affect offspring development.

The physiological and emotional consequences of fatherhood and of being a single mother have been rarely studied in mammals, especially biparental mammals. Males of both biparental and uniparental species show paternal behavior in certain circumstances (Dewsbury, 1985). However, the "paternal" behavior displayed by uniparental species is possibly underpinned by significantly different mechanisms than that of biparental species (Rosenfeld et al., 2013). Thus, it may be more appropriate to study the consequences of paternal experience and single motherhood in monogamous species that are biparental than in polygynous species that are uniparental (Brown et al., 1995).

California mouse (*Peromyscus californicus*) is exclusively monogamous both socially and genetically (Brown, 1993, Gubernick & Teferi, 2000, Ribble, 1991). Fathers engage in all of the same behaviors as mothers, except lactation (Gubernick & Alberts, 1987), and paternal care in this species has been found to greatly enhance pup survival and development both in the field and under challenging lab settings, such as cold temperature or having to work for food (Bredy et al., 2007, Cantoni & Brown, 1997, Dudley, 1974, Gubernick & Teferi, 2000, Gubernick et al., 1993, Wright & Brown, 2002). California mice have a relatively long life expectancy (9-18 months in the field) and produce relatively small litters (1-5 pups) (Gubernick, 1988). They occupy chaparral, breed throughout the year, and frequently breed postpartum (Drickamer & Vestal, 1973). In captivity they have been reported to live as long as 5.5 years (Weigl, 2005); both males and females can breed successfully until 3.5 years of age or later with no decrease in litter size (unpub. obs.). Length of the estrous cycle averages 9 days and ranges from 5 to 20 days (Gubernick, 1988). A postpartum estrus occurs 1-3 days following birth of the pups. The postpartum estrus and the fact that this species is biparental means males engage in paternal care and copulation at the same time, and that females often are simultaneously pregnant and lactating. Average length of gestation while concurrently lactating (34.5 days) was significantly longer than for nonlactating pregnancies (31.6 days) (Gubernick, 1988).

My dissertation research addresses two questions: 1) are there any physiological, morphological or behavioral costs of fatherhood in this biparental system and 2) how does the father in a biparental system influence the mother's physiological and affective

condition? These questions address the trade-offs between reproductive gains and costs in self maintenance, and the interactions between mothers and fathers in biparental breeding systems. I hypothesized that fathers, like mothers, experience energetic costs as well as behavioral and affective changes associated with parenthood; that mothers rearing offspring without assistance from their mates would have poorer morphological, physiological and affective condition, as well as impaired survival and development of pups, compared to mothers housed with their mates; and that the effects of fatherhood and single motherhood would be more pronounced under energetically challenging environmental conditions that increase the cost of parenthood. The specific aims of the dissertation are as follows:

Aim 1. Investigate the metabolic and affective changes in males related to different stages of fatherhood, and identify associated changes in metabolically important hormones.

Aim 2. Characterize the additional influences of chronic stress or a challenging living environment on fathers' physiological and affective condition.

Aim 3. Test whether the removal of fathers would affect the survival of the offspring, as well as mothers' physiological and affective conditions.

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CHAPTER 1

Metabolic and Affective Consequences of Fatherhood in Male California Mice

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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 less 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 California mouse males. 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.

Keywords: reproduction, metabolism, fatherhood, affect, pain, lipid profile

1. Introduction

Parental investment by fathers can greatly increase reproductive success in biparental species by enhancing survival and development of offspring (Bales & Saltzman, 2016, Braun & Champagne, 2014). 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 (Garland, 2014). 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 (Saltzman & Ziegler, 2014), as well as in expression of their respective receptors (Bales & Saltzman, 2016; Perea-Rodriguez et al., 2015). Some of these neuroendocrine changes appear to directly influence the expression of parental care and could potentially influence metabolic and affective functions in fathers (Saltzman & Ziegler, 2014).

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 (Campbell et al., 2009,

Kenkel et al., 2014). At the same time, similar to lactating females (Speakman, 2008), 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 (Campbell et al., 2009). In 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 (Ziegler et al., 2006). This is followed by a drop in body weight during the postpartum period (Achenbach & Snowdon, 2002, Ziegler et al., 2009). 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 (Harris et al., 2011, Saltzman et al., 2015). In addition, experienced California mouse fathers underwent significant increases in body mass across their mates' pregnancy when housed with pups from their previous litter (Saltzman et al., 2015). Finally, California mouse fathers had smaller fat pads than virgin males (Andrew et al., 2016). However, other metabolic and morphological consequences of being a father, such as 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 (Brunton et al., 2008, Slattery & Neumann, 2008).

Stress hyporesponsiveness, as well as reduced anxiety and fearfulness (Lonstein et al., 2014) in mothers, might reduce the likelihood that maternal care will be disrupted by stress, and could be important for offspring development (Slattery & Neumann, 2008) but potentially at a cost to the mother's self-maintenance and surival (Bókony et al., 2009). Thus, dampened affect and stress-reactivity might reflect a trade-off between self-maintenance and reproduction (Bókony et al., 2009; Wingfield & Sapolsky, 2003).

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 (Lieberwirth et al., 2013); however, another study of the same species found lower levels of anxiety-related behaviors in fathers than in virgin males (Kenkel et al., 2014). In California mice, two studies found reduced behavioral responses to stress in fathers compared with non-fathers (Bardi et al., 2011, Chauke et al., 2011). 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 (Bardi et al., 2011). 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 urine exposure, whereas fathers showed no behavioral responses to the same stressor (Chauke et al., 2011). 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 (Chauke et al., 2011; De Jong et al., 2013; Harris & Saltzman, 2013; Harris et al., 2013). 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 (De Jong et al., 2013).

In this study we tested the hypothesis that fathers, like mothers (Bronson, 1985, Speakman, 2008), 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 (Bredy et al., 2007, Cantoni & Brown, 1997, Dudley, 1974, Gubernick & Teferi, 2000, Gubernick et al., 1993, Wright & Brown, 2002). 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 (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)) 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. 1.1).

2.3. Tubal ligation

Females in non-breeding pairs underwent bilateral ligation of the oviducts as previously described (Harris & Saltzman, 2013). 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.

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. 1.1).

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 seconds. 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 provided). The food and water bottle were weighed immediately before and after the 4-hour 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 ~40g 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-hour 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 minutes, 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. (2003). 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 minutes, 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 minutes) 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 (13300 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. (2011), Good et al. (2003), and Harper and Austad (2006). 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. 1.2). 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. 1.2). 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 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. 1.3). 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 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 on 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., Vendruscolo et al., 2004, Weaver et al., 2007) (Fig. 1.1). 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 stimulate 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 x 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 the open-field test, as measures of anxiety (Archer, 1973, Bronikowski et al., 2001, Coleman et al., 1998, Flint et al., 1995). 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 second for a total of 6 minutes. LabAnalyst software (www.warthog.ucr.edu) was used for baseline correction and calculation of activity duration (Malisch et al., 2009). 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 minutes 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* (Kolb, 2010) 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. 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-hour test period, and the amount of each liquid consumed was calculated as the difference between initial and final mass. The amount of consumed 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/I))+log(glucose (mg/dl))), both based on fasted glucose and insulin levels (Borai et al., 2011, Bowe et al., 2014).

All traits were analyzed by analysis of covariance (ANCOVA) using SPSS. Age, time from pairing to measurement, and/or other potentially relevant variables 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 1.3). 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 also found no group differences (all P > 0.459) (Table 1.2).

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 1.2). The data on the two successive testing days for each male were highly correlated and not significantly different between days (Table 1.1).

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 1.1), as has been seen in other mammals and with other organs (Coleman et al., 1998, Idelman, 1978). VM had significantly lower average testis masses than did NB (P = 0.00003) and BM (P = 0.00047) (Fig. 1.4). No other organ masses differed between reproductive groups based on *a priori* contrasts (Table 1.2).

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 1.1).

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 1.1). 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 1.2) (Fig. 1.5A).

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. 1.5B). 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 1.2).

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 = 2.38×10^{-11}), but no significant group differences (Table 1.2). 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 1.2). 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 1.2). 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 1.2).

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 1.2).

Log_{10}-transformed latencies to begin eating on the two successive testing days were not correlated and not significantly different (Table 1.1). 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 1.2).

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 1.1). 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 1.2).

3.2.6. High-fat-diet preference

The three reproductive groups did not differ significantly in their preference for high-fat diet (Table 1.2). 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 1.1). 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 1.2).

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 1.1). 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 1.2). 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 1.1). An ANCOVA of rank-transformed mean values with age, time since pairing, and body mass as covariates indicated no differences between groups (Table 1.2).

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 1.1). 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 conducted on squared data, with age, time since pairing, and body mass as covariates. *A priori* contrasts indicated no significant differences between VM, NB or BM (Table 1.2).

Numbers of fecal pellets on the two successive testing days were highly correlated (r = 0.702) and not significantly different from one another (Table 1.1). 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, 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 more pellets than BM (P = 0.003; Table 1.2, Fig. 1.6).

3.3.3. Saccharine preference

The three reproductive groups did not differ significantly in their preference for saccharin (Table 1.2). 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 1.3).

4. Discussion

We hypothesized that in the biparental California mouse, fathers, like mammalian mothers, experience increased metabolic demands (Speakman, 2008) and affective changes (Slattery & Neumann, 2008, Wingfield & Sapolsky, 2003), 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 1.2). 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 Saltzman & Ziegler, 2014), 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 (Harris et al., 2011, Saltzman et al., 2015). 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 (Saltzman et al., 2015). 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 (Blouin et al., 2008, Harada et al., 2016, Lovejoy et al., 1995, Nettleship et al., 2007, Page et al., 2005, Sainsbury & Zhang, 2012, Ziegler et al., 2009). 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 (Breed & Taylor, 2000, Kenagy & Trombulak, 1986, Preston et al., 2003). 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., Gammie et al., 2003, Surwit et al., 1988). 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 (Wilcox, 2005). 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 (Borai et al., 2011, Bowe et al.,

2014). 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 (Cefalu et al., 1995, Lewis et al., 2002). 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, and 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 (Gubernick & Nelson, 1989, Gubernick et al., 1995, Trainor et al., 2003). Importantly, several of these hormones are known to modulate glucose and lipid metabolism in humans and rodents (reviewed in Santosa & Jensen, 2015, Shen & Shi, 2015, Varlamov et al., 2015). For example, testosterone can increase fat oxidation and lipolysis (Santosa & Jensen, 2015), reduce gluconeogenesis, and increase glycogen synthesis and storage in the liver, thereby lowering circulating glucose levels (Shen & Shi, 2015). Testosterone also improves insulin sensitivity (Varlamov et al., 2015). In our study, NB had significantly higher testis masses than VM. If circulating testosterone levels followed a similar pattern (Eleftheriou & Lucas, 1974), 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 (Santosa & Jensen, 2015), and plasma concentrations of both androgens and progesterone differ across reproductive conditions in male California mice (Trainor et al., 2003). 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 (Roberts et al., 2013). 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 (Strekalova et al., 2004, Willner et al., 1992). 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 (Cryan et al., 2005). 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 (Archer, 1973, Bronikowski et al., 2001, Coleman et al., 1998, Flint et al., 1995). 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 (Saltzman & Ziegler, 2014).

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) (Chauke et al., 2012). 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 (Chauke et al., 2011, Chauke et

al., 2012, Harris et al., 2013) or chronic stressors (De Jong et al., 2013). 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 (Chauke et al., 2012). New fathers and expectant fathers also spent more time sniffing and touching a wire mesh ball containing a newborn pup than virgin males (De Jong et al., 2009). 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 (Bardi et al., 2011). 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 (Brunton et al., 2008, Slattery & Neumann, 2008).

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 (Vendruscolo et al., 2004). For example, manipulations that reduce fear/anxiety (e.g., administration of benzodiazepines) can attenuate the analgesia elicited by stressful situations (Helmstetter, 1993). 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 (Saltzman et al., 2015). 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 (McNab & Morrison, 1963), 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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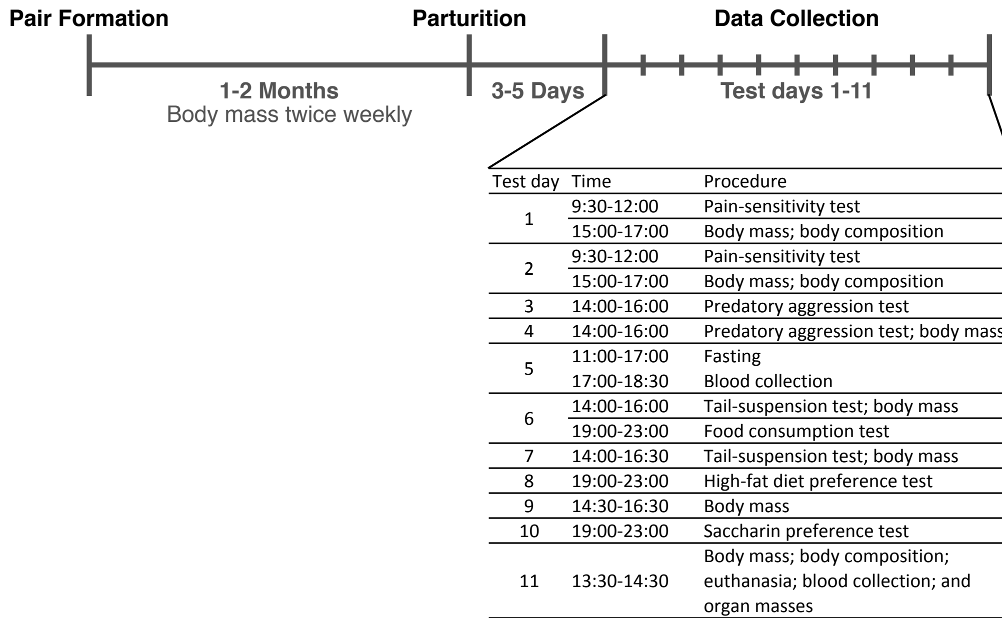
Figures and tables



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. 1.1. Timeline of experimental procedures.

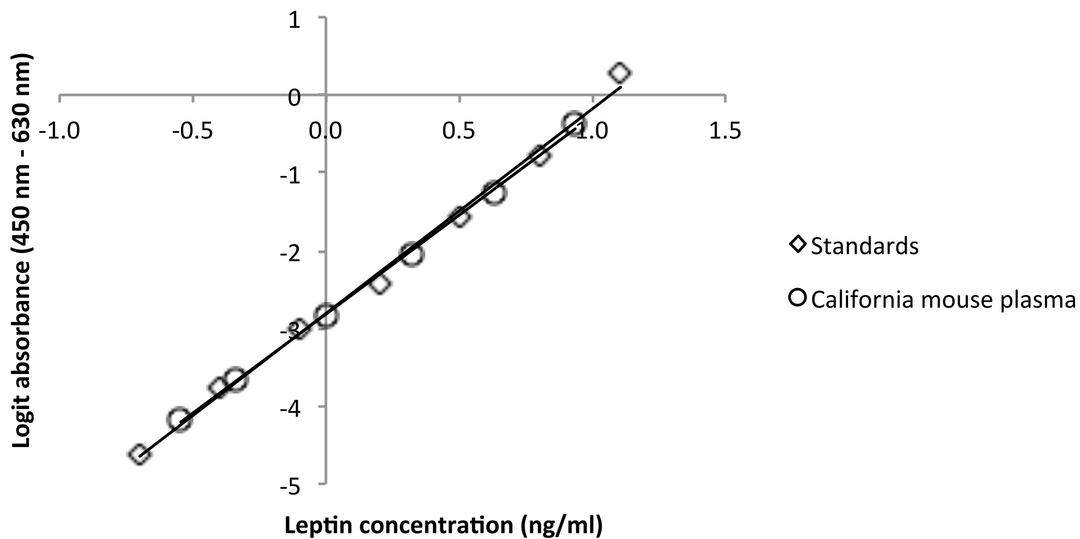


Fig. 1.2. Parallelism of leptin levels from serial dilutions of California mouse plasma (circles) and standards from a commercially available mouse leptin ELISA kit (squares). Data were transformed using the log-logit method. California mouse plasma: $y = 2.5443x - 2.8192$. Standard curve: $y = 2.6274x - 2.8088$. Difference between slopes: $P=0.444$.

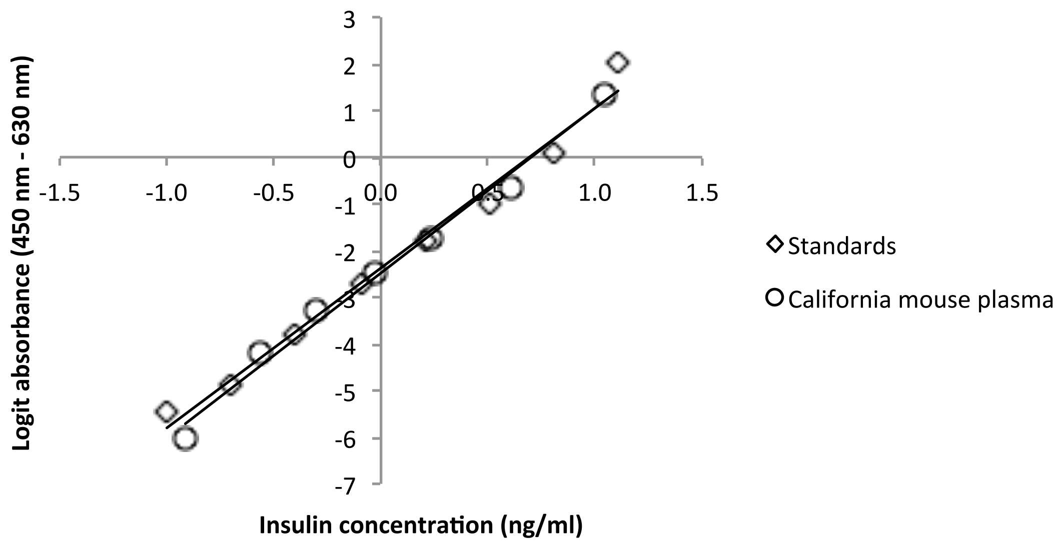


Fig. 1.3. Parallelism of insulin levels from serial dilutions of California mouse plasma (circles) and standards from a commercially available mouse insulin ELISA kit (squares). Data were transformed using the log-logit method. California mouse plasma: y = 3.5358x - 2.4810. Standard curve: y = 3.4276x - 2.3737. Difference between slopes: P=0.679.

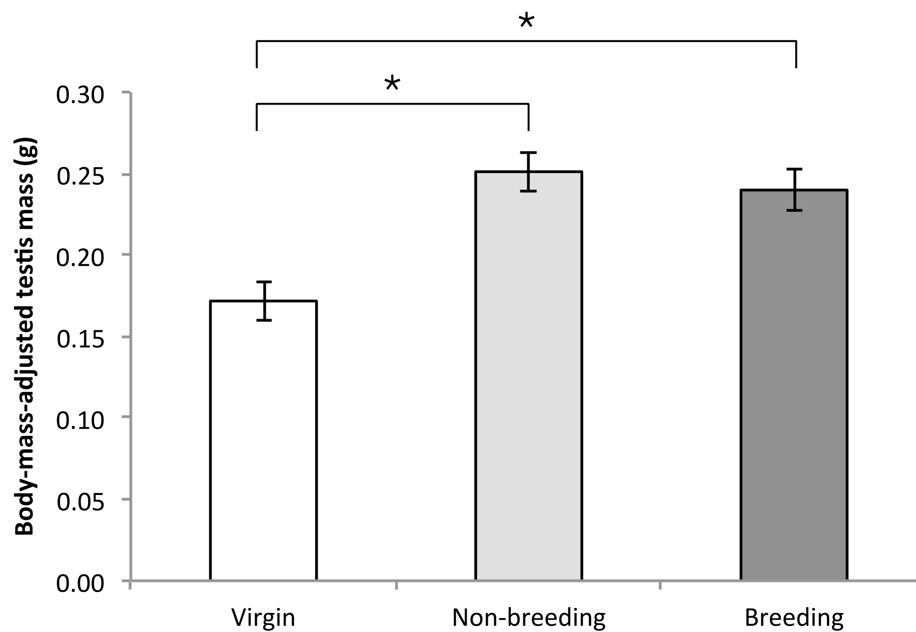


Fig. 1.4. Estimated marginal means (EMM) and associated standard errors (SE) of testis mass (mean of left and right testes). *A priori* contrasts indicated that virgin males had significantly smaller testis masses than did non-breeding (P = 0.00003) and breeding males (P = 0.00047). Values are estimated marginal means and standard errors, adjusting for variation in body mass, age, and time since pairing.

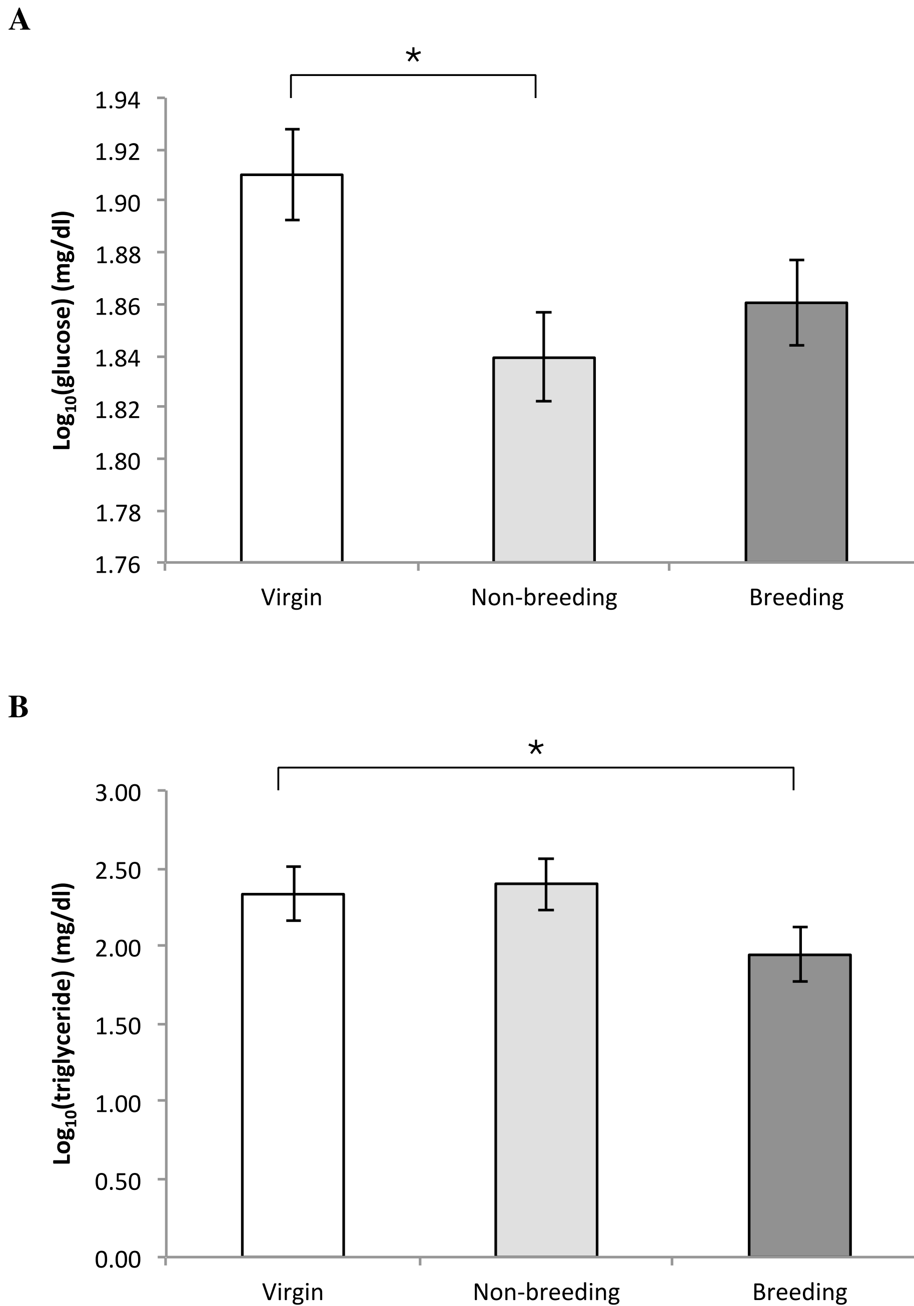


Fig. 1.5. A) EMM ± SE $\log_{10}$-transformed fasted blood glucose levels. *A priori* contrasts indicated a significant difference between virgin and non-breeding males (P = 0.007). **B)** EMM ± SE $\log_{10}$-transformed fasted triglyceride levels. Values for breeding males were significantly lower than for virgin males (P = 0.005) and nominally lower than for non-breeding males (P = 0.011). Values are estimated marginal means and standard errors, adjusting for variation in handling time, age and time since pairing.

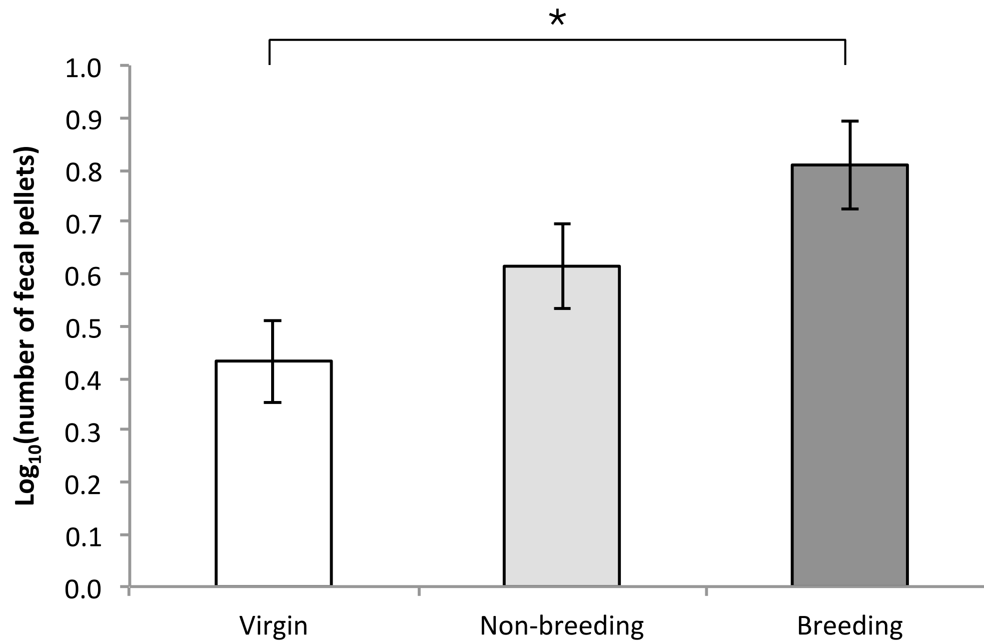


Fig. 1.6. EMM ± SE log_{10}-transformed number of fecal pellets expelled in tail-suspension tests. Mean values from two trials on each mouse were used for comparing groups. *A priori* contrasts indicated a significant difference between virgin and breeding males (P = 0.003). Values are estimated marginal means and standard errors, adjusted for variation in body mass, age and time since pairing.

Table 1.1. 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	---	none	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	second	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.

Table 1.2. 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.
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.

									Virgin (VM)		Non-breeding (NB)		Breeding (BM)	
Trait	Unit	Transform	D.F.[&]	F[&]	Group P[&]	P VM vs. NB	P VM vs. BM	P NB vs. BM	EMM	SE	EMM	SE	EMM	SE
Pain-sensitivity: Latency	second	log_{10}	2,37	3.063	0.059	<u>0.020</u>	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	second	**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	**<u>0.003</u>**	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

[&] 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.

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.

Table 1.3. Least-squares linear regressions of each trait on litter size, as well as relevant covariates (as described in the text), for the breeding males. B = partial regression slope, 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 by the homeostatic model assessment of insulin resistance, QUICKI = insulin sensitivity calculated by the quantitative insulin check index of insulin sensitivity.

Trait	Transform	N	F	df	P	B
Pain-sensitivity: Latency	none	15	2.802	14	0.125	10.468
Pain-sensitivity: Number of Urine Pools	none	14	3.114	14	0.108	0.383
Pain-sensitivity: Number of Fecal Pellets	none	15	4.644	14	0.057	1.205
Predatory Aggression: Latency to Attack Cricket	$\log_{10}$	15	2.996	14	0.114	-0.254
Predatory Aggression: Latency to Eat Cricket	none	12	5.316	11	0.055	-8.237
Predatory Aggression: Mass of Cricket Consumed	**0.5	15	4.031	14	0.076	0.131
Tail-suspension: Duration of Mobility	**2	13	1.850	12	0.211	5.759
Tail-suspension: Number of Fecal Pellets	none	14	0.001	13	0.973	0.064
Hematocrit	none	13	0.901	12	0.367	1.302
Glucose	none	15	0.001	14	0.973	0.162
TC	**3	15	0.003	14	0.956	26702.169
HDL	$\log_{10}$	15	0.459	14	0.517	-0.031
TRG	$\log_{10}$	15	0.113	14	0.746	-0.065
Non-HDL	none	14	0.115	13	0.745	2.245
TC/HDL	**0.1	14	0.654	13	0.445	0.008
Circulating Leptin	none	14	4.357	13	0.066	0.643
Circulating Insulin	none	11	0.477	10	0.516	0.468
HOMA-IR	none	11	0.780	10	0.407	3.090

QUICKI	none	11	0.004	10	0.952	-0.003
Food Consumption	none	14	8.361	13	0.018	0.586
Water Consumption	**2	14	6.289	13	0.033	5.202
High-fat Diet Preference	**0.2	14	0.148	13	0.708	0.076
Sweet'N Low Preference	**0.5	13	1.802	12	0.212	0.152
Fat Mass	none	14	7.647	13	0.020	2.687
Lean Mass	**2	14	2.316	13	0.159	185.463
Percent Fat Mass	**0.5	14	5.680	13	0.038	0.561
Percent Lean Mass	none	14	5.862	13	0.036	-3.723
Heart Mass	$\log_{10}$	14	0.123	13	0.734	-0.013
Triceps Surae Mass	**0.1	14	2.614	13	0.140	-0.006
Testes Mass	none	14	1.136	13	0.314	-0.026
Kidneys Mass	none	14	1.201	13	0.302	-0.024
Prepartum Body Weight Slopes	none	15	0.200	14	0.663	0.021
Postpartum Body Weight Mean	**2	14	9.112	13	0.013	514.335

Nominally significant P values (0.007 < P < 0.05) are underlined. No P values were significant (P < 0.007) when adjusted for the False Discovery Rate.

CHAPTER 2

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

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Abstract

Reproduction strongly influences energetic demands, metabolism, and morphology in female mammals. In species in which males assist with rearing offspring, reproduction might be associated with similar changes in fathers. We examined effects of an energetic and physical 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 (breeding males), an ovariectomized female treated with estrogen and progesterone to induce estrus (non-breeding males), or an untreated ovariectomized female (virgin males). Within each group, half of the animals were housed under standard laboratory conditions and half in cages requiring them to climb wire mesh towers to obtain food and water; in addition, these latter animals were fasted for 24 hours every third day. Body and fat mass showed a housing condition x reproductive group interaction: the challenge condition increased body and fat mass in both virgin and non-breeding males, but breeding males were unaffected. Males housed under the physical and energetic challenge had higher blood lipid content, compared to controls. The challenge condition also decreased maximal aerobic capacity and related traits (hematocrit and relative triceps surae mass), but increased pain sensitivity and number of fecal boli excreted during tail-suspension tests (a measure of anxiety). These results indicate that our physical and energetic challenge paradigm had numerous metabolic and morphological effects, but these effects were largely unaffected by reproductive condition.

Keywords: anxiety, body composition, fatherhood, metabolism, paternal care,

reproduction

1. 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 ($\dot{V}O_2max$), 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 [$\dot{V}O_2max$]) 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.

2. Materials and 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 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).

2.2. 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; 10-11 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. 2.1). 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.

2.3. 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.

2.4. 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).

2.5. Measurements

2.5.1. Morphology

2.5.1.1. 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 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. 2.1).

2.5.1.2. 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.

2.5.1.3. 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.

2.5.2. Metabolism, energetics, and related behaviors

2.5.2.1. 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.

2.5.2.2. 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.

2.5.2.3. Maximum aerobic metabolic rate (days 11 and 12)

Maximal oxygen consumption ($\dot{V}O_2max$) 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. $\dot{V}O_2$max was determined as highest $\dot{V}O_2$ averaged over 1 min. We tested $\dot{V}O_2$max for repeatability across the two days of tests, and the higher of the two values for each animal was used for analysis.

2.5.2.4 Blood metabolic profiles

2.5.2.4.1. 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). 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). 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.

2.5.2.4.2. 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).

2.5.2.4.3. 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, (insulin (mU/l)*glucose (mmol/l)/22.5), and the quantitative insulin check index of insulin sensitivity (QUICKI, 1/(log(insulin (mU/I))+log(glucose (mg/dl))), both based on fasted glucose and insulin levels (Borai et al., 2011, Bowe et al., 2014).

2.5.2.4.4. 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.

2.5.3. Behavioral indicators of pain sensitivity, depression, and anxiety

2.5.3.1. 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).

2.5.3.2. 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.

2.5.3.3. 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.

2.6. 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 2.1), and mean values were used for subsequent analyses (except for $\dot{V}O_2max$). 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."

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. 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 2.2).

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 2.2, Fig. 2.2).

3.1.2. 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 2.2, Fig. 2.3). 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 ($P = 4.90\times10^{-7}$). 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. 2.4).

3.1.3. 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 2.1), 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 ($P = 3.9\times10^{-5}$), and lower average testis mass ($P = 3.33\times10^{-7}$) than did CTRL. No other organ masses differed among reproductive groups or between housing conditions, and no interactions occurred (Table 2.2). 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. 2.4).

3.2. Metabolism/energetics and related behaviors

3.2.1. 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 2.1). 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 2.1). CHAL had higher fasted TC ($P = 1.01\times10^{-8}$), HDL ($P = 0.017$) and rank-transformed triglyceride levels ($P = 0.0001$) than CTRL (Table 2.2), 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.

3.2.2. 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 ($P = 4.72\times10^{-8}$) and a significant difference between housing conditions ($P = 0.006$), with CHAL having higher leptin levels than CTRL (Table 2.2); 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 (Table 2.2). 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 2.2).

3.2.3. 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 2.2). 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.

3.2.4. 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 2.2).

3.2.5. 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 2.2). In general, males did not consistently prefer high-fat diet over standard diet (grand mean ± SE = 0.660 ± 0.042).

3.2.6. Maximum aerobic metabolic rate

Measurements of $\dot{V}O_2max$ 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 ($P < 3\times10^{-5}$) and a significant difference between housing conditions ($P < 0.0015$), with CHAL lower than CTRL (Table 2.2). $\dot{V}O_2max$ was not affected by reproductive group or by an interaction between housing condition and reproductive group.

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

3.3.1. 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 2.1). 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 2.2). 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 2.1). 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 2.2). The numbers of fecal boli expelled on the two successive testing days were also positively correlated (r = 0.340; Table 2.1). We found no significant difference between reproductive groups and no significant housing condition x reproductive group interaction (Table 2.2).

3.3.2. 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 2.1). 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 2.2).

Numbers of fecal boli expelled on the two successive testing days were correlated (r = 0.399) and not significantly different from one another (Table 2.1). 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 2.2: 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.

3.3.3. 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 2.2). In general, males did not consistently prefer the saccharin solution over water (grand mean ± SE = 0.650 ± 0.037).

3.4. 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).

4. 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.

4.1. 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 the fact 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 consistently available *ad lib*.

4.2. 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, but not in the current one, 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). The biological significance, if any, of the difference in insulin and leptin sensitivity under the two housing conditions is unclear.

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 these effects did not differ between fathers, virgins, and males housed with tubally ligated female mates (De Jong et al., 2013). In the present study, however, corticosterone levels, measured >26 h after food was returned to the challenged males from the previous 24-h fast, did not differ between males housed under standard lab conditions and energetically challenging conditions. This finding was unexpected, and the reason for it is unclear.

4.3. 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 would expect that animals in challenged conditions would show lower pain sensitivity and more anxiety-like behavior (number of fecal boli) during tail-suspension tests; however, in our study, while animals in the challenge condition showed higher pain sensitivity, they showed more anxiety-like behavior during tail-suspension tests. The mechanism and functional significance of this difference between our expectation and the result are unknown.

4.4. $\dot{V}O_2$max and correlates

$\dot{V}O_2$max is a measure of maximal aerobic metabolic rate, with higher $\dot{V}O_2$max indicating a greater capacity for sustained, aerobically supported exercise. Endurance exercise training can increase $\dot{V}O_2$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 $\dot{V}O_2$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 $\dot{V}O_2$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 $\dot{V}O_2$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 $\dot{V}O_2max$ 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).

4.5. 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.

5. Conclusions

In conclusion, we found that housing male California mice under physically and energetically challenging conditions broadly affected the animals' morphology, metabolism, and affective behavior, whereas fatherhood had very few effects on these measures. 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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Figures and tables

	Day of birth	PPD1	PPD2	PPD3	PPD4	PPD5	PPD6	PPD7	PPD8	PPD9	PPD10	PPD11	PPD12	PPD13	PPD14	PPD15	PPD16
				Test day 1	Test day 2	Test day 3	Test day 4	Test day 5	Test day 6	Test day 7	Test day 8	Test day 9	Test day 10	Test day 11	Test day 12	Test day 13	Test day 14
Morning			**Remove food**	**Return food**	Pain sensitivity	Pain sensitivity **Remove food**	**Return food**		**Remove food**	**Return food**		**Remove food**	**Return food**	Body mass $\dot{V}O_2max$	Body mass $\dot{V}O_2max$ **Remove food**	**Return food**	
Afternoon				Body mass Body comp.	Body mass Body comp.				Glucose and lipid profiles		Tail suspension Body mass	Tail suspension Body mass				Body mass Body comp.	Decapitation Blood collection Organ masses
Night					Saccharin preference			Food consumption						High-fat diet preference			

Fig. 2.1. 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 CHAL condition.

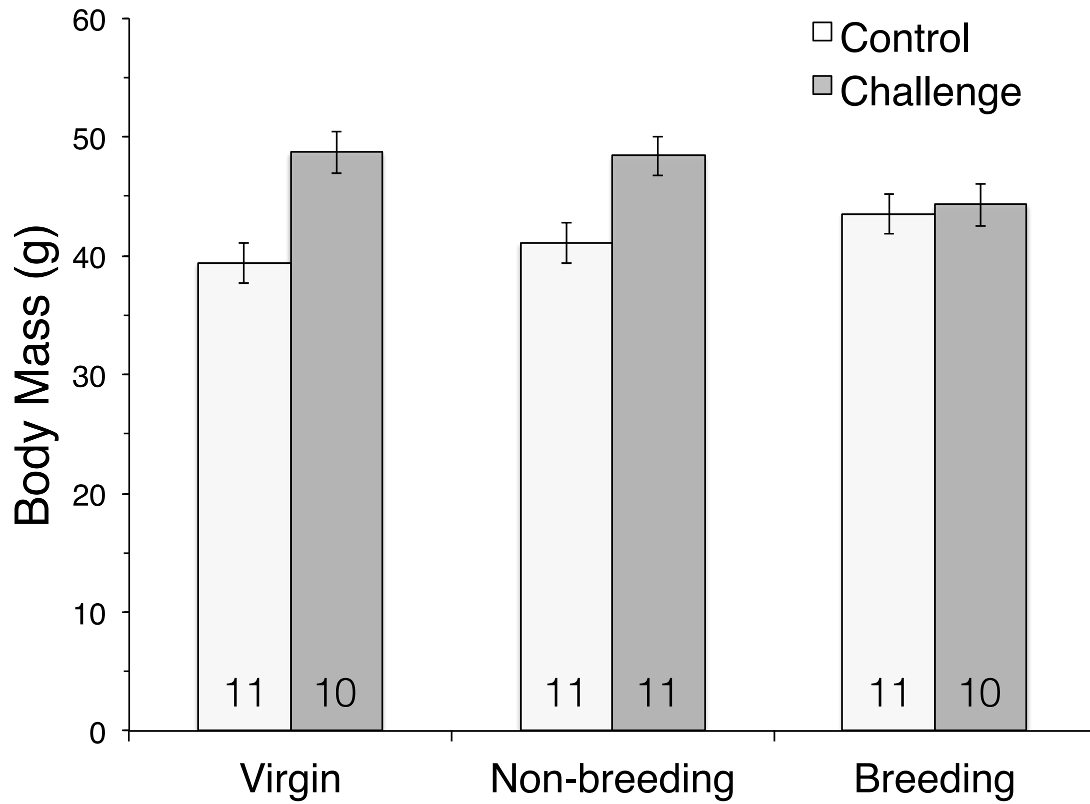

Fig. 2.2. Estimated marginal means (EMM) and standard errors (SE) of male postpartum body mass, adjusted for age. ANCOVA revealed an interaction between housing and reproductive conditions ($P = 0.037$) and a main effect of housing condition ($P < 0.001$). Sample sizes are shown in the bars.

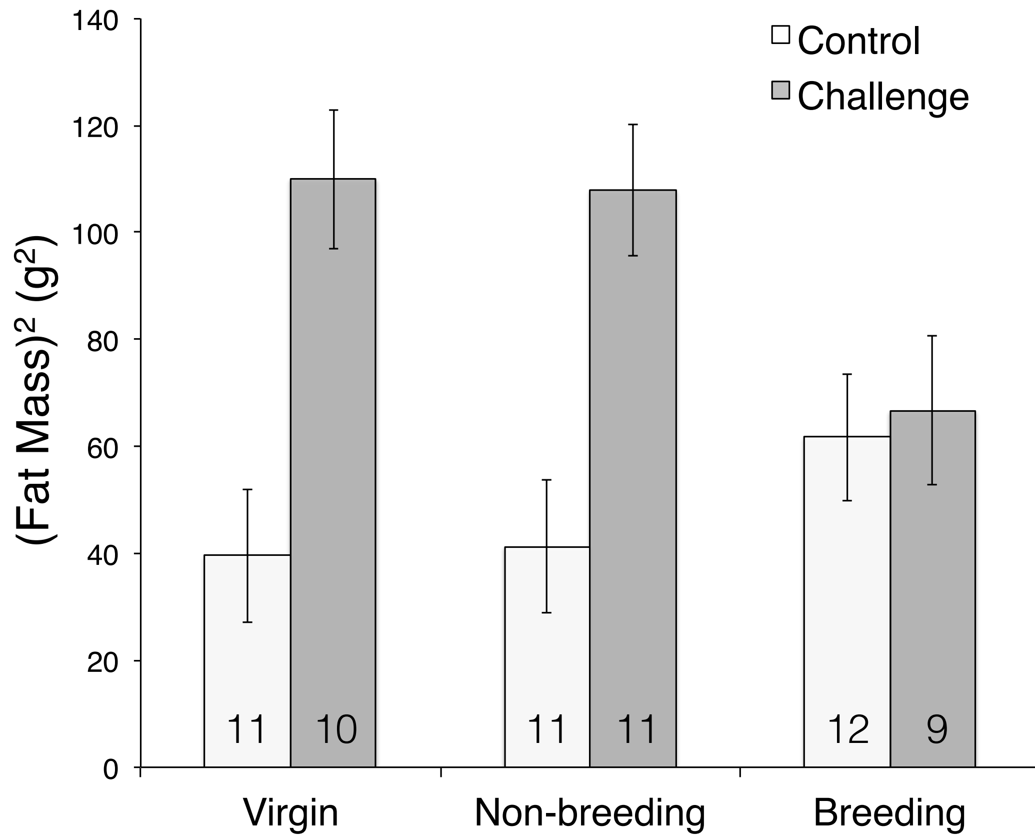

Fig. 2.3. EMM ± SE of squared fat mass, adjusted for variation in age. ANCOVA revealed an interaction between housing and reproductive conditions ($P = 0.019$) and a main effect of housing condition ($P < 0.0001$). Sample sizes are shown in the bars.

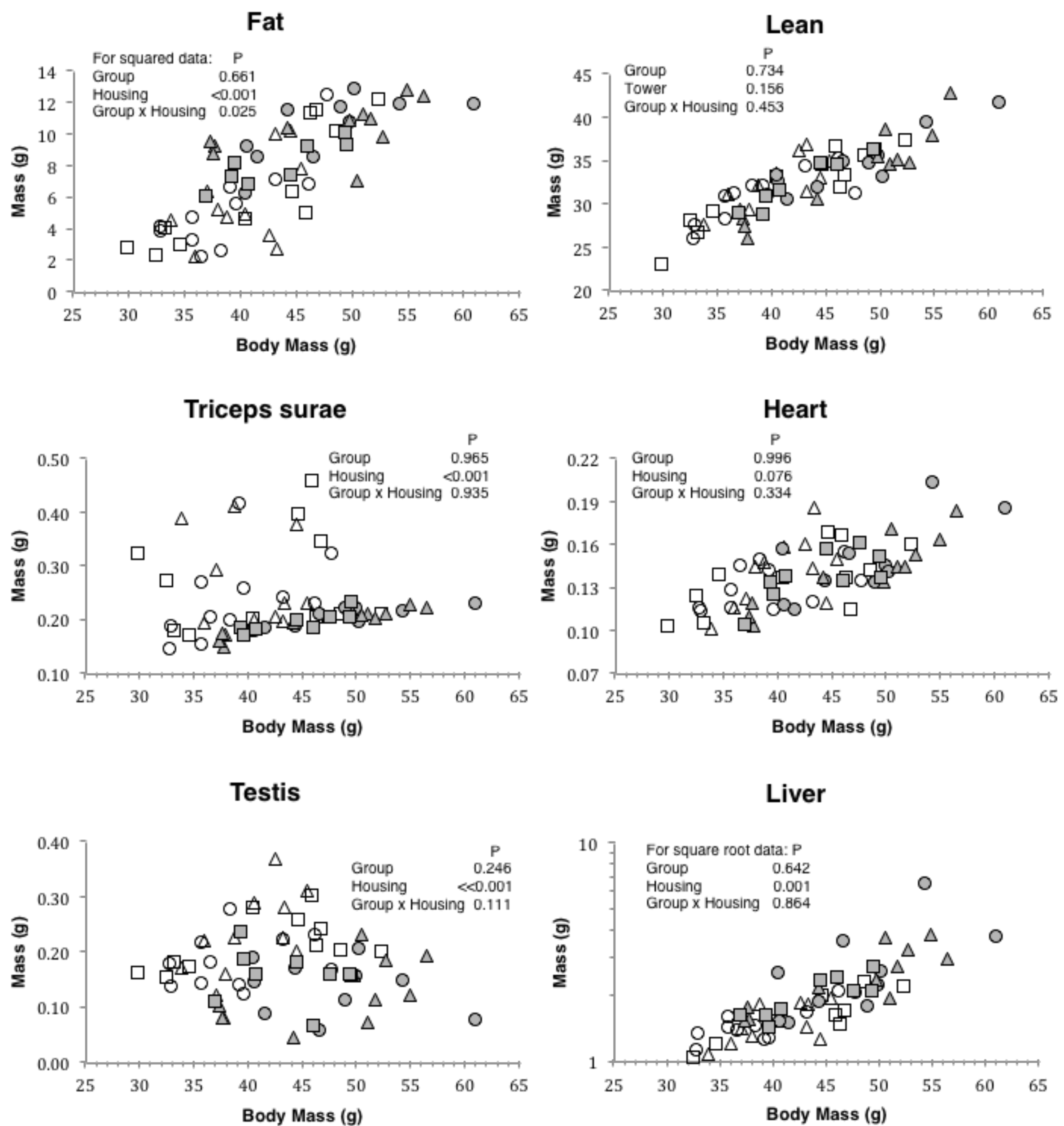

Fig. 2.4. Organ masses and body composition of males. Fat mass, triceps surae mass, testis mass and liver mass all differed significantly between Control (open shapes, n=34) and Challenge (closed shapes, n=30) conditions, but lean mass and heart mass did not. Fat mass and triceps surae mass also showed higher variance among Challenge individuals than among Control animals. Liver mass is shown on a $\log_{10}$-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 2.1. 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 ($P \leq 0.019$, 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.

Trait	Unit	Transform	N of paired observations	r of correlation	P of correlation	t of paired t-test	P of paired t-test
Pain-sensitivity: Latency	second	$\log_{10}$	57	0.313	**0.018**	0.827	0.412
Pain-sensitivity: Number of Urine Pools		none	65	0.454	**1.44E-04**	0.341	0.734
Pain-sensitivity: Number of Fecal Pellets		none	65	0.455	**1.38E-04**	-0.741	0.462
Tail-suspension: Duration of Immobility	minute	none	64	0.722	**1.67E-11**	-1.289	0.202
Tail-suspension: Number of Fecal Pellets		none	64	0.399	**0.001**	-0.768	0.445
$\dot{V}O_2$max	ml min^{-1}	none	64	0.879	**1.39E-21**	0.972	0.335
Glucose	mg dl^{-1}	none	65	0.970	**1.28E-40**	-0.337	0.737
Fat Mass	gram	none	61	0.992	**3.56E-55**	1.845	0.070
Lean Mass	gram	none	61	0.966	**3.02E-36**	-1.035	0.305
Percent Fat Mass	%	none	61	0.994	**5.76E-59**	4.515	**3.00E-05**
Percent Lean Mass	%	none	61	0.977	**3.67E-41**	-0.542	0.590
Triceps Surae Mass	gram	none	64	0.851	**5.85E-19**	-1.023	0.310
Testis Mass	gram	none	64	0.977	**2.64E-43**	1.704	0.093
Adrenal Mass	gram	none	63	0.858	**2.67E-19**	7.575	**2.16E-10**
Kidney Mass	gram	none	64	0.985	**2.72E-49**	4.245	**7.30E-05**

Table 2.2. 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 ($P \leq 0.019$, 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.

				F			P		
	Unit	Trans-form	D.F.[&]	Group	Condition	Group * Condition	Group	Tower	Group * Condition
Pain-sensitivity: Latency	second	log_{10}	55	0.528	8.635	0.248	0.593	**0.005**	0.782
Pain-sensitivity: Number of Urine Pools		rank	56	1.279	5.476	0.296	0.286	0.023	0.745
Pain-sensitivity: Number of Fecal Boli		rank	56	0.073	1.000	0.218	0.930	0.322	0.804
Tail-suspension: Duration of Immobility	minute	none	56	0.047	2.476	0.187	0.954	0.121	0.830
Tail-suspension: Number of Fecal Boli		rank	56	0.773	6.117	1.187	0.466	**0.016**	0.313
$\dot{V}O2max$	ml min^{-1}	none	55	0.342	12.008	0.386	0.712	**0.001**	0.681
Hematocrit	%	none	57	0.521	9.690	1.912	0.597	**0.003**	0.157
Glucose	mg dl^{-1}	none	57	0.197	0.389	0.798	0.822	0.535	0.455
TC	mg dl^{-1}	none	47	2.660	48.172	1.238	0.080	**1.01E-08**	0.299

HDL	mg dl^{-1}	none	42	0.350	6.215	0.200	0.707	**0.017**	0.820
TRG	mg dl^{-1}	rank	51	0.488	17.163	2.194	0.617	**1.29E-04**	0.122
Circulating leptin	ng ml^{-1}	log$_{10}$	54	0.597	8.336	1.548	0.554	**0.006**	0.222
Circulating insulin	ng ml^{-1}	log$_{10}$	56	0.295	3.618	0.147	0.746	0.062	0.863
Circulating corticosterone	ng ml^{-1}	log$_{10}$	54	1.246	2.900	0.438	0.296	0.094	0.648
HOMA-IR		log$_{10}$	58	0.376	12.453	0.117	0.688	**0.001**	0.890
QUICKI		log$_{10}$	58	0.458	11.410	0.109	0.635	**0.001**	0.897
Food Consumption	gram	rank	56	0.569	3.602	1.705	0.569	0.063	0.191
High-fat Diet Preference	%	squared	54	0.337	1.413	0.054	0.716	0.240	0.948
Sweet'N Low Preference	%	rank	57	0.649	0.811	0.889	0.526	0.372	0.417
Fat Mass	gram	squared	57	0.461	18.178	4.249	0.633	**7.60E-05**	**0.019**
Lean Mass	gram	none	57	0.235	2.157	1.209	0.791	0.147	0.306
Percent Fat Mass	%	squared	57	0.292	17.159	2.328	0.715	**1.43E-04**	0.105
Percent Lean Mass	%	rank	57	0.409	32.186	3.284	0.666	**4.90E-07**	0.045
Heart Mass	gram	none	56	0.004	3.266	1.117	0.996	0.076	0.334
Liver Mass	gram	square root	54	0.447	11.353	0.147	0.642	**0.001**	0.864

Spleen Mass	gram	rank	52	0.214	2.159	0.493	0.808	0.148	0.613
Triceps Surae Mass	gram	rank	54	0.036	20.110	0.067	0.965	**3.90E-05**	0.935
Testis Mass	gram	none	55	1.441	33.685	2.291	0.246	**3.33E-07**	0.111
Adrenal Mass	gram	log_{10}	54	1.123	0.793	2.764	0.271	0.185	0.071
Kidney Mass	gram	log_{10}	55	0.417	0.285	0.587	0.661	0.596	0.559
Prepartum Body Mass Slopes		none	57	0.138	15.245	3.508	0.872	**2.52E-04**	0.037
Postpartum Body Mass Mean	gram	none	58	0.088	4.295	0.317	0.916	0.043	0.730

CHAPTER 3

Behavioral, Morphological, and Endocrine Effects of Single Motherhood on Mothers in the Biparental California Mouse

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Abstract

Being a mother is energetically costly for mammals and is associated with pronounced changes in mothers' physiology, morphology and behavior. In ~5% of mammals, fathers assist their mates with rearing offspring and can enhance pup survival and development. Although these beneficial consequences of paternal care can be mediated by direct effects on offspring, they might also be mediated indirectly, through beneficial effects on mothers. We tested the hypothesis that fathers in the monogamous, biparental California mouse (*Peromyscus californicus*) reduce the burden of parental care on their mates, and therefore that females rearing offspring with and without assistance from their mates will show differences in endocrinology, morphology and behavior, as well as in survival and development of pups. We found that pups' survival and development in the lab did not differ between those raised by a single mother and those reared by both mother and father. Both single and paired mothers had higher lean mass and/or lower fat mass and showed more anxiety-like behavior in open-field tests and tail-suspension tests, compared to non-breeding females. Single mothers had higher body-mass-corrected liver and heart masses, but lower ovarian and uterine masses, than both paired mothers and non-breeding females. Single motherhood also induced a flattened diel corticosterone rhythm and a blunted corticosterone response to stress, compared to non-breeding females. These findings suggest that absence of a mate induces morphological and endocrine changes in mothers, which might result from increased energetic demands of pup care and could potentially help maintain normal survival and development of pups.

1. Introduction

Maternal care is costly for female mammals and is associated with morphological, physiological, and behavioral changes in mothers (Lonstein, 2007, Slattery & Neumann, 2008, Speakman, 2008). In small mammals, mothers often undergo declines in fat stores during lactation. They can also experience organ remodeling, such as growth of the alimentary tract and associated organs such as the liver and pancreas, which may be necessary to meet the high demands of lactation (Jolicoeur et al., 1980, Kennedy et al., 1958). Consequences of the costs of motherhood include reductions in thermogenesis and physical activity, bone loss, and disruption of sleep patterns (Speakman, 2008). Mothers also exhibit blunted hormonal, neural and behavioral responses to stressors during late pregnancy and lactation in some mammals (Brunton et al., 2008, Lightman et al., 2001, Slattery & Neumann, 2008). Furthermore, pregnant and lactating rats, mice and possibly humans exhibit reduced anxiety and fearfulness (Lonstein, 2007, Lonstein et al., 2014, Slattery & Neumann, 2008). After becoming mothers, for example, rats (*Rattus norvegicus*) and lab mice (*Mus spp*.) have reduced acoustic startle responses, increased locomotion in open-field tests, increased time spent in the open arms of the elevated plus maze and reduced fleeing from an intruder.

Although most mammals are uniparental, with only the mothers caring for the offspring, approximately 5% are biparental, with fathers helping rear their young (Kleiman & Malcolm, 1981). Fathers in biparental species can reduce the energetic burden of motherhood. Depending on the species, paternal care in mammals can include

a variety of behaviors, including retrieving, defending, playing with, grooming and huddling offspring, as well as providing them with food and a nest (Kleiman & Malcolm, 1981). In some species, such as the California mouse (*Peromyscus californicus*), fathers spend as much time interacting with their offspring as do mothers (Bester-Meredith et al., 1999, Woodroffe & Vincent, 1994). In prairie voles (*Microtus ochrogaster*), fathers' paternal behavior may compensate for low levels of maternal care (Perkeybile et al., 2013): offspring receive more care from fathers when mothers display low care.

Fathers in biparental species can influence offspring survival and development. For example, in California mice, paternal care has been found to greatly enhance survival and development of offspring both in the field and under energetically challenging lab conditions, such as low ambient temperature or having to work for food (Bredy et al., 2007, Cantoni & Brown, 1997, Dudley, 1974, Gubernick & Teferi, 2000, Gubernick et al., 1993, Wright & Brown, 2002). Father absence can also influence offspring's cognitive, emotional, and reproductive behavior. In California mice, permanent removal of the father on postnatal day 3 decreases spatial learning ability (Bredy et al., 2004), and in mandarin (*M. mandarinus*) and prairie voles, paternally deprived offspring are more anxious and less social on multiple measures (Cao et al., 2014, Jia et al., 2009, Wang et al., 2012, Yu et al., 2012). Therefore, pups reared by mothers without their mates may have impaired survival and development, as well as changes in behavior, compared to pups reared by both their mother and father, especially under energetically challenging conditions.

Although the negative consequences of losing the father can be mediated by direct effects on offspring, they might also be mediated indirectly, through negative effects on mothers. However, the effects of fathers on their mates have received very little study. Exceptions include studies in Djungarian hamsters (*Phodopus campbelli*) showing that removal of the mate might influence pup survival by altering mothers' thermoregulatory abilities (Scribner & Wynne-Edwards, 1994, Walton & Wynne-Edwards, 1997, Wynne-Edwards & Lisk, 1989), as well as a recent study in prairie voles showing that mothers losing their mates have altered emotionality (i.e., stress-responsiveness, depression, anxiety and fearfulness), including increased anxiety-related behavior in the elevated plus maze and more passive stress-coping in the forced-swim test (Bosch et al., 2017).

The California mouse is monogamous and biparental in both the field and lab, and fathers engage in all the same parental behaviors as mothers except nursing, and to a similar extent (Gubernick & Alberts, 1987). Because biparental care is relatively rare in mammals, most research in the California mouse has focused on the biology of paternal behavior, while less has focused on maternal care (Bester-Meredith et al., 2017). In this study we tested the hypothesis that California mouse mothers rearing offspring without assistance from their mates would have poorer morphological, physiological and affective condition, as well as impaired survival and development of pups, compared to mothers housed with their mates. In addition, we hypothesized that these differences would be more pronounced under energetically challenging environmental conditions that increase the cost of motherhood. We predicted that single mothers would have lower body and fat mass, show more anxiety- and depression-like behavior, and display altered

profiles of a metabolically important, stress-responsive hormone, corticosterone (CORT), compared to mothers housed with their male mate. Finally, we anticipated that these differences between single and paired mothers would be more pronounced, or might only occur, under energetically challenging conditions.

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 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 22° C and humidity was about 57%. Cages were checked once daily and changed weekly.

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).

2.2. Experimental design

Mice were weaned at 27-31 days of age, prior to the birth of younger siblings, and housed in same-sex groups of 3-4 related and/or unrelated, age-matched individuals. At 92-111 days of age (102.6 ± 0.5 days, mean ± SE; young adulthood), 88 females were randomly assigned to two categories. Non-breeding females (NB, n=29) were housed with a male that had been vasectomized (see below) 7 days before pairing, and breeding females (n=59) were housed with intact males. These latter males were vasectomized 16-21 days after pairing (13 to 32 days before birth of the first litter) to prevent postpartum pregnancies. Pair mates were no more closely related to each other than first cousins.

When its first litter was born, each breeding female was randomly assigned to one of two conditions: single mothers (SM, n=29), whose male mates were removed 1 day after the birth of the first litter, or paired mothers (PM, n=30), whose mates remained with them throughout the entire lactation period. All mothers (and NB, at a comparable time point) were then left undisturbed for 2-3 days until the beginning of the 25-day testing period (Fig. 3.1). At postpartum day 27 or 28, females and pups were weighed and their body composition was assessed (see below). Pups were also checked daily for eye opening (Harris et al., 2013, Zhao et al., 2018).

Approximately half of the pairs in each reproductive group (n=42: 14 NB, 14 PM, 14 SM) were housed under standard lab conditions throughout the experiment (CTRL). The remaining pairs (n=46: 15 NB, 16 PM, 15 SM) were housed from the time of pair formation in cages in which they were required to climb towers in order to gain access to

food and water (CLIMB; see below). Thus, we had a total of 6 experimental groups (3 reproductive groups × 2 housing conditions).

2.3. Climbing towers

A climbing-exercise paradigm modified from previous studies of lab mice (*Mus musculus*) (Lionikas & Blizard, 2008, Mori et al., 2003), rats (*Rattus norvegicus*) (Notomi et al., 2001) and California mice (Zhao et al., 2018) was used to subject the animals in the CLIMB condition to a mild physical challenge. Mice were housed in cages with wire lids connected to two wire mesh (mesh openings: ~5 mm × 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.

2.4. Vasectomies

Male mice were vasectomized 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 vas deferens was sutured and cut. All reproductive structures were repositioned back in the abdominal cavity, the 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).

Following surgery, males to be paired with non-breeding females were housed individually in a standard cage for 7 days until pair formation. For breeding pairs, male and female pair mates were housed for the first 7 days after surgery on opposite sides of a standard cage divided in half by a steel mesh barrier, with food and water available in each half, to prevent direct physical interaction during post-surgical recovery (Harris & Saltzman, 2013). Males and females in non-breeding pairs were also housed in divided cages for 7 days at a matched time point to control for any effects of separation from the pair mate. At the end of the experiment, all females were dissected to check for pregnancy. None had visible fetuses.

2.5. Measurements

2.5.1. Morphology

2.5.1.1. Body mass

From the time of pair formation until the birth of the first litter in each breeding pair, or a matched time point for NB, females were weighed to the nearest 0.01 g at 14:00-16:00 h twice weekly, at 3- to 4-day intervals, to assess overall health, habituate the animals to handling, and monitor pregnancies through patterns of mass gain. Females were also weighed at 14:00-16:00 h on test days 1, 4, 8, and 13, as well as at 11:40-12:30 h on test day 12 (Fig. 3.1).

2.5.1.2. Body composition (days 1, 4, 8 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, 2018). Each scan lasted approximately 90 s, and animals were not sedated or anesthetized. 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).

2.5.1.3. Euthanasia and organ collection (day 14)

Females were decapitated between 13:30 and 16:00 h. Trunk blood was collected in a heparinized weighing boat, and organs [heart, liver, spleen, leg muscles (left and right triceps surae), ovaries (left and right), uteri, adrenal glands (left and right) and kidneys (left and right)] were removed and weighed as previously described (Zhao et al., 2017, 2018).

2.5.2. Behavioral indicators of neophobia, anxiety, and depression

2.5.2.1. Novel-object test (day 4)

Novel-object tests were administered between 14:00 h and 16:00 h to characterize neophobia. Each female's cage mate(s) (male mate and/or pups) were removed from the home cage, housed in a clean cage, and female's cage mate(s) were moved to a separate room. After 5 min, a golf ball (diameter: 4.8 cm) was placed in the corner of the home cage furthest from the female. Behavioral responses to the novel object were video-

recorded for 5 min. Immediately after testing, the female was weighed, scanned for body composition (see above), and then reunited with its cage mate(s) in the home cage. Behavioral parameters scored included latency to approach to within 2 cm of the golf ball, bouts of approaching the ball, total duration of immobility, and total durations of time spent sniffing and touching the ball (Chauke et al., 2012).

2.5.2.2. Open-field test (day 8)

Tests were administered between 14:00 h and 16:00 h to characterize anxiety-like behavior. The open-field arena was a 1.0 m × 1.0 m square with a height of 0.4 m, constructed of opaque black plastic and placed on a clean sheet of white butcher paper to enhance contrast between the arena floor and the darkly colored mice as previously described (Perea-Rodriguez et al., 2018). Tests were recorded by a video camera (GoPro HERO SESSION, GoPro Inc., San Mateo, CA, USA) suspended above the arena. After each test, the arena was disinfected and the butcher paper replaced. The open-field arena was located in an environmental chamber maintained at 1400 lux with two overhead white lights; temperature and humidity were maintained at ~23°C and ~70%, respectively. For each test, the female subject was placed in the center of the arena and video-recorded for 10 min. Immediately following testing, the female was weighed, scanned for body composition (see above), and then reunited with its cage mate(s) in the home cage.

Exploratory behaviors in the open-field arena were quantified using TopScanLite software (Clever Sys Inc., Reston, Virginia, USA). The arena was divided into two

concentric regions in the software: an inner square, measuring 0.5 × 0.5 m, in the center of the arena, and an outer region extending 0.5 m from each wall to the perimeter of the inner square. Bouts of crossing the boundary between inner and outer regions, total distance moved, distance moved in each region, and duration of time spent in each region were determined. We also recorded the number of fecal boli produced during the 10 min of testing. Distance traveled, duration in the inner region of the arena, and number of crossing bouts were considered negative indicators of anxiety-like behavior, whereas number of fecal boli were considered positive indicators (Gould et al., 2009).

2.5.2.3. Tail-suspension test (day 12)

We performed the tail-suspension test both to characterize depression-like behavior (Cryan et al., 2005) and as an acute stressor for assessment of the CORT stress response. Between 11:30 and 12:35 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, 2018). 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 Warthog LabHelper (http://www.warthog.ucr.edu). Activity was recorded every 0.004 s. Warthog LabAnalyst (http://www.warthog.ucr.edu) 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 as a positive indicator of anxiety.

2.5.2.4. Saccharin preference test (day 6)

Females' 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, a common marker of depression (Gibson, 2006, Pecoraro et al., 2004), as previously described (Zhao et al., 2017, 2018). Briefly, each mouse's cage mate(s) were removed from the home cage at the time of lights-off (19:00 h). The female remained alone in its home cage for 4 h, 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.

2.5.3. Blood collection (test days 2, 3, 10, 12)

Blood was collected at 03:50-04:10 h on test day 2, 19:50-20:10 h on test day 3 and 11:50-12:10 h on test day 10 for analysis of basal CORT concentrations across the diel cycle, and immediately after tail-suspension tests (11:30-12:35 h) on test day 12 for assessment of the CORT response to an acute stressor. Animals were anesthetized with isoflurane, and blood was collected from the retro-orbital sinus into heparinized capillary tubes within 3 min after either initial disturbance to the cage (basal samples) or the end of tail-suspension test (post-stress samples). Blood was centrifuged immediately for 12 min (13,300 rpm, 4 °C), and plasma was removed and stored at -80 °C.

2.5.4. Corticosterone radioimmunoassay

Plasma corticosterone concentrations were 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 1:100 to 1:1600 in order to ensure that values were contained within the curve. All samples were diluted and run in duplicate, and the averages of the duplicates were used in data analysis. Intra- and inter-assay coefficients of variation, calculated using kit-provided controls, were 2.19% and 5.31% for the high control, and 9.48% and 7.82 for the low control, respectively.

2.6. Statistical analysis

Data were analyzed by analysis of covariance (ANCOVA) or repeated-measures ANOVA, and Fisher's LSD for post hoc tests using SPSS. Age and/or other potentially relevant variables (see Results) were used as covariates. For each analysis, residuals were checked for skewness and visually inspected for outliers. Plasma CORT data were also tested for sphericity using Mauchly's test, and all other traits were tested for homogeneity of variance using Levene's test. Dependent variables were transformed as needed; however, data are presented as non-transformed values in the text and figures for ease of interpretation. For paired (right and left) organs, values from the two organs were compared using a paired t-test and a Pearson correlation to gauge repeatability (Table

3.1), and mean values were used for subsequent analyses. For the breeding pairs, we also performed ANOVAs to determine if litter size, pups' age of eye opening, and body composition at weaning age differed between pups reared by single mothers and the ones reared by paired mothers.

Excluding such nuisance variables as age, this study generated 250 P-values, 61 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 inter-related. 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 Incorporated, Cary, NC, USA). Based on this procedure, the 43 smallest P-values would have adjusted P-values <0.05 (the highest being 0.016). For simplicity, all P-values reported in the text and figures are raw values, not adjusted for multiple comparisons; however, we refer to P-values ≤0.016 as 'significant' and those between 0.016 and 0.05 as 'nominally significant' (Andrew et al., 2016, Zhao et al., 2017, 2018). All tests were two-tailed.

3. Results

3.1. Morphology

3.1.1. Body mass

We analyzed each individual body-mass value during the test period (days 1, 4, 8, 12 and 13) and also the mean postpartum body mass from all test days except day 12; body mass on day 12 was excluded because it was measured immediately after the tail-suspension

test. However, analyses that included test day 12 generated similar results. ANCOVA (with age as a covariate) found no main effect of housing condition, reproductive group, or interaction between housing condition and reproductive group for either mean body mass or body mass on test days 1, 4, 8 or 13. On test day 12, body mass differed nominally among reproductive groups ($P = 0.046$); post-hoc tests revealed that SM had significantly higher body mass than NB ($P = 0.014$; Table 3.2).

3.1.2. Body composition

Neither rank-transformed fat mass on any test days nor mean fat mass differed between housing conditions or among reproductive groups, when using age as a covariate. Similar results were found when mean or individual fat mass was expressed as percentage of total body mass, except that rank-transformed percent fat mass on test days 8 and 13 showed a main effect of reproductive group ($P = 0.014$ and $P = 0.031$ respectively), with SM having lower percent fat mass than both PM ($P = 0.043$) and NB ($P = 0.005$) on test day 8, and SM having lower percent fat mass than NB ($P = 0.011$) on test day 13. Note that mean and individual fat mass, as well as percent fat mass, had significant heterogeneity of variance that was not eliminated by transforms (with NB having higher variability than SM and PM), but the differences among groups are evident.

Square root-transformed mean postpartum lean mass nominally differed among reproductive groups ($P = 0.029$; Fig. 3.2) when analyzed by ANCOVA with age as a covariate, with SM having higher lean mass than NB ($P = 0.008$). Lean mass also differed among reproductive groups for test days 4, 8 and 13 (all $P < 0.005$). On each of

these days, both SM and PM had significantly higher lean mass than NB (all $P < 0.002$). No differences were found among groups for test day 1.

When expressed as a percentage of total body mass, mean percent lean mass did not differ among reproductive groups. However, percent lean mass on test days 4 and 13 differed among reproductive groups ($P = 0.008$ and $P = 0.050$, respectively), with SM having higher percent lean mass than NB ($P = 0.002$) on test day 4, and both SM and PM having nominally higher percent lean mass than NB ($P = 0.042$ and $P = 0.022$, respectively) on test day 13. Percent lean mass did not differ among groups on any other day. Neither lean mass nor percent lean mass was influenced by a main effect of housing condition or a housing condition × reproductive group interaction (Table 3.2).

3.1.3. Organ masses

Body mass was used as a covariate in all organ-mass analyses. For all paired organs (triceps surae, ovaries, adrenals and kidneys), the two organs showed high correlations within individual animals, similar to findings in male California mice (Andrew et al., 2017; Zhao et al. 2017, 2018). Right kidneys were significantly heavier than left kidneys (Table 3.1), as has been seen in other mammals and with other organs (e.g. Coleman et al., 1998, Idelman, 1978), while right adrenals were significantly lighter than left adrenals. Three females from the CLIMB condition had much higher triceps surae mass than the other CLIMB females and were excluded from analysis as outliers.

ANCOVA (with age and body mass as covariates) found positive effects of body mass (all $P < 0.001$) and significant or nominally significant effects of reproductive

groups on heart mass ($P = 0.023$), liver mass ($P < 0.001$), mean triceps surae mass ($P = 0.008$), mean ovary mass ($P = 0.002$) and uterus mass ($P = 0.004$) (Table 3.2; Fig. 3.2); however, no main effects of housing condition or housing condition × reproductive group interactions were found.

Post-hoc tests revealed that SM had significantly higher heart mass ($P = 0.006$) and lower rank-transformed triceps surae mass than NB ($P = 0.002$). Log_{10}-transformed liver mass was higher in SM than in both PM ($P = 0.046$) and NB ($P < 0.001$) and was higher in PM than NB ($P = 0.023$). Finally, SM had lower ovary and uterus masses than both PM ($P = 0.003$ and $P = 0.001$, respectively) and NB ($P = 0.001$ and $P = 0.042$, respectively; Fig. 3.2). No other organ masses differed among reproductive groups or between housing conditions, and no interactions occurred (Table 3.2).

3.2. Behavioral indicators of neophobia, anxiety, and depression

3.2.1. Novel-object test

Neither housing condition, reproductive group, nor their interaction affected females' latency to approach to within 2 cm of the novel object, number of approaches, duration of time spent sniffing and touching the novel object, or duration of immobility (Table 3.2).

3.2.2. Open-field test

ANCOVA (with age as a covariate) revealed a significant difference among reproductive groups in number of fecal boli produced ($P = 0.014$), with both SM and PM producing more boli than NB ($P = 0.009$ and $P = 0.012$, respectively; Fig. 3.3). Number of fecal boli

was not influenced by a main effect of housing condition or a housing condition × reproductive group interaction.

Neither housing condition, reproductive group, nor their interaction affected females' distances traveled during the open-field test (total distance and distance within each region), duration of time spent in the inner or outer region, or number of crossing bouts (Table 3.2).

3.2.3. Tail-suspension test

ANCOVA (with age and body mass as covariates) revealed a positive effect of body mass ($P = 0.012$) on the duration of immobility, but we found no significant differences between housing conditions or among reproductive groups, and the housing condition × reproductive group interaction was not significant.

As in the open-field test, number of fecal boli expelled differed significantly among reproductive groups, with both SM and PM producing nominally more boli than NB ($P = 0.036$ and $P = 0.030$, respectively). Number of fecal boli was not influenced by a main effect of housing condition or a housing condition × reproductive group interaction (Table 3.2; Fig. 3.3).

3.2.4. Saccharin preference

ANCOVA (with age as a covariate) showed that females housed in the CLIMB condition had nominally lower preference for saccharin compared to females in the CTRL condition ($P = 0.030$). Preference for saccharin solution did not differ among

reproductive groups and was not affected by a housing condition × reproductive group interaction (Table 3.2; Fig. 3.4).

3.3. Plasma corticosterone concentrations

3.3.1. Baseline corticosterone

Baseline CORT data were analyzed via repeated-measures ANOVA with time of sample (04:00, 20:00, and 12:00 h) as a within-subjects factor, and housing condition and reproductive group as between-subjects factors. The data were not normally distributed mainly because of 8 outliers (2 CTRL SM, 2 CTRL PM, 2 CTRL NB, and 2 CLIMB SM) for samples collected at 04:00 h (outlier values were ~10-fold higher than others samples, for no obvious biological reason). Therefore, data from the outliers were discarded, and the remaining data were $\log_{10}$-transformed to meet normality assumptions; however, analysis of $\log_{10}$-transformed data yielded similar results whether the outliers were included or not. Here we report results of analyses with the outliers omitted.

Plasma CORT concentrations (Fig. 3.5) varied across the three times of day (main effect of time: $F_{2,112} = 242.111$, $P < 0.001$), in a pattern that was influenced by both housing condition (time × housing condition interaction: $F_{2,112} = 3.761$, $P = 0.026$) and reproductive group (time × reproductive condition interaction: $F_{2,112} = 3.445$, $P = 0.011$). CORT levels were highest around the beginning of the active period (20:00 h, 1 h after lights-off) and lowest near the beginning of the inactive period (04:00 h, 1 h before lights-on). Neither the main effects of housing condition or reproductive group, the

interaction between housing condition and reproductive group, nor the 3-way interaction between time, housing condition and reproductive group was significant.

Post-hoc tests for the interaction between time and housing condition revealed that at 04:00 h, females in standard cages had significantly higher CORT levels than females in towered cages ($P = 0.009$). Post-hoc tests for the interaction between time and reproductive condition revealed that at 20:00 h, both single mothers and paired mothers had lower CORT levels than non-breeding females ($P = 0.002$ and $P = 0.040$, respectively). In addition, at 12:00 h, single mothers had nominally higher baseline CORT levels than non-breeding females ($P = 0.030$). No other pairwise comparisons were significant.

3.3.2. Corticosterone response to stress

Data were analyzed using repeated-measures ANOVA with stress condition (baseline CORT at 12:00 h, post-stress CORT at 12:00 h, immediately after a 6-min tail-suspension test) as a within-subjects factor, and housing condition and reproductive group as between-subjects factors. The tail-suspension test significantly increased $\log_{10}$-transformed plasma CORT (main effect of stress: $F_{1,66} = 345.425$, $P < 0.001$), and this effect differed among reproductive groups (stress × reproductive group interaction: $F_{1,66} = 4.484$, $P = 0.015$; Fig. 3.6). As described above, post-hoc tests indicated that baseline CORT levels at 12:00 h were significantly higher in paired mothers than in non-breeding females. However, post-stress CORT levels were significantly lower in single mothers ($P = 0.011$), but not paired mothers ($P = 0.130$), compared to non-breeding females. None of

the remaining main effects (housing condition, reproductive group) or interactions (stress × housing condition, stress × housing condition × reproductive group) were significant.

3.4. Pup development

Neither litter size at weaning (range: 1–3 pups, mean ± SE = 1.9 ± 0.1) nor day of eye opening of first pup (16.4 ± 0.4 days) differed between offspring of single mothers and paired mothers or between the CTRL and CLIMB conditions. We also found no difference in pups' body mass or body composition at the time of weaning (postpartum day 27 or 28) (Table 3.3).

4. Discussion

Maternal care is costly for female mammals and is associated with morphological, physiological, and behavioral changes in mothers (Lonstein, 2007, Slattery & Neumann, 2008, Speakman, 2008). In biparental species, fathers can improve offspring survival and development. Pups reared by mothers alone may have impaired survival and development, especially under challenging conditions (Bredy et al., 2007, Cantoni & Brown, 1997, Dudley, 1974, Elwood & Broom, 1978, Gubernick & Teferi, 2000, Gubernick et al., 1993, Piovanotti & Vieira, 2004, Wang & Novak, 1992, Wright, 2006). Fathers can also help reduce the energetic burden of motherhood (Bester-Meredith et al., 1999, Kleiman & Malcolm, 1981, Perkeybile et al., 2013, Woodroffe & Vincent, 1994). However, it is unknown if the adverse effects of paternal deprivation on offspring are mediated indirectly by negative effects on mothers. To study the effects of mate loss on

mothers, we measured physiological, morphological and behavioral traits in single mothers, paired mothers, and non-breeding female California mice housed under both standard laboratory conditions and physically challenging conditions, in which mice had to climb wire-mesh towers to obtain food and water. We found that motherhood and/or losing mates shortly after giving birth had several morphological, physiological and behavioral effects on females, but these effects were not modulated by housing condition.

4.1. Effects of housing condition

In this study, we evaluated the effects of single motherhood in two housing conditions, standard laboratory housing and physically challenging conditions. As females housed in towered cages had to spend more time and energy to climb and forage for food, we expected to see more severe effects of being single under the challenging condition. However, we found no other effects of towered cages, aside from changes in baseline CORT and saccharine preference, suggesting that this housing paradigm is neither highly stressful nor particularly enriching for California mice.

Housing under challenging conditions had a moderate effect on baseline CORT concentrations. As previously found in California mice (Harris et al., 2012) and many other species (Dallman et al., 1987, Dickmeis, 2009), CORT levels were lowest at the end of the active period (04:00 h), intermediate midway through the inactive period (12:00 h), and highest around the start of the active period (20:00 h). Although this pattern was seen in females in all three reproductive conditions, mice in standard cages had significantly higher CORT levels than females in towered cages at 04:00 h. A

previous study in our lab showed that chronic variable stress could increase baseline CORT levels in male California mice (Harris et al., 2013). Clearly, housing in towered cages in the present study was not stressful enough to increase CORT levels, but instead decreased the CORT trough. An increase in glucocorticoid levels at the circadian trough is commonly seen under chronic stress, which, in humans, might facilitate development of the metabolic syndrome (Dallman et al., 2000) and depression (Meijer et al., 1997) in the long term. Therefore, a lower trough indicated that the towered cages might be even less stressful than the standard cages.

We also found that females housed in towered cages had significantly lower preference for saccharine than those housed under standard conditions. Low preference for sweetened water or other rewarding substances is an indicator of anhedonia, a component of depression-like behavior (Schrader, 1997). Stressful environmental conditions, such as restricted access to food, overnight illumination, cage tilt, and intermittent white noise, can decrease responsiveness to rewards in lab rats (Willner et al., 1992) and mice (Strekalova et al., 2004). Environmental enrichment, similarly, can influence animals' preference for rewarding stimuli. For example, rats housed in enriched environments show reduced cocaine self-administration (Puhl et al., 2012). It is possible that the required amount of climbing in the towers in our study might not have been energetically demanding and might even have served as enrichment, in view of the California mouse's semi-arboreal habit in the wild (Ribble, 1992). However, whether the towered cages in our study acted as a stressor or as enrichment is not clear. In a study of male California mice we found other effects of being housed in these towered cages

(Zhao et al., 2018), including increase in body mass and body fat, but those animals were also food-restricted every third day, which likely accounted for the effects of housing conditions.

4.2. Effects of motherhood

Compared to non-breeding females, both single mothers and paired mothers produced significantly more fecal boli in the open-field test and the tail-suspension test, which is considered an indicator of higher anxiety level (Archer, 1973, Bronikowski et al., 2001, Colman et al., 2007, Flint et al., 1995). Motherhood in several uniparental mammals influences females' responses to stress: mothers exhibit blunted hormonal, neural and behavioral responses to a multitude of stressors during pregnancy and lactation (Brunton et al., 2008, Lightman et al., 2001, Slattery & Neumann, 2008), including reduced anxiety and fearfulness (Lonstein, 2007, Slattery & Neumann, 2008), potentially to protect maternal care from being inhibited by stress (Brunton et al., 2008). In contrast, we found evidence that mothers were more anxious than non-breeding females. This might indicate a difference between uniparental and biparental animals, but more studies on other biparental species are needed to test this hypothesis. Alternatively, since we found no other effects of single motherhood on behavior in the novel-object, open-field or tail-suspension tests, the difference in production of fecal boli might reflect changes in bowel function associated with lactation (Elias & Dowling, 1976, Hammond, 1997).

Mothers in our study, both paired and single, had higher lean mass on test days 4, 8, and 13 than the non-breeding females. We also found lower percent fat and higher

percent lean in single mothers and, in some cases, in paired mothers than in non-breeding females on one or more test days. Although no measures of body composition differed significantly among reproductive groups on test day 1 (3-4 days after the mothers gave birth), the differences were mostly significant or nominally significant after test day 4, indicating that lactation increased mothers' lean mass while decreasing fat stores, but that this effect did not become apparent within the first few days after parturition.

Energy balance in female mammals is strongly influenced by reproduction; females have high energy demands during pregnancy and, especially, lactation (Boland et al., 2001, Speakman, 2008). Motherhood can induce changes in body composition by increasing utilization of fat stores, especially in capital breeders, which rely primarily on fat stores to meet the energetic demands of reproduction (Bonnet et al., 1998). Our observed effects of motherhood on body fat and lean content in California mice are similar to those in capital breeders. Whether California mice are capital or income breeders (increase food intake to meet higher energetic demands) has yet to be investigated, but one study suggested that females in the congeneric species *P. leucopus* (white-footed mouse) are likely to be income breeders (Millar, 1975).

4.3. Effects of single motherhood

In small mammals, mothers (especially lactating females) have high basal and/or resting metabolic rate and experience growth of the alimentary tract in order to meet the high energy demands of lactation (Speakman, 2008). Although we did not measure metabolic rate in this study, we found that single mothers had significantly higher heart mass than

non-breeding females. Heart mass is usually positively correlated with basal metabolic rate (Chappell et al., 1999, Daan et al., 1990, Meerlo et al., 1997), daily energy expenditure (Dlugosz et al., 2012), and maximal oxygen consumption (Andrew, 2017). Measures of mothers' metabolic rate in future studies would be helpful to further clarify the physiological effects of single motherhood.

Liver is a key organ that governs glucose and lipid metabolism as well as numerous other functions (Samuel & Shulman, 2016). In our study, mothers had heavier livers than non-breeding females, consistent with findings from other species that lactation can induce hepatic growth (Speakman, 2008). In addition, we found that liver mass was higher in single mothers than in paired mothers, which might indicate that single mothers have higher energy demands during lactation than paired mothers.

Although both heart mass and liver mass can correlate positively with metabolic rate and energetic demands, thickened heart muscle and liver enlargement can also have pathological causes and/or consequences, such as hypertension and fatty liver disease, in both humans and lab rodents (Samuel & Shulman, 2017, van Nierop et al., 2013). In addition, heart and liver can influence each other's functioning, and many metabolic factors can affect both organs simultaneously (Møller & Bernardi, 2013). Thus, the proximate causes and consequences of heart and liver enlargement in single mothers are not clear.

Triceps surae is an important muscle in running behavior, and the mass of gastrocnemius and/or triceps surae typically is positively related to sprinting performance in mice (Dohm et al., 1994, Syme et al., 2005). Research in exercise physiology indicates

that voluntary exercise (e.g., wheel-running) in mice leads to increases in skeletal muscle masses (Soffe et al., 2016). Single mothers in our study had significantly lower triceps surae mass than the non-breeding females, suggesting some differences in their muscle physiology, possibly due to changes in their behavior, such as less locomotion and more huddling with the pups.

We did not observe differences in triceps surae mass between females in standard and towered cages, which almost certainly differed in locomotion. In lab mice, towers similar to the ones used in the present study have revealed a strain × exercise interaction in the forelimb biceps brachii muscle, but no effect of climbing exercise was found on hindlimb muscles (Lionikas & Blizard, 2008, Mori et al., 2003). In contrast, 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). The different findings from lab rats, mice, and California mice might be accounted for by possible species differences in behavioral responses to the towers and/or by differences in the duration of housing in towered cages.

In addition, we found that single mothers had significantly lower ovarian and uterine masses compared to both paired mothers and non-breeding females, possibly due to lack of stimulation from a male mate. Masses of reproductive organs in females are commonly used as indicators of reproductive function (Kumar et al., 2000, Kumar, 2005, Lee et al., 1998) and can be influenced by several reproductive hormones (e.g. luteinizing hormone, follicle-stimulating hormone, estrogen; Dewailly et al., 2006; Dupont et al., 2000; Halpin & Charlton, 1988; Kumar et al., 1997). Secretion of these hormones, in

turn, can be elicited by interactions with males, including mating behaviors (Edwards, 1970). Additionally or alternatively, reduced ovarian and uterine masses in single mothers might have resulted more directly from increased energetic demands on these females and shunting of metabolic resources to other, more essential organs such as heart and liver. Indeed, energetic demands can greatly influence female reproductive function at the level of the ovaries and uterus through changes in lipid metabolism (Bellefontaine & Elias, 2014, Fontana & Torre, 2016).

We found no effects of single motherhood on behavior in the novel-object, open-field or tail-suspension tests. In contrast, a recent study in prairie voles found that mothers whose male mates were removed on post-pairing day 18, prior to birth of their first litter, showed altered emotionality, including increased anxiety-related behavior in the elevated plus maze and more passive stress-coping in the forced-swim test, which might reflect increased signaling in the brain's corticotropin-releasing factor system (Bosch et al., 2017). Interestingly, we found effects of being a single mother on CORT responses to stress, but not their behavioral indicators of emotionality. This is possibly a species difference between California mice and prairie voles. The timeline regarding the male-female separation might also explain the different findings in the two studies – while single mothers in the present study were separated from their male mate on the first day after parturition, approximately 35 days after pair formation, the prairie vole mothers were separated from their mate on post-pairing day 18, prior to giving birth. The earlier and longer separation from their mate might be more stressful for the prairie vole mothers, which might result in more severe emotional effects of single motherhood.

4.4. Corticosterone

Together with many other hormonal changes during lactation, postpartum females in uniparental species and humans can have elevated glucocorticoid levels in order to maintain milk production and increase food intake, facilitating shifts in feeding behavior and energy utilization to meet increased metabolic demands (Tu et al., 2005, Woodside et al., 2012). In the present study, we found that single mothers had higher baseline CORT levels at 12:00 h compared to non-breeding females. However, we found that at 20:00 h (the peak of the CORT rhythm), both single and paired mothers had significantly lower CORT levels than non-breeding females, which generated a more flattened CORT rhythm in mothers. Interestingly, lactating rats also had a more flattened CORT rhythm than virgin females, although the flattening was through an elevated nadir CORT level (Walker et al., 1992).

In addition to baseline CORT concentrations, CORT responses to an acute stressor differed among reproductive groups. A 6-minute tail-suspension test midway through the active period markedly elevated plasma CORT levels in all animals, as expected, but this effect was blunted in single mothers, compared to non-breeding females: single mothers had significantly higher CORT levels under baseline conditions, but significantly lower CORT levels immediately after the stressor. Neither group differed significantly from paired mothers either in basal CORT levels at 12:00 or in stress-induced CORT levels, consistent with a previous study in our lab (Chauke et al., 2011).

Lactating females in several species (e.g., rats, sheep) are less likely than non-lactating females to exhibit glucocorticoid elevations in response to stressful stimuli (Tu

et al., 2005). Our findings of blunted CORT responses in single but not paired mothers might indicate that, with help from the male mate, motherhood in biparental species differs in fundamental ways from motherhood in uniparental species, therefore not requiring marked changes in HPA axis function. Removal of the mate might increase the demands on mothers in biparental species, thereby mimicking the situation in uniparental species and eliciting changes in HPA activity, similar to findings in several bird species (Wingfield & Sapolsky, 2003). For example, removal of the mate might alter mothers' food intake, activity levels, sleep patterns, thermoregulatory abilities (Walton & Wynne-Edwards, 1997), or patterning of nursing bouts, all of which could potentially affect baseline CORT levels and responses to stress.

4.5. Pups

None of the developmental measures that we monitored in pups – survival, age of eye opening, and body mass and body composition at the age of weaning – differed between pups reared by single mothers and those reared by both mother and father. This result indicates that single mothers were able to provide a similar quality of parental care and raise a similar quality and number of offspring, compared to paired mothers, both under standard housing conditions and when housed in towered cages. However, it is possible that more severe effects of having a single mother on the offspring could be revealed under more challenging conditions. Losing fathers in California mice has been found to impair pup survival and development in the field and in energetically challenging laboratory settings, but not under standard lab conditions (Bredy et al., 2007, Cantoni &

Brown, 1997, Dudley, 1974, Gubernick & Teferi, 2000, Gubernick et al., 1993, Wright & Brown, 2002). Therefore, it appears that mothers in this species can compensate for the loss of a mate by providing more maternal care, under benign living conditions but not under demanding conditions, in order to maintain offspring survival and quality. In contrast, in prairie voles, single mothers showed no evidence of strong parental compensation in response to the lack of the father, indicating a minimal effect of family structure on maternal behavior but a large effect on the total amount of pup care (Ahern et al., 2011, Bosch et al., 2017), although effects on pup's survival, development or body composition were not reported in the same study.

5. Conclusion

In conclusion, this was one of the first studies to evaluate effects of loss of a mate on mothers in a biparental mammal. Effects of single motherhood on mothers' behavior, morphology, and physiology suggest that rearing offspring alone is energetically costly and can induce organ remodeling and changes in HPA activity, even when animals are housed in benign laboratory conditions with abundant food and water, mild temperature and humidity, and absence of predators. Although single mothers in the present study were able to maintain a similar quality of offspring and showed no differences in behavioral indicators of emotionality compared to paired mothers, it is possible that more severe effects of being a single mother on the females, and/or effects of having a single mother on the offspring, could be revealed under more challenging conditions.

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Figures and tables

Day of Expt.	~ 59	~ -45	~ (-29)-(-24)	0	1	3	6	9	12	14	15	17	18	20	21	23	24	26	27
						Test day 1	Test day 2	Test day 3	Test day 4	Test day 5	Test day 6	Test day 7	Test day 8	Test day 9	Test day 10	Test day 11	Test day 12	Test day 13	Test day 14
Time					09:00-10:00 h	14:00-16:00 h	04:00 h	20:00 h	14:00-16:00 h	20:00-22:00 h	19:00-23:00 h	14:00-16:00 h	14:00-16:00 h	8:00-10:00 h	12:00 h	2:00-4:00 h	11:30-12:35 h	14:00-16:00 h	14:00-16:00 h
Procedure(s)	Vasectomize NB males	Pair formation, move half of pairs to climbing cages.	Vasectomize SM & PM males; move all pairs to divided cages for for 1 week	Birth	Remove SM fathers	Body comp.	Blood sample (basal CORT)	Blood sample (basal CORT)	Novel object, body comp.	Behavioral obs.	Saccharine preference test	Behavioral obs.	Open-field test, body comp.	Behavioral obs.	Blood sample (basal CORT)	Behavioral obs.	Tail-suspension, blood collection (post-stress CORT), body mass	Body comp.	Dissection

Fig. 3.1. Experimental timeline. Adult females were weighed twice weekly from pair formation to parturition, and pups were checked daily for eye-opening from birth to the appearance of eye-opening.

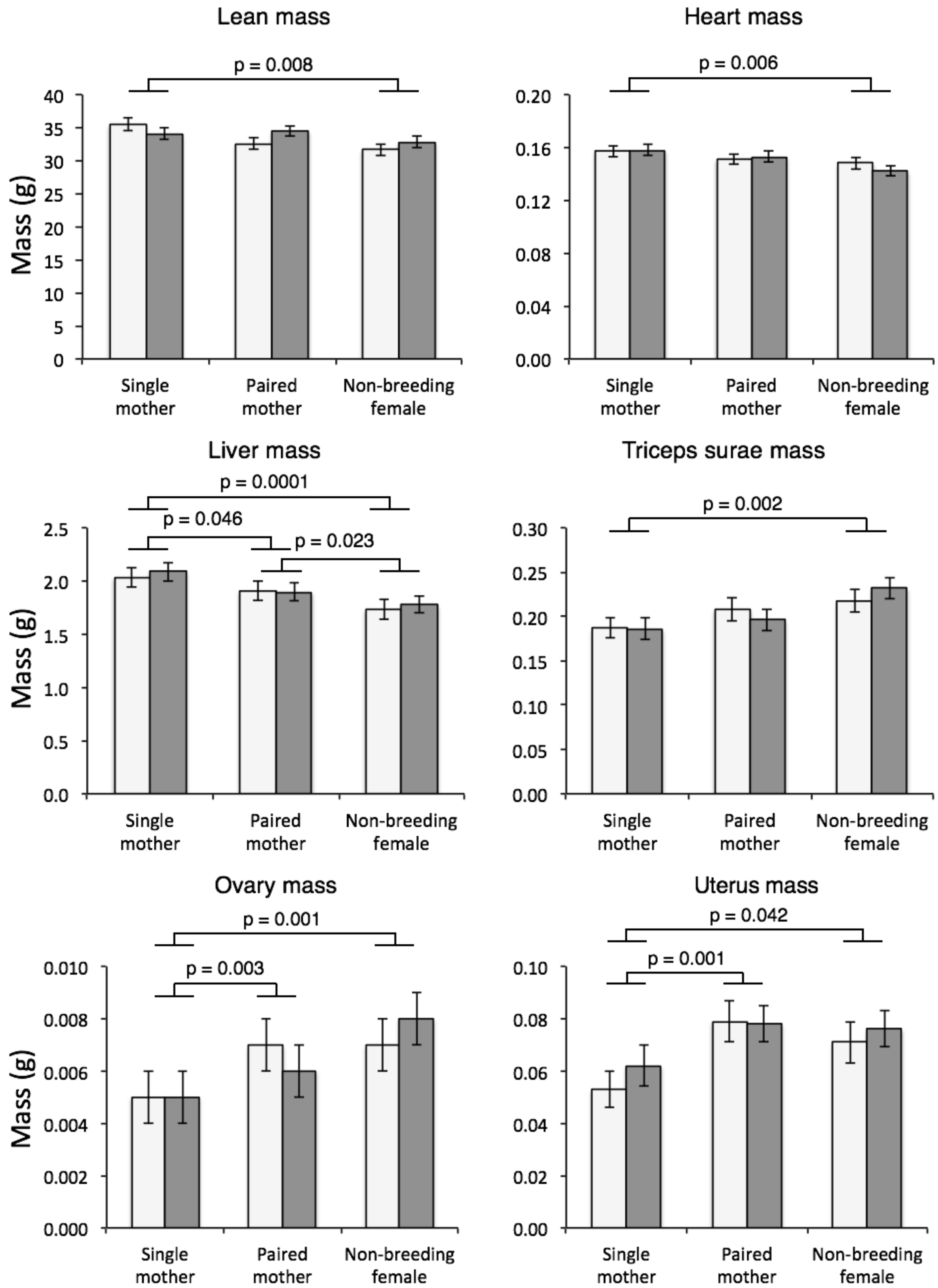
Lean mass
p = 0.008
Mass (g)
40
35
30
25
20
15
10
5
0
Single mother
Paired mother
Non-breeding female
Heart mass
p = 0.006
0.20
0.16
0.12
0.08
0.04
0.00
Liver mass
p = 0.0001
p = 0.046
p = 0.023
2.5
2.0
1.5
1.0
0.5
0.0
Triceps surae mass
p = 0.002
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ovary mass
p = 0.001
p = 0.003
0.010
0.008
0.006
0.004
0.002
0.000
Uterus mass
p = 0.042
p = 0.001
0.10
0.08
0.06
0.04
0.02
0.00

Fig. 3.2. Estimated marginal means (EMM) and associated standard errors (SE) of total lean mass and organ masses of female California mice. Ovary and triceps surae masses are mean masses of the two paired organs. Statistical results shown are for pairwise comparisons among reproductive groups; neither the main effect of housing condition nor the housing condition × reproductive group interaction was significant. Light gray, standard cages; dark gray, towered cages.

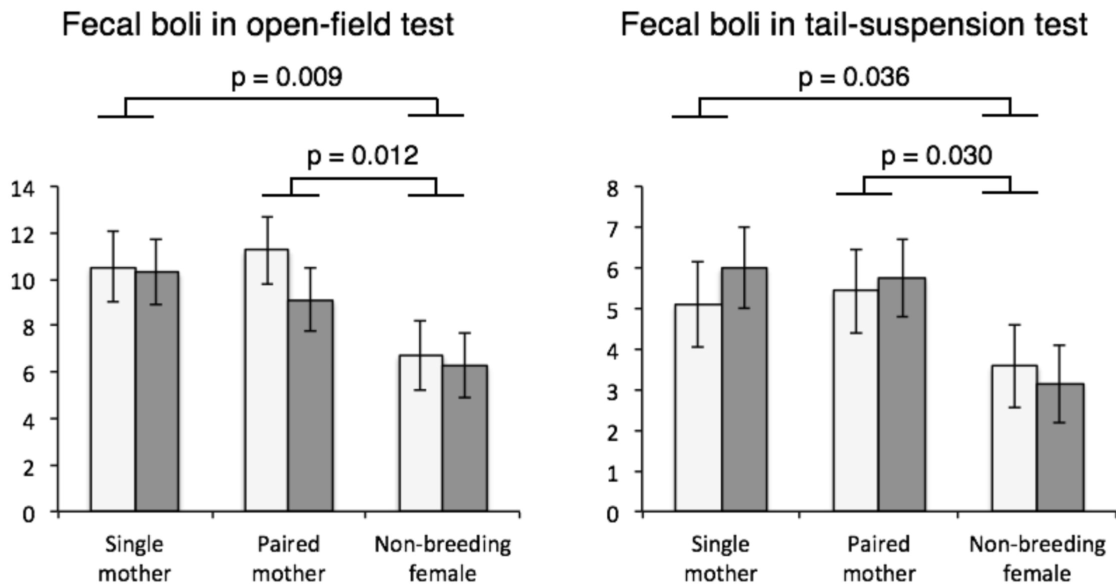

Fig. 3.3. EMM ± SE number of fecal boli produced during 10-minute open-field tests and 6-minute tail-suspension tests. Statistical results shown are for pairwise comparisons among reproductive groups; neither the main effect of housing condition nor the housing condition × reproductive group interaction was significant. Light gray, standard cages; dark gray, towered cages.

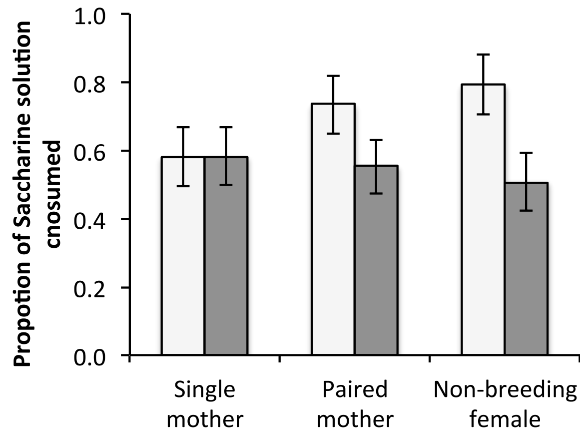

Fig. 3.4. EMM ± SE preference for saccharine during a 4-hour test. Light gray, standard cages (CTRL); dark gray, towered cages (CLIMB). CLIMB vs. CTRL conditions: P = 0.030.

A B

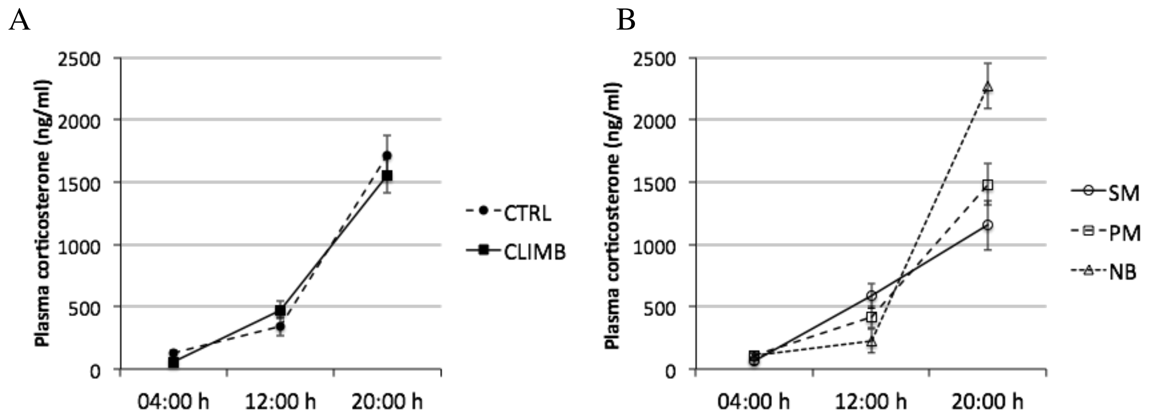

Fig. 3.5. EMM ± SE baseline plasma corticosterone concentrations across the diel cycle in female California mice. A: CORT levels of all females housed in standard cages (CTRL) or in cages with towers requiring the mice to climb to obtain food and water (CLMB), collapsed across reproductive conditions. There was an interaction between time and housing condition with females in standard cages having significantly higher CORT levels than females in towered cages at 04:00 h ($P = 0.009$). B: CORT levels in all single mothers (SM), paired mothers (PM), and non-breeding females (NB), collapsed across housing conditions. There was an interaction between time point and reproductive condition ($P = 0.011$) with both single mothers ($P = 0.002$) and paired mothers ($P = 0.040$) having significantly lower CORT levels than non-breeding females at 20:00 h. In addition, at 12:00 h, single mothers had nominally higher baseline CORT levels than non-breeding females ($P = 0.030$).

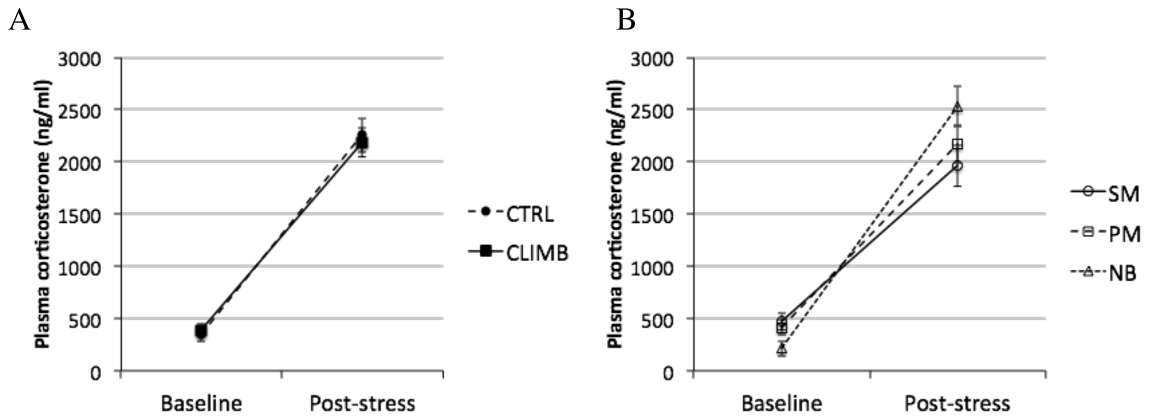

Fig. 3.6. EMM ± SE plasma corticosterone concentrations at 12:00 h under baseline conditions and immediately after a 6-minute tail-suspension test. The tail-suspension test significantly increased plasma CORT. A: CORT levels in all females housed in standard cages (CTRL) or in cages with towers requiring the mice to climb to obtain food and water (CLMB), collapsed across reproductive conditions. B: CORT levels of single mothers (SM), paired mothers (PM), and non-breeding females (NB), collapsed across housing conditions. Baseline CORT levels at 12:00 h were significantly higher in paired mothers than in non-breeding females (P = 0.048), while post-stress CORT levels were significantly lower in single mothers (P = 0.011), but not paired mothers, compared to non-breeding females. None of the remaining main effects (housing condition, reproductive group) or interactions (stress × housing condition, stress × housing condition × reproductive group) were significant.

Table 3.1. Results of Pearson correlations and paired t-tests comparing values for paired organ masses. Positive t values indicate that left>right. Significant P values ($P \leq 0.016$, when modified for Adaptive False Discovery Rate) are both bold and underlined.

Organ	Unit	Transform	N of paired observations	r of Pearson correlation	P of Pearson correlation	t of paired t-test	P of paired t-test
Triceps surae	gram	none	80	0.860	**<u>1.66E-24</u>**	1.111	0.270
Ovary	gram	none	79	0.774	**<u>5.77E-17</u>**	0.026	0.979
Adrenal	gram	none	80	0.818	**<u>1.85E-20</u>**	5.816	**<u>1.22E-07</u>**
Kidney	gram	none	80	0.930	**<u>1.44E-35</u>**	-1.989	0.0501

Table 3.2. Results of ANCOVAs comparing the three reproductive groups in the two housing conditions. Significant P values (P≤0.016, when modified for Adaptive False Discovery Rate) are both bold and underlined. Nominally significant P values (0.016<P<0.05) are underlined but not bold.

				Repro. Group		Housing Condition		Repro. Group * Housing Condition	
	Unit	Transform	D.F.	F	P	F	P	F	P
Novel object: latency to approach novel object	second	rank	81	0.679	0.510	0.210	0.648	0.116	0.890
Novel object: bouts of approaching object		**0.5	81	0.035	0.966	1.075	0.303	0.541	0.584
Novel object: duration of immobility	second	$\log_{10}$	81	1.198	0.312	0.199	0.658	0.512	0.603
Novel object: durations of sniffing and touching object	second	$\log_{10}$	81	0.854	0.430	0.319	0.574	1.146	0.323
Saccharine preference (proportion of total fluid consumption)		none	80	0.391	0.678	4.874	0.030	1.438	0.243
Open field: number of crossing bouts		$\log_{10}$	74	0.861	0.427	0.148	0.702	0.267	0.766
Open field: duration in outer region	second	rank	73	0.023	0.978	0.038	0.845	1.499	0.230
Open field: duration in inner region	second	**0.5	74	0.273	0.762	0.290	0.592	1.521	0.225
Open field: distance traveled in outer region	meter	**0.5	74	2.622	0.079	0.184	0.669	0.179	0.836

Open field: distance traveled in inner region	meter	**0.5	68	0.266	0.767	0.225	0.637	0.126	0.882
Open field: total distance traveled in inner region	meter		71	1.318	0.274	0.389	0.535	0.012	0.988
Open field: number of fecal boli		none	80	4.497	**0.014**	0.620	0.433	0.264	0.769
Open field: number of urine pools		**0.5	80	0.411	0.665	0.584	0.447	0.197	0.822
Tail suspension: duration of mobility	minute	none	81	0.512	0.602	0.070	0.793	0.141	0.869
Tail suspension: number of fecal boli		none	77	3.142	0.049	0.101	0.752	0.229	0.796
Heart mass	gram	none	72	3.989	0.023	0.071	0.790	0.457	0.635
Liver mass	gram	$\log_{10}$	72	8.545	**4.68E-04**	0.321	0.573	0.181	0.835
Spleen mass	gram	$\log_{10}$	72	0.224	1.526	0.725	0.125	0.847	0.166
Triceps surae mass (mean of left and right)	gram	rank	69	5.245	**0.008**	0.118	0.732	0.295	0.745
Ovary mass (mean of left and right)	gram	**0.5	71	6.955	**0.002**	0.007	0.932	0.386	0.681
Uterus mass	gram	$\log_{10}$	71	5.846	**0.004**	0.749	0.390	0.215	0.807

Adrenal mass (mean of left and right)	gram	rank	72	1.205	0.306	0.046	0.830	0.285	0.753
Kidney mass (mean of left and right)	gram	none	72	0.276	0.759	0.003	0.960	0.531	0.590
Postpartum body mass (mean of body masses 1-5)	gram	none	80	0.786	0.459	0.541	0.464	1.398	0.253
Postpartum body mass 1	gram	$\log_{10}$	76	0.595	0.554	0.215	0.645	1.286	0.282
Postpartum body mass 2	gram	none	79	0.223	0.801	0.096	0.757	1.630	0.203
Postpartum body mass 3	gram	none	81	2.175	0.120	1.553	0.216	1.671	0.195
Postpartum body mass 4	gram	none	78	3.203	0.046	1.203	0.276	0.984	0.378
Postpartum body mass 5	gram	none	68	2.086	0.132	0.877	0.352	0.714	0.494
Postpartum fat mass (mean of fat masses 1-4)	gram	rank	81	1.055	0.353	0.089	0.766	2.201	0.117
Postpartum fat mass 1	gram	rank	76	1.381	0.258	0.103	0.749	0.649	0.526
Postpartum fat mass 2	gram	rank	80	2.815	0.066	0.000	0.996	2.223	0.115
Postpartum fat mass 3	gram	rank	80	1.890	0.158	0.025	0.874	2.276	0.109
Postpartum fat mass 4	gram	rank	73	1.740	0.183	0.047	0.830	3.024	0.055
Postpartum lean mass (mean of lean masses 1-4)	gram	**0.5	81	3.686	0.029	0.470	0.495	1.977	0.145

Postpartum lean mass 1	gram	rank	76	2.524	0.087	0.084	0.772	1.155	0.321
Postpartum lean mass 2	gram	rank	80	11.200	**0.003**	0.955	0.763	0.360	0.345
Postpartum lean mass 3	gram	**0.5	80	7.433	**0.001**	1.331	0.252	0.640	0.530
Postpartum lean mass 4	gram	**0.5	80	7.422	**0.001**	1.315	0.255	0.642	0.529
Percent fat mass (mean of percent fat masses 1-4)		rank	81	2.959	0.058	0.394	0.532	1.713	0.187
Percent fat mass 1		rank	76	1.658	0.197	0.059	0.809	0.480	0.621
Percent fat mass 2		rank	80	4.477	**0.014**	0.005	0.947	1.474	0.235
Percent fat mass 3		rank	79	3.644	0.031	0.085	0.771	2.251	0.112
Percent fat mass 4		rank	72	1.352	0.265	0.248	0.620	2.105	0.129
Percent lean mass (mean of percent lean masses 1-4)		rank	81	1.804	0.171	0.046	0.831	1.713	0.187
Percent lean mass 1		rank	75	0.218	0.804	0.011	0.918	0.456	0.635
Percent lean mass 2		rank	80	5.153	**0.008**	0.001	0.975	1.446	0.242
Percent lean mass 3		rank	79	2.785	0.068	0.118	0.733	1.773	0.177
Percent lean mass 4		rank	72	3.133	0.0496	0.124	0.725	2.393	0.099

Table 3.3. Results of ANOVAs comparing the pups reared by single mothers and paired mothers in the two housing conditions (standard cages, towered cages).

				Repro. Group		Housing Condition		Repro. Group * Housing Condition	
	Unit	Transform	D.F.	F	P	F	P	F	P
Litter size at weaning	pup	none	55	1.414	0.239	0.118	0.732	0.006	0.937
Pups' age of eye opening (eye opening of the first pup of the litter)	day	none	55	0.228	0.635	0.198	0.658	0.685	0.411
Pups' fat mass (mean of all pups in litter)	gram	$\log_{10}$	51	0.146	0.704	1.281	0.263	1.087	0.302
Pups' lean mass (mean of all pups in litter)	gram	none	51	0.559	0.458	1.944	0.169	0.252	0.618
Pups' percent fat mass (mean of all pups in litter)		$\log_{10}$	51	0.032	0.859	0.226	0.637	0.856	0.359
Pups' percent lean mass (mean of all pups in litter)		rank	51	0.009	0.923	0.944	0.336	0.455	0.503
Pups' body mass (mean of all pups in litter)	gram	none	53	1.144	0.290	3.107	0.084	0.278	0.600
Pups' body mass (total body mass of litter)	gram	none	53	2.609	0.112	3.426	0.070	0.021	0.884

Conclusion

My dissertation addresses possible trade-offs between reproduction and self-maintenance in a biparental breeding system. In biparental species, both mother and father can contribute to better survival of the offspring. As a result of being a mother, females may experience physiological and morphological costs. Since males assist with caring for the young in biparental species, I investigated possible costs of fatherhood in the males themselves (Aims 1 and 2). I also studied how fathers can influence mother's physiological and affective condition, by testing the effects of losing the male mate on mothers (Aim 3). I hypothesized that fathers, like mothers, would experience energetic costs as well as behavioral and affective changes associated with parenthood; that mothers rearing offspring without assistance from their mates would have poorer morphological, physiological and affective condition, as well as impaired survival and development of pups, compared to mothers housed with their mates; and that the effects of fatherhood and single motherhood would be more pronounced under energetically challenging environmental conditions that increase the cost of parenthood.

Overall, this dissertation revealed several behavioral, morphological, and physiological consequences associated with reproduction and parenthood in California mice. Results from Aims 1 and 2 suggest that cohabitation with a female and/or fatherhood induced changes of blood glucose and lipid profile, as well as altered physiological responses to energetic challenge. Additionally, Aim 3 provides some evidence that male mates can have moderate effects on the mothers' physiology and that single motherhood can induce organ remodeling and endocrine changes, but not affective

changes, which might have allowed pup survival and development to be maintained as normal.

The predicted costs of fatherhood and the predicted effects of losing a male mate on mothers and offspring were based on the ideas that 1) in biparental species, fathers can contribute substantially to the rearing of offspring, 2) a large part of this contribution is by lightening the burden of motherhood, 3) the contribution from the father is expensive enough that it reduces the father's amount of energy or other resources available for self-maintenance and that single mothers can experience costs of losing the male's contribution, and 4) the costs of fatherhood in males and of single motherhood in females can be revealed by the behavioral, morphological, and physiological measures I examined. Data from this dissertation suggest that some of these scenarios may not occur in California mice under the housing conditions used in these studies. The few physiological, morphological, and affective changes that I observed in first-time California mouse fathers, in combination with results of our previous studies (Andrew et al., 2016), suggest that being a father is not necessarily particularly expensive, at least in this biparental rodent. In addition, losing the male mate did not result in lower survival rate or delayed development of offspring. Single motherhood induced morphological changes in several organs as well as endocrine changes, but other potential effects of single motherhood, such as lower body mass, lower fat mass, behavioral measures of anxiety or depression, were not found.

Although we found few costs of fatherhood and affective impacts of single motherhood in California mice, they may be found in some other biparental rodents. For

example, in another well-studied biparental rodent, the prairie vole, some evidence indicates that fatherhood is energetically costly (Campbell et al., 2009) and that single motherhood can result in altered emotionality (Bosch et al., 2017). In addition, Aim 3 might reveal some differences between uniparental and biparental species. Although motherhood has been shown to induce many physiological and affective effects in females, these effects have been studied mainly in uniparental species, which naturally do not have any help from a male mate in the care of offspring (Slattery & Neumann, 2008, Speakman, 2008). Differences between uniparental and biparental species, and between different biparental species, might be due to species' unique ecological environments in the wild, evolutionary pathways by which these biparental breeding systems arose, as well as neuroendocrine and physiological mechanisms of initiating parental behaviors. More studies comparing different species can better reveal the ultimate (e.g., ecological, evolutionary) and proximate (e.g., neuroendocrine, sensory) basis of parenthood, in order to better understand the different consequences of parenthood in various breeding systems.

These studies found little evidence that an environmental challenge (periodic food restriction + climbing towers in males, climbing towers in females) modulated the effects of parenthood or single parenthood. It is certainly possible, however, 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 on males and more severe impacts of losing a mate on single mothers and their offspring. Indeed, in California mice, the benefits of biparental care for the offspring are better revealed under

energetically challenging conditions, such as when parents must work for food or are housed under cold temperature (Bredy et al., 2007, Cantoni & Brown, 1997, Dudley, 1974, Gubernick & Teferi, 2000, Gubernick et al., 1993, Wright & Brown, 2002). Future studies should therefore characterize effects of parenthood on parents in a more challenging environment that better mimics their natural living conditions.

It would also be informative to analyze the mechanisms and consequences of the physiological changes found in this dissertation. For example, although both heart mass and liver mass can correlate positively with metabolic rate and energetic demands, thickened heart muscle and liver enlargement can also have pathological causes and/or consequences, such as hypertension and fatty liver disease in both humans and lab rodents (Samuel & Shulman, 2017, van Nierop et al., 2013). Therefore, a better understanding of the proximate causes and consequences of heart and liver enlargement in California mouse single mothers is potentially important in improving the health of parents. In addition to insulin, leptin, and corticosterone, it is to be tested whether parents undergo changes in other metabolically important hormones (e.g., adiponectin, ghrelin), and whether the physiological effects of being a virgin or being a single mother could be manipulated through hormonal treatments. Furthermore, mothers in small mammals and humans can experience changes in their bone density during lactation (Speakman, 2008); however, whether single mothers have more severe changes in bone, as well as differences in content and/or production of milk, compared to paired mothers, is unknown.

This research is some of the first work investigating the physiological and affective consequences of parenthood for fathers, as well as some of the first examining the effects of fathers on their female mate's condition. It provides important implications for the understanding of trade-offs between reproduction and self-maintenance in a biparental breeding system. In addition, it can help to elucidate possible physiological connections between reproductive behaviors and ecological conditions. Species in the genus *Peromyscus* exhibit various breeding systems and are distributed widely in different ecological environments in North America. Researchers have long been interested in investigating the tradeoffs between reproduction and other behaviors in species with different breeding systems in certain ecological contexts (Millar, 1975, Stephens et al., 2009). Therefore, this work may also contribute to phylogenetic studies of how different breeding systems co-evolved with various energetic strategies. Moreover, a better understanding of reproductive physiology as well as how it is influenced by environmental challenge in this biparental species might contribute to conservation efforts in captive and also, potentially, wild animals with similar life history strategies. Finally, motherhood and fatherhood can have far-reaching effects on health and longevity in human parents. Although associations of fatherhood and single motherhood with health and longevity appear to be mediated largely by sociocultural variables (Bartlett, 2004, Beatson-Hird et al., 1989), investigations of the biological processes underlying these effects could help with understanding and improving parent's health, and might provide insights into child health and development (Daryanani et al., 2016, Florez et al., 2011).

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