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Acute effects of corticosterone injection on paternal behavior in California mouse (*Peromyscus californicus*) fathers

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ABSTRACT

Glucocorticoids are thought to mediate the disruption of parental behavior in response to acute and chronic stress. Previous research supports their role in chronic stress; however, no study has experimentally tested the effects of acute glucocorticoid elevation on paternal behavior. We tested the prediction that acute corticosterone (CORT) increases would decrease paternal behavior in California mouse fathers and would lead to longer-term effects on reproductive success, as even short-term increases in CORT have been shown to produce lasting effects on the hypothalamic-pituitary-adrenal axis. First-time fathers were injected with 30 mg/kg CORT, 60 mg/kg CORT or vehicle, or left unmanipulated. Interactions between the male and its pup(s) were recorded 1.5–2 h after injection and scored for paternal and non-paternal behavior. Treatment groups were combined into control (unmanipulated + vehicle, n = 15) and CORT (30 mg/kg + 60 mg/kg, n = 16) for analysis based on resulting plasma CORT concentrations. CORT treatment did not alter paternal or non-paternal behaviors or any long-term measures (male body mass or temperature, pup growth rate, pup survival, interbirth interval, number or mass of pups born in the second litter). Fathers showed a significant rise in body mass at day 30 postpartum, followed by a decrease in body mass after the birth of the second litter; however, this pattern did not differ between the CORT and control groups. In summary, acute elevation of plasma CORT did not alter direct paternal behavior, body mass, or reproductive outcomes, suggesting that acute CORT elevation alone does not overtly disrupt paternal care in this biparental mammal.

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Introduction

The glucocorticoids, steroid hormone end-products of the hypothalamic-pituitary-adrenal (HPA) axis, play a major role in mediating the physiological and behavioral changes that occur in response to stressors. These hormones, which include cortisol and corticosterone, are known to affect multiple homeostatic and organismic systems (e.g., blood glucose levels, mood, cognition, metabolism; McEwen, 2005; Sapolsky et al., 2000) as well as several types of behavior, including reproductive behavior (both sexual and parental; see Wingfield and Sapolsky, 2003 for a review). Therefore, numerous authors have hypothesized that increased glucocorticoid concentrations in response to stress, both acute and chronic, may signal parents to invest in themselves over their offspring, thus mediating the trade-off between self-maintenance and reproduction (Breuner and Hahn, 2003; Ricklefs and Wikelski, 2002; Wasser and Barash, 1983; Wingfield and Sapolsky, 2003; Wingfield et al., 1998). Under adverse and energetically challenging ecological or organismic circumstances, decreasing investment in offspring might increase a parent's chances of survival and its lifetime reproductive success at the expense of current reproductive effort (Breuner and Hahn, 2003; Silverin, 1986, 1998; Wingfield and Sapolsky, 2003; Wingfield et al., 1998).

Experiments designed to test the effects of glucocorticoids on parental behavior have typically utilized chronic stress or chronic glucocorticoid manipulation. Findings from these studies suggest that chronic stress can negatively impact parental care and that this effect is mediated, at least in part, by persistent increases in glucocorticoid concentrations. Effects of chronic glucocorticoid implantation on parental behavior by both mothers and fathers (maternal and paternal care) have been studied most extensively in birds. Data from the avian literature indicate that prolonged circulation of high glucocorticoid concentrations results in decreased parental effort (Breuner et al., 2008). For example, glucocorticoid implantation in mothers and/or fathers in several species led to decreased time on the nest (Kitaysky et al., 2001), less time spent in the territory (Breuner and Hahn, 2003), decreased feeding of young and/or nest abandonment (Silverin, 1986, 1998; Spée et al., 2011). For mammalian species no data are available on the effects of chronic stress or glucocorticoid elevation in fathers; however, studies of female mammals have yielded similar findings to those obtained in birds. Data from female rats (*Rattus norvegicus*), for example, suggest that various forms of chronic stress, such as wet bedding and forced foraging (Léonhardt et al., 2007) or decreased nesting material (Ivy et al., 2008), can decrease maternal behavior. As in birds, this effect appears to be mediated, at

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least in part, by chronic glucocorticoid elevations. For example, repeated injection of synthetic glucocorticoid in common marmoset (*Callithrix jacchus*) mothers caused mothers to carry their infants less than vehicle-injected mothers (Saltzman and Abbott, 2009).

Much less is known about the effects of acute stress or glucocorticoid manipulation on parental behavior: very few studies have investigated this relationship in mothers, and to date no studies have been conducted on fathers. Acute stressors have been shown to disrupt maternal behavior in female rats (Roth and Sullivan, 2005; Sukikara et al., 2010; Yamada et al., 2002) and pigtail macaques (*Macaca nemestrina*; Maestripieri and Carroll, 1998). The mechanism by which this occurs is not known, but glucocorticoids are a likely candidate.

In a recent review of the trade-off between self-maintenance and reproduction under stressful conditions, Breuner et al. (2008) emphasized the need for more data on acute manipulations. They argued that drawing an ecologically relevant line between what constitutes acute vs. chronic stress in a free-living organism can be difficult, and that an acute paradigm more closely mimics natural stress reactivity (Breuner et al., 2008). They further suggested that future studies should include more direct measurements of reproductive output and survival combined with manipulation of acute glucocorticoid elevation, as "exogenous glucocorticoid treatment should be one of the best ways to test relationships between acute stress reactivity and performance measures" (Breuner et al., 2008, p. 293), and should more directly test for a trade-off between self-maintenance and reproductive effort/outcome in the face of stress.

In this study, therefore, we aimed to 1) experimentally determine the effects of acute glucocorticoid elevation on parental behavior, separate from effects of acute stress, and 2) measure any possible longer-lasting fitness effects. Due to the lack of data on male mammals, and because paternal care is practiced by 6–10% of mammalian species, including humans (Kleiman and Malcolm, 1981), and can be important for survival and development of offspring (e.g., Ovtscharoff et al., 2006; Piovanotti and Vieira, 2004; Schradin and Pillay, 2004), we chose to manipulate glucocorticoid levels in first-time fathers of the monogamous, biparental California mouse (*Peromyscus californicus*). In this species, care by both parents maximizes offspring survival, accelerates offspring development, and increases parents' reproductive success both in the lab and in the field, especially under challenging conditions (Bester-Meredith and Marler, 2001; Bredy et al., 2004; Cantoni and Brown, 1997a,b; Dudley, 1974; Frazier et al., 2006; Gubernick and Teferi, 2000; Gubernick et al., 1993; Wright and Brown, 2002). Therefore, if parental care by either the mother or the father is disrupted, decreases in offspring quality and survival, as well as in parental fitness, are likely to occur.

To determine the effects of acute glucocorticoid elevation we injected corticosterone (CORT) or vehicle, or performed no manipulations, in first-time California mouse fathers, and characterized the acute effects on paternal care and general activity. In order to quantify possible longer-term fitness effects of acute CORT treatment, we characterized changes in the male (body mass over time, body temperature), the female pairmate (interbirth interval, second litter size), and their offspring (body mass over time, survival to weaning). We chose these specific long-term measures because recent studies have suggested that even a single acute stressor can have persistent effects on the HPA axis (Lynn et al., 2010; Malisch et al., 2010), and CORT is known to exert metabolic effects that can be manifest as changes in body mass (Baxter, 1976; Strack et al., 1995). In addition, if CORT caused a reduction in male parental care, it is possible that the pups would grow more slowly, or that the female pairmate would compensate by investing more in care, possibly resulting in a longer interbirth interval or a decrease in the number of pups born in the second litter. This study, to our knowledge, is the first to experimentally test whether glucocorticoids inhibit paternal behavior in mammalian fathers, and to measure the effects of an acute increase in glucocorticoids in a male mammal on longer-term reproductive outcomes.

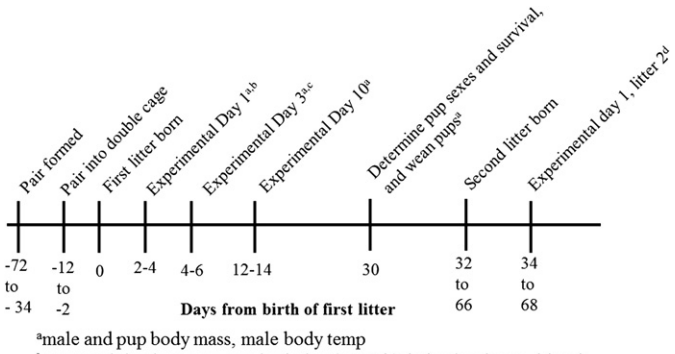
Methods

Animals

Mice were bred in our colony at the University of California, Riverside (UCR) and were descended from an original stock purchased in 2007 from the Peromyscus Genetic Stock Center, University of South Carolina (Columbia, SC). The colony was maintained on a 14:10 light:dark cycle, with lights on at 05:15 h and lights off at 19:15 h. Ambient temperature was approximately 23 °C with a humidity of about 65%. Mice were housed in standard shoe-box style, polycarbonate cages (44×24×20 cm) lined with aspen shavings; cotton wool was provided for nesting material. Food (Purina 5001 rodent chow) and water were provided *ad libitum*. Cages were cleaned once per week unless otherwise noted. In our colony, siblings are never mated with one another, and first-cousin matings are avoided whenever possible. Animals were weaned at 27–32 days of age (prior to the birth of younger siblings), ear-punched for individual identification, and housed in same-sex groups of 2–4 mice until they were pair-housed with a female for the experiment at 90–164 days of age (114.7 ± 3.4 days, mean ± SEM). Prior to the start of the experiment, beginning when animals were housed in male–female pairs, mice were weighed twice weekly to assess overall health and to detect pregnancy. UCR has full AAALAC accreditation, and all procedures were approved by the UCR IACUC and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*.

Design

The experimental design is summarized in Fig. 1. Beginning approximately 1 week prepartum, when the female showed steady weight gain (6–10 g), each pair was housed in a double cage consisting of two standard cages connected via clear plastic Crittertrail® tubing forming a z shape (35×5 cm). Animals had the opportunity to move freely between the two cages; both cages contained food, water, and aspen shavings, but initially only one side contained cotton wool. These cages allowed the male more behavioral options than standard housing (e.g., avoiding female and pups; Brown, 1993; Schradin, 2007). After each pair's first litter of pups was born, the male was randomly assigned to one of four conditions: high CORT (60 mg/kg; n = 8), low CORT (30 mg/kg; n = 8), vehicle (oil; n = 8), and unmanipulated controls (n = 7). CORT doses were based on a pilot study indicating that these low (30 mg/kg) and high (60 mg/kg) doses produced circulating CORT levels similar to the endogenous levels occurring during the circadian peak (1500–1800 ng/ml) or following acute stress exposure during lights-on (2200–2700 ng/ml) in this species, respectively (unpub. data). *P. californicus* is nocturnal, so lights-



[a]male and pup body mass, male body temp
[b]**DayE1**: injection or no manipulation (08:00h), behavioral tests, blood sample (10:00h)
[c]**DayE3**: injection or no manipulation (08:00h), blood sample (10:00h), transfer to single cage
[d]male and pup body mass

Fig. 1. Experimental timeline.

on corresponds to the species' inactive period of the day. Treatment groups did not significantly differ on any starting variables, including male and female body mass at pairing, length of time in double cage prepartum, latency from pair formation to birth of first litter, number of pups in first litter, and male and female ages at birth of first litter (data not shown).

Two to four days after the birth of the first litter (2.7 ± 0.1 days postpartum; hereafter referred to as day E1 of the experiment), at approximately 08:00 h, the male was injected subcutaneously with 60 mg/kg CORT, 30 mg/kg CORT, or vehicle and immediately returned to its family, or was left unmanipulated. Exactly 90 min after injection, the family, in its double cage, was placed on an observation surface in the colony room, allowed to acclimate for 10 min, and then videotaped for 10 min in the double cage (family test). Immediately after the family test, the male was isolated in the half of the double cage containing the smaller amount of cotton nesting material, allowed to acclimate for 5 min, and then presented with one of its own pups and videotaped for 5 min (retrieval test). The pup was always placed in the corner farthest from the father. Immediately following the retrieval test (2 h after injection) the male was removed and anesthetized with isoflurane gas, and a blood sample was collected from the retro-orbital sinus for plasma CORT analysis (see below). The male's body temperature (T_B) was determined, and the male and the pups were weighed. Two days later (day E3 of the experiment), the male was injected with the same dose of CORT or oil, or again left unmanipulated, and blood was collected 2 h later, time-matched to the blood sample on day E1, to control for possible effects of behavioral testing on CORT levels on day E1. Body mass of the male and pups, and male T_B, were determined as on day E1. After data collection on day E3, the family was moved permanently into a standard single cage.

Seven days after the second injection (11.9 ± 0.2 days postpartum, or day E10 of experiment) the male and litter of pups were weighed and the male's T_B was recorded. Thirty days postpartum (PP30), the male and pups were weighed, pup sexes were determined, the pups were weaned into virgin groups, and pup survival was recorded. Interbirth interval to the second litter was recorded. After the birth of the second litter, the male and newborn pups were weighed (matched to number of days postpartum for day E1 from litter 1) and then returned to the colony population.

To determine whether changes in fathers' CORT concentrations from day E1 to day E3 in the control groups (see below) were due to handling/behavioral observation rather than an innate hormonal change in new fathers, a separate group of undisturbed, first-time fathers (age 199.0 ± 9.5 days; n = 8) was blood-sampled on the same schedule and at the same time of day (10:00 h) as the experimental animals. Blood sample 1 (corresponding to day E1) occurred 2.1 ± 0.2 days postpartum, and blood sample 2 (corresponding to day E3) at 4.1 ± 0.2 days postpartum.

Corticosterone injections

Crystalline CORT (92% pure, C2505, Sigma Aldrich, St. Louis, MO) was dissolved in 100% ethanol over low heat until no crystals were visible. Sterile sesame oil (10 ml; Hain Celestial Group, Boulder, CO) was then added to the CORT/ethanol mixture, the heat was turned off, and the solution was mixed thoroughly. The mixture was then transferred to a vacuum drying oven (approximately 50 °C) for 18–24 h, or placed on a heated stir plate in a hood overnight to evaporate off the ethanol. CORT concentration was 15 mg/ml for the 60 mg/kg dose and 7.5 mg/ml for the 30 mg/kg dose. A fresh solution was prepared for each animal. Oil for the vehicle control group was prepared in the same way but without addition of hormone. Injection doses were based on animal body mass on the day prior to injection, and the injection volume ranged between 0.12 and 0.21 ml.

Family test

Data from the family test provided a measure of paternal care, but also allowed us to examine male activity level, as well as interactions between the male and his mate and pup(s). The double-cage design enabled us to determine if CORT-treated males spent more time away from the mate and pups compared to controls; additionally, we could determine if any non-paternal behaviors were altered by CORT treatment.

All behavioral tests were videotaped and later scored using JWatcher event-recorder software (Blumstein and Daniel, 2007) with an ethogram developed by our laboratory (de Jong et al., 2009, 2010). Family tests were videotaped for 10 min, and durations of paternal behaviors (huddle, lick/groom, nursing posture, sniff pup) and non-pup-related behaviors (autogroom, dig) were recorded. Numbers of jumps (all four paws off cage floor) and rears (front paws off cage floor) performed by fathers were tallied across the 10 min. Jumps were scored as a measure of activity, and rears are generally thought to be an index of rodent emotionality (more rears being associated with exploratory behavior and/or an alert state; Espejo, 1997).

Location and activity of fathers, and location of mothers, were noted via instantaneous scans every 30 s. Location of each parent was categorized as 1) in contact with at least one pup, 2) within 10 cm of any pup but with no physical contact, 3) in the same cage as at least one pup but not touching any pups and greater than 10 cm from any pup, 4) not in the same cage as any pup. We also determined whether or not the male and female were in the same side of the double cage during each scan. Distance to pup was estimated using reference to a pre-measured strip of paper. Activity of the male was categorized as 1) locomoting (walking, running, jumping), 2) resting (sitting quietly or sleeping) or 3) stationary movement (not locomoting, but active, i.e. autogrooming, digging, grooming mate, eating, drinking). A composite score for paternal care, including total duration of huddling, licking/grooming pup, and nursing posture was calculated to provide an overall measure of paternal care (e.g., de Jong et al., 2009). In addition to scoring paternal care as a composite, we calculated the number of paternally responsive and non-paternally responsive males, defined as those that engaged in some form of the composite behaviors (score ≥ 1.5 s), and those that did not (no time spent licking pup, huddling pup, or in the nursing posture). The above criterion was chosen because there was a clear separation in the data, males either performed the composite behaviors or not.

Retrieval test

The retrieval test was used to more directly measure paternal behavior, as the testing session took place in only one half of the double cage, with only a male and one of its pups present. Both pup-related (latency to contact pup, sniff pup, nursing posture, huddle, lick/groom pup, manipulate pup, and carry pup) and non-pup-related behaviors (autogroom, jump, rear, dig, locomotion) were scored during the 5-min retrieval test. Durations of autogroom, dig, locomotion, sniff pup, nursing posture, huddle pup, lick/groom pup, manipulate pup and carry pup were recorded continuously, and jumps and rears were counted over the 5 min. Durations of three paternal behaviors (nursing posture, lick/groom pup, huddle) were summed to yield a paternal behavior composite score to provide an overall measure of paternal care (see de Jong et al., 2009). Composite scores were used for two different measures. First, males were labeled as paternally responsive or not (using the 1.5 s criterion from the family test), and second, a more stringent requirement was imposed to determine the percentage of paternal males in each group. Males labeled as paternal spent at least 100 s engaging in any combination of licking the pup, huddling the pup, or performing nursing posture; non-paternal males spent less than 75 s performing these behaviors.

The time limits were chosen based on a clear split in the data set between 72 and 100 s.

Blood collection

Mice were anesthetized with isoflurane and blood samples (140 μl) were collected from the retro-orbital sinus using heparinized microhematocrit tubes. Time from disturbance of the cage or end of the test to collection of the blood sample was less than 3 min, with one exception (range: 63–229 s, mean ± SEM: 103.23 ± 32.85 s). Blood samples were centrifuged for 12 min (13,300 rpm, 4 °C), and plasma was removed and stored at −80 °C until assay.

Corticosterone radioimmunoassay

Plasma was assayed in duplicate for corticosterone using an ^{125}I double-antibody radioimmunoassay kit (#07-120102, MP Biomedicals, Costa Mesa, CA) that our lab has validated for this species (Chauke et al., 2011). Intra- and inter-assay coefficients of variation (CVs) were 6.58% and 12.14%, respectively. Samples from each individual mouse were analyzed in the same assay run, and treatment conditions were balanced across assays to minimize assay-induced variation.

Body temperature

Because CORT can increase metabolic rate and alter energy partitioning, exogenous application of the hormone could potentially cause an increase in body temperature, and fathers might avoid huddling with their pups solely for thermoregulatory reasons, as has been observed in female hamsters (Walton and Wynne-Edwards, 1998). Therefore, males' body temperature was determined using a digital infant thermometer (Aldi, Batavia, IL), coated with petroleum jelly and inserted into the rectum to a depth of approximately 25 mm. Exact depth of insertion was recorded.

Statistical analysis

All behaviors were analyzed with non-parametric tests (Mann–Whitney U, Wilcoxon signed-rank, Fisher's exact) unless otherwise noted. Plasma CORT concentrations and latency to contact pup were $\log_{10}$-transformed prior to analysis to obtain normality, and were analyzed parametrically. Log_{10}- transformed CORT data were analyzed using repeated-measures ANOVA, and latency to contact pup was analyzed using an independent-samples *t*-test. Body mass, temperature, and other litter parameters were analyzed with ANOVA. Analyses were performed using SPSS 15.0 (IBM Corporation, Somers, NY). All statistical tests were 2-tailed, and $P<0.05$ was considered significant. In cases where multiple comparisons were performed (in both the family test and the retrieval test), a false discovery rate analysis (Pike, 2011) was completed to correct for alpha inflation within each set of video data; both unadjusted and adjusted P values (q values) are reported. All values are presented as mean ± standard error of the mean unless otherwise stated.

Results

Corticosterone

Four-group analysis

Plasma CORT data for the four treatment groups are displayed in Fig. 2A. A two-way repeated-measures ANOVA (day × treatment group) revealed a significant main effect of day ($F_{1,27}=22.77$, $P<0.001$) and treatment group ($F_{3,27}=60.01$, $P<0.001$) as well as a day × treatment interaction ($F_{3,27}=7.42$, $P=0.001$). Plasma CORT levels did not differ between vehicle-treated and unmanipulated

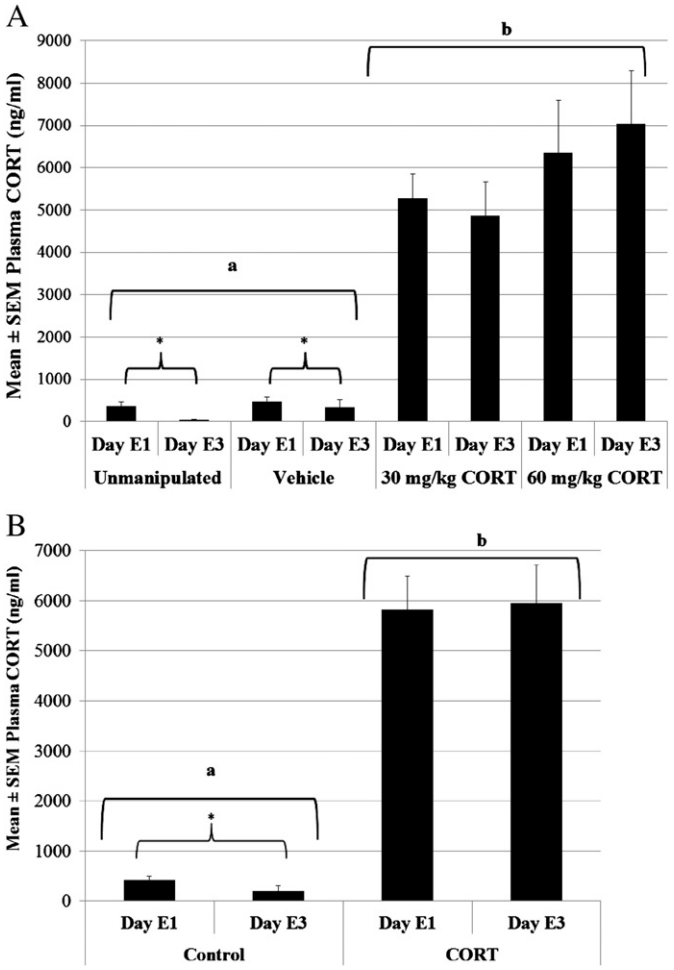


Fig. 2. Mean plasma corticosterone levels following treatment and behavioral tests (day E1) or treatment alone (day E3) in control and CORT-treated groups. Data were $\log_{10}$-transformed for analysis, but non-transformed values are presented here for ease of interpretation. A: CORT concentrations in each of the four original treatment groups. CORT concentrations did not differ between the unmanipulated (n = 7) and vehicle groups (n = 8), or between the 30 and 60 mg/kg CORT groups (n = 8 per group) on either day. Plasma CORT levels were higher on both day E1 and day E3 in the 30 and 60 mg/kg CORT groups when compared to the unmanipulated and oil groups (a vs b, $P<0.0001$). Both unmanipulated and vehicle-treated males had higher CORT on day E1 than on day E3 (*$P<0.005$). B: Mean plasma CORT levels for the two combined groups, Control (unmanipulated and vehicle; n = 15) and CORT (30 mg/kg and 60 mg/kg CORT; n = 16). Control males, but not CORT males, had higher CORT on day E1 than on day E3 (*$P<0.001$). However, CORT-treated males had higher plasma CORT than Control males on both day E1 and day E3 (a vs b, $P<0.001$).

males on either day E1 ($t_{13}=0.45$, $P=0.65$) or day E3 ($t_{13}=2.02$, $P=0.053$; Tukey's LSD tests). In addition, the 30 and 60 mg/kg CORT groups did not differ from each other on day E1 ($t_{14}=0.17$, $P=0.87$) or on day E3 ($t_{14}=0.82$, $P=0.42$; Tukey's LSD tests). On both days E1 and E3, however, unmanipulated and vehicle-treated males had significantly lower CORT levels than the 30 and 60 mg/kg CORT groups ($P<0.001$ in all cases; Tukey's LSD). Both unmanipulated and vehicle-injected males had higher CORT levels on day E1 (injection or no manipulation, followed by behavioral tests) compared to day E3 (injection or no manipulation; Tukey's LSD analysis for simple main effects of treatment group, $t_6=5.59$, $P<0.001$; $t_7=3.34$ $P=0.002$, respectively). Plasma CORT levels did not differ significantly between day E1 and day E3 in either of the CORT-treated groups (30 mg/kg: $t_7=0.73$, $P=0.472$; 60 mg/kg: $t_7=0.33$, $P=0.747$). Moreover, CORT levels did not change from blood sample 1 (equivalent to E1) to blood sample 2 (equivalent to E3) in the separate, unmanipulated first-time fathers from our breeding colony (sample 1 vs. sample 2: 41.85 ± 9.60 vs. 55.25 ± 16.19 ng/ml; $t_7=0.85$ $P=0.42$; paired *t*-test).

Two-group analysis

Because mean CORT levels and variance did not differ between the 30 mg/kg and 60 mg/kg CORT groups, or between the vehicle and unmanipulated groups, the animals were combined into two treatment groups, CORT (30 mg/kg + 60 mg/kg CORT groups; n = 16) and control (unmanipulated + vehicle groups; n = 15), for the remaining analyses (Fig. 2B). Repeated-measures ANOVA on these two groups again revealed a main effect of day ($F_{1,29} = 20.71$, $P < 0.0001$), a main effect of treatment group ($F_{1,29} = 171.25$, $P < 0.0001$) as well as a day × treatment group interaction ($F_{1,29} = 17.35$, $P < 0.0001$). Plasma CORT levels were higher on day E1 than on day E3 in the control group ($t_{14} = 6.05$, $P < 0.001$; Tukey's LSD), but not in the CORT group ($t_{15} = 0.28$, $P = 0.78$; Tukey's LSD). The control group had significantly lower CORT levels on both day E1 and day E3 when compared to the CORT group (day E1: $t_{29} = 11.16$, $P < 0.001$; day E3: $t_{29} = 11.23$, $P < 0.001$; Tukey's LSD).

Behavior

Family test

Family tests were conducted not only to investigate paternal behavior, but also to elucidate any effects of CORT injection on overall activity patterns, as well as on male proximity to the pup(s) and pairmate in the home cage. Results of the family test are presented in Table 1. None of the paternal or non-paternal behaviors differed significantly between the CORT and control groups, and neither aggressive displays nor antagonistic behavior was observed in any instance during these tests. Females spent significantly more of the 30-second scans in contact with the pups than did males (0.16 vs. 1.00, $Z = -3.885$, $p = 0.0001$, Wilcoxon signed-rank test); however, despite females being in contact with the pups almost continually, 46.67% (7/15) of control and 31.25% (5/16) of CORT-treated males were categorized as paternally responsive (spent more than 1.5 s licking or huddling the pup or performing nursing posture). The proportion of paternally responsive males did not differ between the CORT-treated and control groups (Fisher's exact test, $P = 0.473$).

Retrieval test

While the family-test behavioral data represented an overall view of the males' activity and location in relation to the pups and the female, the retrieval test provided a more refined measure of direct paternal behavior, focusing only on interactions between a male and one of its offspring. Results of the retrieval test are presented in Table 2. All males participated in at least one measure of pup-related behavior, and aggressive or antagonistic behaviors were never observed. Overall, males were more attentive to the pups with the female absent from the cage: when the composite scores from the family test and retrieval test were compared, fathers, regardless of treatment condition, significantly increased the amount of time spent engaging in direct paternal care ($Z = -3.466$, $P = 0.001$, Wilcoxon signed-rank test). The percentage of paternally responsive males (≥1.5 s of composite behavior) did not differ between treatment groups ($P = 0.999$, Fisher's exact test), and both groups showed an increase from the family-test values, as 93.75% (15/16) of CORT-treated males interacted paternally with their pups (compared to 31.25% in the family test), while 100% (15/15) of control males did so (up from 46.67%). The one father in the CORT-treated group that did not engage in any of the composite behaviors nonetheless carried and sniffed its pup. In addition to labeling males as paternally responsive or not, we used a more stringent definition to separate paternal and non-paternal males. The proportion of paternally-behaving males (i.e., those that spent ≥100 s of composite behavior) did not differ between CORT-treated (56.25%, 9/16) and control groups (80.00% 12/15; $P = 0.252$, Fisher's Exact test).

CORT-treated fathers took longer to initially contact pups in the retrieval test than did control fathers (25.53 ± 7.46 vs. 7.48 ± 1.91 s, respectively, $t_{29} = 2.45$, $P = 0.021$); however, this difference was not statistically significant after we applied the false discovery rate (FDR) correction ($q = 0.294$). CORT treatment resulted in a marginal

Table 1
Behavioral data from the 10-min family test on experimental day 1 in CORT-treated (n = 16) and control (n = 15) California mouse fathers.

Behavior	Description	Median (range)		P[f]	r[g]	q[h]
		Control	CORT			
% paternally responsive[a]		46.67% (7/15)	31.25% (5/16)	0.473	–	0.815
Paternal[b]	Composite[c]	0.00 (0.00–578.10)	0.00 (0.00–196.68)	0.224	0.218	0.725
	Huddle pup	0.00 (0.00–541.04)	0.00 (0.00–132.48)	0.276	0.196	0.725
	Lick pup	0.00 (0.00–151.49)	0.00 (0.00–54.41)	0.634	0.085	0.815
	Nursing posture	0.00 (0.00–0.00)	0.00 (0.00–9.78)	0.333	0.173	0.771
	Sniff pup	0.00 (0.00–5.51)	0.00 (0.00–7.89)	0.456	0.134	0.815
Non-pup-related	Autogroom[b]	20.19 (0.00–130.71)	50.48 (0.00–238.84)	0.233	0.214	0.725
	Dig[b]	1.64 (0.00–118.35)	3.43 (0.00–77.34)	0.648	0.082	0.815
	Jump[d]	7.0 (0.0–195.0)	1.5 (0.0–26.0)	0.276	0.195	0.725
	Rear[d]	21.0 (0.0–82.0)	7.0 (0.0–89.0)	0.577	0.100	0.815
Male location[e]	Touch pup(s)	0.16 (0.00–1.00)	0.16 (0.00–1.00)	0.903	0.022	0.903
	Within 10 cm but not touching pup(s)	0.00 (0.00–0.16)	0.05 (0.00–1.00)	0.097	0.298	0.725
	In same cage but not within 10 cm of pup(s)	0.11 (0.00–0.79)	0.21 (0.00–0.84)	0.660	0.079	0.815
	In different cage than pup(s)	0.32 (0.00–1.00)	0.24 (0.00–0.74)	0.367	0.162	0.771
Female location[e]	Touch pup(s)	1.00 (0.05–1.00)	1.00 (0.21–1.00)	0.767	0.053	0.895
	Within 10 cm but not touching pup(s)	0.00 (0.00–0.32)	0.00 (0.00–0.05)	0.863	0.031	0.903
	In same cage but not within 10 cm of pup(s)	0.00 (0.00–0.42)	0.00 (0.00–0.42)	0.886	0.026	0.903
	In different cage than pup(s)	0.00 (0.00–0.47)	0.00 (0.00–0.68)	0.195	0.233	0.725
Male and female location[e]	Both in same cage	0.53 (0.00–1.00)	0.61 (0.21–1.00)	0.577	0.0964	0.815
Activity[e]	Locomotion	0.34 (0.00–1.00)	0.18 (0.00–0.68)	0.270	0.198	0.725
	Rest/sleep	0.32 (0.00–1.00)	0.35 (0.00–1.00)	0.952	0.011	0.908
	Stationary	0.21 (0.00–0.53)	0.34 (0.00–0.74)	0.248	0.207	0.725

[a] Percentage of males whose composite duration was ≥1.5 s. Fisher's Exact test.
[b] Total duration (seconds).
[c] Composite consists of nursing posture (N), huddle (H), lick pup (L).
[d] Number of occurrences.
[e] Proportion of 30-second instantaneous scans during which behavior occurred.
[f] Uncorrected P-values from Fisher's exact (paternally responsive) and Mann–Whitney U (all other calculations) tests.
[g] Effect size, calculated as $Z/\sqrt{N}$.
[h] Adjusted p value from FDR correction.

Table 2
Behavioral data from the 5-min retrieval test on experimental day 1 in CORT-treated (n = 16) and control (n = 15) California mouse fathers.

Behavior	Median (range)		P[e]	r[f]	q[g]
	Control	CORT			
Latency to contact pup (seconds)[a]	7.48 (0.37–26.99)	25.53 (0.80–114.40)	0.021	0.890	0.315
% paternally responsive[b]	100% (15/15)	93.25% (15/16)	0.999	–	0.999
% paternal[b]	80.00% (12/15)	56.25% (9/16)	0.252	–	0.540
Paternal[c]					
Composite	207.77 (0.95–271.27)	147.55 (0.00–233.30)	0.082	0.312	0.432
Huddle pup	50.40 (0.00–222.31)	41.73 (0.00–193.35)	0.579	0.100	0.743
Lick pup	68.04 (0.952–230.88)	37.90 (0.00–177.31)	0.206	0.227	0.515
Nursing posture	0.00 (0.00–51.38)	0.00 (0.00–41.27)	0.131	0.272	0.432
Sniff pup	26.94 (10.32–67.83)	34.94 (8.65–67.73)	0.693	0.071	0.743
Manipulate pup	0.00 (0.00–59.79)	0.00 (0.00–128.61)	0.643	0.083	0.743
Carry pup	0.00 (0.00–72.60)	0.00 (0.00–40.17)	0.492	0.122	0.743
Non-pup-related					
Autogroom[c]	10.23 (0.00–130.96)	9.36 (0.00–40.17)	0.551	0.107	0.743
Dig[c]	0.00 (0.00–71.00)	0.00 (0.00–162.43)	0.661	0.079	0.743
Locomotion[c]	8.79 (0.99–55.60)	18.37 (0.00–191.59)	0.144	0.263	0.432
Jump[d]	0.0 (0.0–9.0)	0.0 (0.0–44.0)	0.517	0.116	0.743
Rear[d]	2.0 (0.0–17.0)	5.0 (0.0–44.0)	0.129	0.272	0.432

[a] Latency to contact was $\log_{10}$-transformed and analyzed by *t*-test (displayed as mean and range on table); other behaviors were analyzed using Mann–Whitney U tests.
[b] Paternally responsive males were defined as those males engaging in ≥1.5 s of composite behavior (for comparison with family test); paternally behaving males were defined as those with composite scores ≥100 s. Percentages were compared using Fisher's exact test. Composite consists of nursing posture (N), huddle (H), lick pup (L).
[c] Total duration (seconds).
[d] Number of occurrences.
[e] P-values obtained prior to FDR correction.
[f] Effect size, calculated by $Z/\sqrt{N}$.
[g] Adjusted P-values from FDR calculation.

decrease in proportion of time that fathers spent engaging in direct paternal behavior (nursing posture + huddle pup + lick/groom pup), but this effect did not reach statistical significance, even before FDR correction ($U = 76$, $z = -1.74$, $P = 0.082$, Mann Whitney U test). Acute increase in circulating CORT levels did not affect the duration of time fathers engaged in autogrooming, locomotion, digging or pup-directed sniffing, nor did it alter the number of jumps or rears performed (Table 2).

Male body mass and body temperature

Males were weighed seven times throughout the experiment (at pairing, day prior to first injection (day E0), days E1, E3, E10, PP30, and 2–4 days after birth of the second litter; see Fig. 1). Fathers experienced systematic patterns of change in body mass over the experiment, as repeated-measures ANOVA yielded a significant main effect of day on male mass ($F_{6,156} = 6.25$, $P < 0.0001$); however, CORT treatment had no effect on this pattern (main effect of treatment: $F_{1,26} = 1.73$, $P = 0.201$; time × treatment interaction: $F_{6,156} = 0.17$, $P = 0.985$). Regardless of treatment (CORT or control), males showed a small decrease in body mass from day E0 to E10 (E0 vs. E1, E0 vs. E3, E0 vs. E10; $P < 0.05$ for each comparison), a spike in mass at PP30 ($P < 0.05$ for each group compared to all previous time points for that group), and a return to day E0 mass after the birth of the second litter (Tukey's LSD for all post-hoc analyses listed above; Fig. 3).

Body temperature was significantly correlated with thermometer depth on all occasions ($r = 0.492–0.736$, $P < 0.05$, $n = 21–24$), so residuals were used for analysis. CORT treatment did not affect body temperature ($F_{1,14} = 0.25$, $P = 0.627$; data not shown). Moreover, body temperature did not differ significantly across days ($F_{3,42} = 0.28$, $P = 0.837$), nor was there a significant day × treatment interaction ($F_{3,42} = 0.78$, $P = 0.511$).

Litter and pup parameters

Pup mass

Pups from litter 1 were weighed four times (days E1, E3, E10, and PP30), and pups from litter 2 were weighed once (2–4 days postpartum, time-matched to day E1 of litter 1). Each pup was weighed, but analyses used mean per-pup mass for each litter, to control for differences in litter size. As expected, all litter 1 pups gained body mass throughout the experiment; repeated-measures (day × treatment group) ANOVA for litter 1 showed a main effect of day on per-pup mass ($F_{3,78} = 895.24$, $P < 0.0001$). Per-pup mass increased significantly from day E1 to weaning at PP30 regardless of father's treatment condition, with body mass at each time point being significantly higher than the previous time point for pups of both control and CORT-treated fathers ($P < 0.05$, Tukey's LSD for all comparisons; Fig. 4). Father's treatment group (CORT or control) did not affect per-pup mass ($F_{1,26} = 0.96$, $P = 0.336$), nor was there a time × treatment interaction ($F_{1,26} = 0.18$, $P = 0.680$). A paired-samples *t*-test indicated that day E1 per-pup mass from litter 1 and litter 2 did not differ ($t_{27} = 0.53$, $P = 0.60$; see Fig. 4). For litter 1, there was no correlation between mother's day 1 mass and per-pup mass at day E1 ($r = 0.12$, $P = 0.546$) or day E3

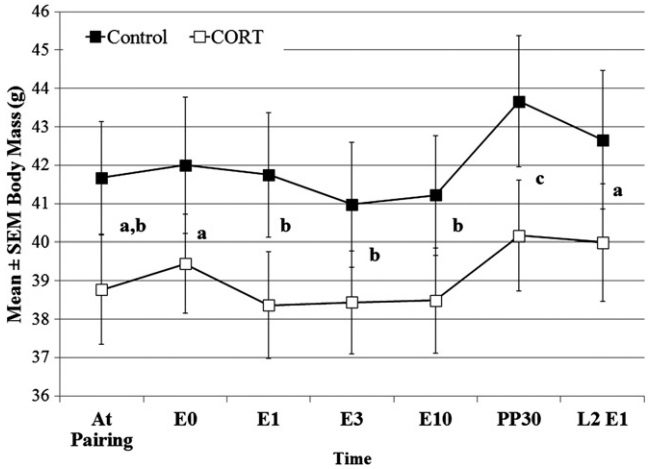


Fig. 3. Body mass in control (n = 15) and CORT (n = 16) males over days of the experiment. CORT treatment did not affect body mass at any time point measured, nor was there an interaction between CORT treatment and time. Statistical results presented are for the main effects of time. Time points with different letters are significantly different from one another ($P < 0.05$).

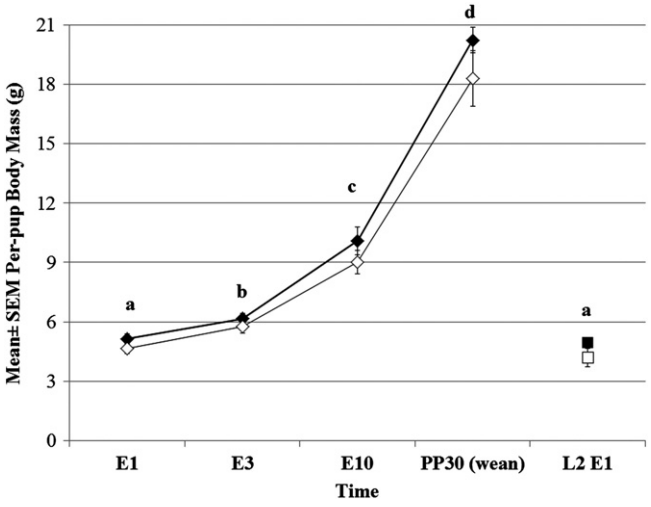


Fig. 4. Mean per-pup body mass over time of pups of control (filled symbols) and CORT-treated (open symbols) fathers. Data from both the first litter (diamonds) and the second litter (squares) are presented. Per-pup mass was not affected by fathers' treatment group, nor was there an interaction between time and treatment. Statistical results are shown for the main effect of time; time points with different letters are significantly different from one another ($P<0.05$).

($r=0.17$, $P=0.370$); therefore, mother's body mass was not used as a covariate for per-pup mass at any time point.

Litter size

CORT treatment of males did not affect the number of pups in litter 2, as litter size did not differ between the CORT and control groups ($t_{28}=1.57$, $P=0.128$, independent-samples *t*-test). The number of pups born increased significantly, however, from the pairs' first litter to second litter (1.9 ± 0.1 vs. 2.3 ± 0.1, respectively; $F_{1,28}=7.00$, $P=0.013$, paired-samples *t*-test), regardless of treatment.

Interbirth interval

CORT treatment of fathers did not affect the latency to the birth of litter 2 (interbirth interval; $t_{28}=0.09$, $P=0.926$, independent-samples *t*-test). Latency to birth of litter 1 (43.8 ± 1.7 days from pair formation) was significantly longer than latency to birth of litter 2 (39.2 ± 1.8 days from previous parturition, $F_{1,28}=6.58$, $P=0.016$) regardless of treatment condition, as there was no time×treatment interaction ($F_{1,28}=0.01$, $P=0.916$).

Discussion

Both acute and chronic stressors have been shown to disrupt parental behavior. The disruption in response to chronic stress appears to be mediated at least in part by elevated glucocorticoid concentrations, and glucocorticoids have also been implicated in mediating the decrease in parental behavior in response to acute stress; however, this possibility has yet to be tested experimentally. The present study was the first to determine the effects of acutely elevated CORT concentrations on paternal behavior and longer-term reproductive parameters in a male mammal. Contrary to the hypothesized inhibitory effects of glucocorticoids on paternal behavior (e.g., Moore and Hopkins, 2009; Wingfield and Sapolsky, 2003), we found no evidence that acute CORT elevation inhibits direct paternal care in this biparental rodent. We did find some evidence that CORT treatment decreases responsiveness to pup stimuli, as CORT-treated animals approached their own pups more slowly than control animals in retrieval tests; however, this effect was not statistically significant after we controlled for false discovery rate. CORT treatment had no other detectable effects on either paternal or non-paternal behavior of first-time California mouse fathers, either while they were housed with their mate and pups under undisturbed conditions (family test) or during a brief interaction between the father and one of its pups (retrieval test).

One possible explanation for the absence of differences between CORT-treated and control fathers' paternal behavior in the family test is that control males had low composite scores for direct paternal care in this test, making it difficult to detect a possible decrease in CORT-treated fathers. Low levels of paternal behavior during the family test were likely due to the mothers' presence during the testing period; mothers spent significantly more time in contact with the pups than fathers, thereby preventing the fathers from engaging in long bouts of care. In contrast to their low composite scores for direct paternal care, control males had considerably higher scores for other behaviors in the family test, which potentially could have been decreased by CORT treatment; however, CORT treatment did not alter the amount of time that fathers spent in proximity to the family, nor did it alter any other behaviors measured, and fathers never behaved aggressively towards the female or pups. Additionally, males from both the CORT-treated and control groups engaged in significantly more direct paternal behavior in the retrieval test than in the family test, and the percentage of males behaving paternally (≥100 s of direct paternal behavior) in the retrieval test did not differ between treatment groups. Thus, we conclude that CORT treatment did not overtly alter either direct paternal care (licking, huddling, nursing posture) or more indirect measures of paternal care (proximity to pups, sniff pup, etc.).

It is possible that elevated CORT concentrations do alter the initial response to a pup, as CORT-treated males tended to approach the pup more slowly in the retrieval test than control males; however, this result was not statistically significant after we controlled for alpha inflation. It may be that elevated CORT alters sensory processing or perception of pup-related sensory cues; a follow-up experiment investigating the relationship between elevated CORT and responsiveness to pup-related stimuli would be informative.

We chose to examine paternal behavior 1.5–2 h after injection, due to the time course of steroid hormone/receptor interactions. CORT can bind two cytoplasmic receptor subtypes, mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). After binding, the hormone-receptor complex translocates to the nucleus, where it binds to DNA and alters gene transcription, a process that takes approximately 1–2 h (Hayashi et al., 2004; Lightman et al., 2008). In addition to effects on gene expression mediated by MR and GR, CORT can cause rapid, non-genomic changes in physiology and behavior, mediated by binding to membrane receptors and activation of signal-transduction pathways (Borski, 2000; Joëls and Baram, 2009; Moore and Orchinik, 1994). However, our study did not address the possibility that CORT might have more rapid, non-genomic effects on paternal behavior. This would be an illuminating area for future research. Additionally, this study investigated effects of only CORT rather than full HPA activation; therefore, it remains possible that other hormones in the HPA axis, such as corticotropin-releasing hormone, can acutely alter paternal behavior, as has been found in females (Almeida et al., 1994; Gammie et al., 2004; Pedersen et al., 1991; Saltzman et al., 2011).

Our manipulations were designed to elevate circulating CORT to levels similar to circadian peak values (1500–1800 ng/ml, unpub. data) or post-stressor values during the lights-on phase of the daily cycle (2200–2500 ng/ml, unpub. data) to determine if different behavioral effects result from CORT concentrations that are reached on a daily basis vs. those reached after exposure to a stressor. Recent research suggests that at the circadian peak, plasma CORT occupies MR as well as some GR, whereas post-stressor titers presumably occupy all MR and a larger proportion of GR (for discussion, see Landys et al., 2006). In our study, the achieved concentrations of circulating CORT were higher than targeted levels and presumably resulted in maximal binding of MR, and possibly maximal binding of GR as well. Nonetheless, no negative physiological effects of elevated CORT were observed, and mice appeared to be healthy

and in good condition. We have previously found that male California mice can produce endogenous CORT concentrations of ~4500 ng/ml in response to a stressor during lights-off conditions (unpub. data). Therefore, although the achieved levels of ~5900 ng/ml in this study may represent supra-physiological values, they are only moderately higher than endogenous CORT levels occurring during the physiological stress response.

In addition to effects of acute CORT elevation on paternal behavior, we tested the hypothesis that these elevations would have longer-term effects on male body condition and reproductive outcomes. Recent literature in house mice (*Mus musculus*; Malisch et al., 2010) and bluebirds (*Sialia sialis*; Lynn et al., 2010) has demonstrated that stressors that are presumed to be acute can have more sustained (e.g. greater than 24 h) effects on HPA function. In addition, previous research in California mice has found that the presence of the father can decrease the pairmate's interbirth interval (Cantoni and Brown, 1997a), and during challenging conditions (forced running or cold environment) more pups survive if the father is present (Cantoni and Brown, 1997b; Gubernick et al., 1993), pointing to the father's importance in offspring survival. In this experiment, however, we did not find any effect of CORT treatment of fathers on pup growth or survival to weaning, interbirth interval, number of pups born in the second litter, or the mass of the second litter's pups. The mice in our study lived in a non-challenging environment and had *ad libitum* access to food. Effects of stress can be context-dependent and are exacerbated by challenging conditions (e.g., social conflict or low food availability; Creel, 2001; Walker et al., 2005); therefore, acute elevation of plasma CORT in a more natural, challenging context may produce different results.

Irrespective of paternal care, we predicted that CORT would increase activity levels of mice, as has been noted in other species after either short-term glucocorticoid administration or acute stress (Breuner et al., 1998; Overli et al., 2002; Windle et al., 1997). Glucocorticoids can also increase metabolic rate and glucose concentrations, and can alter energy partitioning (Baxter, 1976; Sapolsky et al., 2000); therefore, we also measured body temperature and body mass in fathers. We found no difference in measures of activity between control and CORT-treated fathers in either behavioral paradigm tested (family and retrieval tests), nor did CORT treatment affect body temperature or body mass.

Regardless of treatment condition, however, fathers showed a significant increase in body mass on postpartum day 30 (when the first litter was weaned) as compared to all preceding time points. Moreover, fathers systematically lost body mass after the birth of the second litter, suggesting that the increase in body mass seen at weaning of the first litter was not simply due to normal, age-related growth. This pattern suggests that the mate's pregnancy and parturition influenced males' body mass, possibly through hormonal changes in males or changes in food consumption. It is unlikely that the pups themselves were directly responsible for the change in paternal body mass, as mice had unlimited access to food and the pups should have been able to find and retrieve their own food pellets and/or nurse from the female. Changes in male body mass across the reproductive cycle have not been reported previously in this species, but biparental cotton-top tamarin (*Saguinus oedipus*) and common marmoset (*Callithrix jacchus*) fathers have been shown to gain body mass over their mate's pregnancy when compared to control males (Ziegler et al., 2006), as have human men (Clinton, 1986). However, the observed increase in mass at the time of weaning in our study contrasts with results found in another monogamous, biparental rodent, the prairie vole (*Microtus ochrogaster*), as male prairie voles from a longitudinal study had their lowest weights when the first litter was weaned (Campbell et al., 2009).

Currently, the mechanism driving changes in male body mass over the female reproductive cycle is not known. Several hormonal changes have been noted in males of biparental mammalian species during the mate's pregnancy and during care of offspring (Wynne-Edwards and Timonin, 2007; Ziegler, 2000), and these endocrine changes could be responsible for changes in body mass. More specifically, levels of estrogen, testosterone, prolactin, and glucocorticoids, all of which can influence metabolic processes (Frühbeck et al., 2001), have been found to change across the mate's pre- and/or postpartum periods, or in response to offspring cues, in biparental males from several species (Berg and Wynne-Edwards, 2001, 2002; Carlson et al., 2006; da Silva Mota et al., 2006; Fleming et al., 2002; Nunes et al., 2001; Reburn and Wynne-Edwards, 1999); however, the directionality and patterning of hormonal change differ across species, and even between different studies of the same species. More information is needed to elucidate the interactions among male body mass changes, hormones, and the female's pregnancy.

Overall, in California mice, we found that acute CORT elevation in new fathers had no detectable consequences for either direct paternal care or pup survival in a laboratory setting. In a recent comparative analysis of the stress response and reproductive behavior across avian species, Bókony et al. (2009) found that glucocorticoid levels are not phylogenetically conserved but evolve as species-specific attributes, suggesting that relationships between parental care and aspects of the stress response may differ greatly among species. In California mice, a one-time acute elevation in CORT, separate from a physiological or psychological stressor and occurring in an otherwise non-challenging environment, does not seem to be sufficient to trigger a decrease in paternal behavior; however, this may not be the case for other species with different life history strategies. California mice mate for life and are genetically monogamous and biparental; they produce few (1–4), well-developed pups per litter, and both parents invest heavily in parental care. These traits may allow this species to be less constrained by the effects of increased glucocorticoid levels and continue to provide care; the idea of greater parental investment when fewer, more-developed offspring are produced is consistent with life history theory (Roff, 1992; Stearns, 1992). Furthermore, members of highly monogamous, biparental species may be more resistant to the effects of increased glucocorticoids on parental care, as compared to uniparental species, since they can depend on help from their pairmate (Wingfield and Sapolsky, 2003). Comparing the effects of acute glucocorticoid elevation on parental care in both biparental and uniparental congeners could shed light on the evolutionary and life-history correlates of the effects of stress on reproduction in this genus.

Finally, while plasma CORT concentrations may serve as a signal by which parents determine when to invest in themselves and when to invest in reproduction (Wingfield and Sapolsky, 2003), it is likely that acute increases in plasma CORT alone may not be sufficient to derail parental investment in certain life history trajectories. Instead, such increases may be only part of the signal, and environmental factors (e.g., inclement weather, food or water shortage, social rivalry, cues from the mate, or a combination of factors) or stressor characteristics (duration, previous stressor history including repeated acute stressors that may lead to allostatic overload or chronic stress; Landys et al., 2006; McEwen and Wingfield, 2003; Stewart, 2006) may be important. Additional work is needed to evaluate these possibilities. Measures of density, location and activity of CORT receptors, as well as corticosteroid-binding globulin activity (see Malisch and Breuner, 2010), would aid in the understanding of interactions among CORT, paternal behavior, and reproductive outcomes.

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