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Reproduction in the Face of Stress: Mediation by the Hypothalamic-Pituitary-Adrenal (HPA) Axis

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

Doctor of Philosophy

in

Evolution, Ecology and Organismal Biology

by

Breanna Nicole Harris

December 2012

Dissertation Committee:
Dr. Wendy Saltzman, Chairperson
Dr. Mark Chappell
Dr. Richard Cardullo
Dr. Theodore Garland, Jr.

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The Dissertation of Breanna Nicole Harris is approved:

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Committee Chairperson

University of California, Riverside

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Chapter 1: **Hypothalamic-pituitary-adrenal (HPA) axis function in the California mouse (*Peromyscus californicus*): Changes in baseline activity, reactivity, and fecal excretion of glucocorticoids across the diurnal cycle.**
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Chapter 5: **Acute effects of corticosterone injection on paternal behavior in California mouse (*Peromyscus californicus*) fathers.**
Breanna N. Harris, Juan Pablo Perea-Rodriguez, and Wendy Saltzman
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DEDICATION

I dedicate this dissertation to my awesome family

ABSTRACT OF THE DISSERTATION

Reproduction in the Face of Stress: Mediation by the Hypothalamic-Pituitary-Adrenal (HPA) Axis

by

Breanna Nicole Harris

Doctor of Philosophy, Graduate Program in Evolution, Ecology, and Organismal Biology
University of California, Riverside, December 2012
Dr. Wendy Saltzman, Chairperson

Stress and glucocorticoids are hypothesized to mediate a trade-off between current and future reproduction, as organisms are predicted to invest less in current reproduction under stressful conditions. In this dissertation I explored whether stress and activation of the hypothalamic-pituitary-adrenal (HPA) axis (resulting in elevated glucocorticoid concentrations) suppress current reproductive behavior in the monogamous, biparental California mouse (*Peromyscus californicus*). Initially, I characterized HPA function under baseline and post-stress conditions (Aim 1). I next tested the hypotheses that older animals are more stress-resistant than younger animals, as older animals have lower residual reproductive value (Aim 2), and that fathers are more stress-resistant than non-fathers (Aim 3). Lastly, I determined whether chronic stress (Aim 4) and acute glucocorticoid elevation, separate from external stress (Aim 5), would decrease paternal care and alter survival and development of offspring. Results demonstrate that HPA function in California mice differs markedly from that of

previously studied rodents (Aim 1), and that changes in residual reproductive value (age; Aim 2) and reproductive status (Aim 3) do not modulate HPA axis activity. Chronic stress reduced paternal behavior (Aim 4), but effects were subtle and not likely mediated solely by changes in circulating corticosterone levels, as corticosterone injection did not alter paternal behavior (Aim 5). Moreover, decreased paternal behavior during chronic stress did not appreciably alter survival, development, growth or HPA function of pups, suggesting that stress-induced decreases in paternal care had no fitness consequences under controlled laboratory conditions. In conclusion, glucocorticoids do not appear to directly mediate the trade-off between current and future reproduction in males of this species; however, stress can decrease paternal behavior. Therefore, while corticosterone concentrations may serve as part of the signal by which parents determine reproductive investment, increases in this hormone alone may not be sufficient to derail current reproductive attempts. Instead, stress-induced changes in other hormones and neuropeptides might be important in regulating stress-related changes in behavior. These experiments provide important insight into interactions between reproductive status and HPA function in a biparental mammal, and could have implications for conservation of biparental species and for understanding effects of stress on human parenting.

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Figure 4.4: Back-transformed plasma corticosterone concentration in pups raised by chronically stressed (CVS; n=14 pups), separation control (SC; n=11 pups) or undisturbed control (UC; n=13 pups) fathers for the first 8-10 days of life. Plasma samples were collected at 1430-1520 h, and predator-odor stress significantly elevated corticosterone levels in pups (P<0.01); however, pups' corticosterone concentrations were not affected by fathers' experimental condition.

Figure 5.1: Experimental design

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DISSERTATION INTRODUCTION

The evolution of trade-offs is a major tenet of life-history theory (Roff 1992, 1996; Stearns 1989, 1992, 2000). Life-history theory posits that resources are limited and available organismal resources are diverted to the processes or behaviors that prove to be the most beneficial in order to maximize lifetime reproductive success (Bronson, 1989; Reznick et al., 1990; Roff, 1992; Salmon et al., 2001; Stearns, 1992, 2000; Wasser and Barash, 1983; Zera and Harshman, 2001). The necessity for organisms to partition limited resources has led to several proposed life-history trade-offs, including a trade-off between investment in current vs. future reproduction (Stearns, 1992, 2000). Investing too heavily in a current reproductive bout, specifically under challenging conditions, could lead to decreases in parental body condition (e.g., energy reserves, immune function), which could ultimately impact survival and future reproductive opportunities (Bronson, 1989; Daan et al., 1990; Roskaft, 1985;Wasser and Barash, 1983; Wingfield and Sapolsky, 2003). Which breeding option (current vs. future) is more favorable depends on a variety of organismal and environmental factors, including, but not limited to, nutritional and health status, somatic growth/body condition, social cues, abiotic and biotic resources, residual reproductive value, current life-history state, and stress (Bronson, 1989; Stearns, 1992).

The underlying mechanisms of life-history trade-offs have received relatively little attention and are largely unknown; however, hormones have been viewed as likely candidates (Ketterson and Nolan, 1999; Moore and Hopkins, 2009; Ricklefs and

Wikelski, 2002; Stearns, 1989, 2000; Zera and Harshman, 2001). Accordingly, the hormonal cascade associated with the endocrine stress response has been suggested to mediate the trade-off between current reproductive effort and future reproductive potential (Greenberg and Wingfield, 1987; O'Reilly and Wingfield, 2001; Ricklefs and Wikelski, 2002; Wingfield et al., 1998). This endocrine response is a likely candidate as the glucocorticoid hormones (released in high concentrations following a stressful event) are known to influence energy partitioning and can bind receptors in the brain to alter behavior (Sapolsky et al., 2000).

Stress is very broadly defined as a threat to homeostasis; however, this definition is overly general, and finding a suitable operational definition has proven difficult. As highlighted by Hans Selye, 'the grandfather of stress research' (1973), "*Everybody knows what stress is and nobody knows what it is.*" Recently, however, Kim and Diamond (2002) have proposed a useful definition that emphasizes important aspects of stress, including predictability and an individual's perception of and control over the stressor. They also emphasize that an aversive stimulus (stress) can be physiological or psychological and that the arousal caused by the stress must be measurable (e.g., electroencephalogram, motor output [behavior], neurochemical measures [glucocorticoids, catecholamines]; Kim and Diamond, 2002). For the purposes of this dissertation the following will be used to define the amorphous concept that is stress: "*Stress is a condition in which an individual is aroused by an **aversive** situation. The magnitude of the stress and its physiological consequences* [i.e., glucocorticoid levels]

are influenced greatly by the individual's ***perception*** *of its ability to* ***control*** *the presence or the intensity of the stimulation"* – Kim and Diamond (2002).

Stress is traditionally measured by an increase in activation of the sympathoadrenomedullary system (SMS) and the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky, 2002). Once a stimulus is perceived as stressful, the SMS is quick to respond, resulting in an almost immediate release of catecholamines: epinephrine from the adrenal medulla and norepinephrine from sympathetic post-ganglionic neurons (Armario, 2006; Sapolsky, 2002). Epinephrine enters the blood stream and exerts rapid effects on heart rate, breathing, blood flow, and other physiological processes associated with the "fight or flight" response (Cannon, 1932).

Concomitantly with SMS activation, the HPA axis is activated. The HPA axis begins in the paraventricular nucleus of the hypothalamus with the neuropeptide corticotropin-releasing-hormone (CRH). Upon neuronal activation, this hormone travels via parvocellular neurons to the median eminence, where it is released, either by itself or in conjunction with arginine vasopressin (AVP), into the portal blood vessels connecting the hypothalamus to the anterior pituitary (Armario, 2006). Release of CRH can be influenced by differing neural pathways, and several brain regions have been implicated in regulation of the stress response (for a list see Herman and Cullinan, 1997). Once released, CRH stimulates the corticotrope cells in the anterior pituitary to synthesize pro-opiomelanocortin (POMC), which is then post-translationally modified into adrenocorticotropic hormone (ACTH) and other products such as melanocyte-stimulating hormone and endorphins (Bicknell, 2008). ACTH enters the systemic blood supply and

binds to G protein-coupled receptors in the zona fasciculata of the adrenal cortex, causing synthesis and release of glucocorticoids (Xia and Wikberg, 1996). Glucocorticoids can then bind to receptors in both the hypothalamus and the anterior pituitary to regulate glucocorticoid secretion in a negative feedback loop (other brain regions, e.g. hippocampus and prefrontal cortex, may also be involved in negative feedback, Herman and Cullinan, 1997). Glucocorticoid release takes 3-10 min from onset of the stressor; therefore, end-products of the HPA axis respond more slowly than catecholamines released by the SMS (Sapolsky, 2002). The HPA axis is highly conserved across vertebrates and is critical for the regulation of organismal homeostasis, both under basal conditions and in response to stress (Denver, 2009; Sapolsky et al., 2000).

Glucocorticoids are necessary for metabolic, brain, and homeostatic regulation on a daily basis (Sapolsky et al., 2000), as these hormones are released in a circadian pattern, with the endogenous glucocorticoid peak occurring just prior to the onset of daily activity (Dallman et al., 1987, 1993; Chrousos, 1998a; Sapolsky et al., 2000; Malisch et al., 2008). As previously stated, the HPA axis and the glucocorticoids are also critically important in the response to stressors (Sapolsky, 2002; Sapolsky et al., 2000). Stressors, defined as stimuli that induce stress, can range from environmental and social demands (e.g., high population density, social instability, predation) and physical or physiological challenges (e.g., injury, sickness, food or water restriction, extreme ambient temperatures) to psychological stressors (e.g., lack of control, unpredictability, novelty, noise, restraint). Stress can also be classified as acute (transient) or chronic (prolonged). The 'stress hormones' or glucocorticoids act on numerous cell types and physiological

systems and affect a large number of organismic functions, including behavior, emotion, cognition, immunity, heart rate, glucose regulation and energy partitioning (they can suppress insulin secretion as well as stimulate gluconeogenesis, lipolysis, glycogenolysis, and proteolysis), metabolism, feeding, locomotion, reproductive physiology and reproductive behavior (Boonstra, 2005; Chrousos, 1998a,b; Dallman et al., 1993; Ferin, 2006; Reeder and Kramer, 2005; Sapolsky, 2002; Sapolsky et al, 2000; Stratakis and Chrousos, 1995; Wingfield and Sapolsky, 2003), all of which make these hormones prime candidates as mediators of trade-offs.

Frequently, suppressing or triaging reproduction under currently unfavorable, stressful conditions is more advantageous than wasting precious energy and resources on reproductive attempts that are likely to fail (Bronson, 1989; Reznick, 1985; Wasser and Barash, 1983; Wingfield and Sapolsky, 2003). Consistent with that concept, it has been shown repeatedly that HPA axis activation and elevated glucocorticoid levels dampen several aspects of reproductive physiology (see Wingfield and Sapolsky, 2003). Reproduction, however, encompasses both reproductive physiology and reproductive behavior. *Reproductive physiology* comprises the internal processes that make fertilization and gestation possible (e.g., gametogenesis, sex steroid production, maintenance of secondary sexual characteristics, and in mammals, pregnancy and lactation), while *reproductive behavior* includes both sexual behavior and parental behavior.

The importance of parental behavior depends on the life-history strategy of the organism, but for all mammals and other taxa that produce altricial young, parental care

is essential to offspring survival (Kleiman and Malcolm, 1981). In mammals, this care is generally provided exclusively by the mother, but in a small fraction of mammalian species, including humans, fathers also make important contributions to the survival and growth of offspring. Fathers, like mothers, can provide food, warmth and protection, and can influence behavioral and neuroendocrine development of offspring (Bester-Meredith and Marler, 2001; Bredy et al., 2004; Cantoni and Brown, 1997a,b; Gubernick and Teferi, 2000; Gubernick et al., 1993; Kleiman and Malcolm, 1981; Lamb, 2003; Schradin and Pillay, 2004; Wright and Brown, 2002). Although glucocorticoids and the stress response have been shown to suppress reproductive physiology, evidence for such an effect on reproductive behavior, specifically on parental behavior, is less conclusive (Breuner et al., 2008; Chrousos, 1998b; Ferin, 2006; Wingfield and Sapolsky, 2003; Wynne-Edwards and Timonin, 2007). However, stress and glucocorticoids are often assumed to inhibit parental behavior (see Wingfield and Sapolsky, 2003).

The effects of stress on maternal behavior in mammals have been investigated, but the mechanisms are not well understood (Ivy et al., 2008; Rees et al., 2004, 2006; Saltzman and Abbott, 2009; Saltzman et al., 2011). To date, very few studies have investigated the effects of stress on paternal care, especially in mammalian fathers. What is known about stress and fathers comes from experiments on birds (Bókony et al., 2008; Bonier et al., 2009; Breuner et al., 2008). Additionally, no studies have experimentally tested whether glucocorticoid administration or prolonged stress inhibits paternal behavior in a male mammal. In this dissertation I explored whether the HPA axis, mainly glucocorticoid hormones, mediates a trade-off between current and future reproduction in

a biparental male mammal. Specifically, my dissertation addressed the extent to which stress and glucocorticoids alter paternal behavior in a first step to addressing this trade-off. Males were chosen as the focal sex because, to date, no study has systematically investigated the effect of stress or glucocorticoid manipulation in a mammalian father.

I chose California mice (*Peromyscus californicus*) as the focal species, as these rodents are both monogamous and biparental in both the lab and the field and therefore represent a logical system in which to study paternal care (King, 1968; Ribble 1991; Ribble and Salvoni, 1990). Additionally, California mice survive and breed well in captivity, and paternal behavior (licking, grooming, retrieving, huddling) is important for survival of pups both in nature and in the lab (Cantoni and Brown 1997a,b; Gubernick and Teferi, 2000; Gubernick et al., 1993). In the wild, *P. californicus* mate for life (Ribble, 1991, 1992; Ribble and Salvoni, 1990). A pair can produce several litters per year under favorable environmental conditions (Cantoni and Brown, 1997a,b; Gubernick, 1988; Gubernick and Alberts, 1987; Ribble, 1991, 1992; Ribble and Salvioni, 1990), as breeding is not dependent on photoperiod or season (Drickamer and Vestal, 1973). Females become reproductively mature around 40 days of age (Gubernick, 1988), and males begin to breed successfully at 60-90 days of age (J. Crossland, pers. comm.; unpub. obs.). Gestation averages about 35 days, the female gives birth to 1-5 altricial pups (mean = 1.75 pups for first litter; unpub. data), and birth is followed by a post-partum estrus 1-3 days later (Gubernick, 1988). California mice have an average life span of 9 to 18 months in the wild (Merritt, 1999) but can live up to 4 years (C.A. Marler , pers. comm.; unpub. obs.). In captivity, both males and females breed successfully until 3 years of age

or later, with no decrease in number of pups born (unpub. obs.). These mice produce corticosterone as their primary glucocorticoid (Ogunsua et al., 1971).

I addressed the possible role of stress and glucocorticoids in a trade-off between current vs. future reproduction from two complementary angles: 1) does reproductive status or age alter activity and reactivity of the HPA axis? and 2) do stress and elevation of glucocorticoid hormones inhibit parental behavior? Both of these questions address plausible ways in which organisms could regulate the trade-off between current and future reproductive behavior, if stress and glucocorticoids are in fact a mediating factor. I hypothesized that fathers would be more resistant to stress than would non-fathers, that older animals would be more stress-resistant than younger animals as they have lower residual reproductive value, and that both chronic stress and glucocorticoid elevation, outside of external stress, would decrease paternal behavior. The specific aims of the dissertation are as follows:

- **Aim 1**: Characterize activity and reactivity of the HPA axis in male and female California mice under baseline and various stressful conditions.
- **Aim 2**: Test the hypothesis that old adult male and female mice are more resistant to stress and have decreased HPA activity and/or reactivity as compared to young adult males and females.
- **Aim 3**: Test the hypothesis that reproductive status alters HPA activity and reactivity in male California mice.
- **Aim 4**: Test the hypothesis that chronic variable stress disrupts paternal behavior and subsequent pup development.
- **Aim 5**: Test the hypothesis that acute corticosterone injection inhibits paternal behavior in California mouse fathers.

Chapter 1

Hypothalamic-pituitary-adrenal (HPA) axis function in the California mouse (*Peromyscus californicus)*: Changes in baseline activity, reactivity, and fecal excretion of glucocorticoids across the diurnal cycle

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ABSTRACT

The California mouse, *Peromyscus californicus*, is an increasingly popular animal model in behavioral, neural, and endocrine studies, but little is known about its baseline hypothalamic-pituitary-adrenal (HPA) axis activity or HPA responses to stressors. We characterized plasma corticosterone (CORT) concentrations in *P. californicus* under baseline conditions across the diurnal cycle, in response to pharmacological manipulation of the HPA axis, and in response to a variety of stressors at different times of day. In addition, we explored the use of fecal samples to monitor adrenocortical activity non-invasively. California mice have very high baseline levels of circulating CORT that change markedly over 24 hours, but that do not differ between the sexes. This species may be somewhat glucocorticoid-resistant in comparison to other rodents as a relatively high dose of dexamethasone (5 mg/kg, s.c.) was required to suppress plasma CORT for 8 h post-injection. CORT responses to stressors and ACTH injection differed with time of day, as CORT concentrations were elevated more readily during the morning (inactive period) than in the evening (active period) when compared to time-matched control. Data from ^{3}H-CORT injection studies show that the time course for excretion of fecal CORT, or glucocorticoid metabolites, differs with time of injection. Mice injected in the evening excreted the majority of fecal radioactivity 2-4 h post-injection whereas mice injected during the morning did so at 14-16 h post-injection. Unfortunately, the antibody we used does not adequately bind the most prevalent fecal glucocorticoid metabolites and therefore we could not validate its use for fecal assays.

INTRODUCTION

The hypothalamic-pituitary-adrenal (HPA) stress response has been characterized in many vertebrates (Denver, 2009; Greenberg and Wingfield, 1987) and is activated when an organism is presented with an actual or potential threat, resulting in the release of glucocorticoids (GCs) from the adrenal cortex. The principal glucocorticoid produced by humans and other primates is cortisol, whereas many rodents, including *P. californicus*, primarily produce corticosterone (Ogunsua et al., 1971). Despite the fact that GCs are often referred to as "stress hormones", the HPA axis is continuously active and GCs at baseline levels have important daily functions (Sapolsky et al., 2000). Baseline levels of GCs exhibit a sinusoidal pattern over the course of the day, with the highest concentrations occurring just prior to the onset of waking in most species (reviewed by Dickmeis, 2009; Landys et al., 2006; Levin and Levine, 1975; Lightman et al., 2008). Glucocorticoids help organisms respond to and recover from stressors, and aid in regulation of inflammation and immune function, gluconeogenesis, brain function, cardiovascular activity, various behaviors, and numerous other processes (Chrousos, 1998; Dallman et al., 1993; Dallman et al., 2002; Malisch et al., 2008; Roozendaal et al., 2000; Sapolsky et al., 2000; Sloviter et al., 1989; Strack et al., 1995).

The California mouse, *Peromyscus californicus*, is a monogamous, biparental rodent that is becoming an increasingly popular animal model. Not only is this species useful for studying parental behavior and its neural and hormonal correlates (Becker et al., 2010; Bester-Meredith and Marler, 2003; Brown, 1993; de Jong et al., 2009, 2010;

Gubernick et al., 1994; Lee and Brown, 2002; Trainor and Marler, 2001), but it is also used in studies on aggression (Bester-Meredith et al., 1999; Silva et al., 2010; Trainor et al., 2008, 2010) and immune function (Glasper and DeVries, 2005; Martin et al., 2006, 2008), as well as in studies investigating relationships between stress and behavior (Bardi et al., 2011; Chauke et al., 2011; Harris et al., 2011; Trainor, 2011). California mice survive and breed well in captivity, and parental behavior by both sexes has been well characterized (Bester-Meredith et al., 1999; Brown, 1993; de Jong et al., 2009, 2010; Gubernick and Alberts, 1987). Since both males and females invest heavily in their offspring (Gubernick et al., 1993; Gubernick and Teferi, 2000), *P. californicus* provides a valuable model for studying the effects of stress on parental care, as well as the effects of parental status on stress responsiveness in both mothers and fathers.

Despite the increasing use of California mice in behavioral, neural, and endocrine studies, little is known about their baseline HPA activity or their HPA response to stressors. Although many aspects of the HPA axis are conserved across vertebrates, several parameters, including circulating GC concentrations, circadian dynamics, and temporal responses to and recovery from stressful stimuli, differ markedly among species (Bonier et al., 2009; Davis and Parker, 1986; Dingemanse et al., 2010; Landys et al., 2006; Romero, 2002; Wingfield, 2005) and even within strains of a single species (Dhabhar et al., 1993; Sarrieau et al., 1998). Thus, before we are able to fully understand and interpret behavioral or physiological data in the context of stress in California mice, we must first characterize normative activity and reactivity of the HPA axis.

Determination of circulating GC concentrations via plasma or serum assay provides the most direct measure of GC levels available to the tissues and can provide important information on immediate HPA responses to perturbations; however, this approach presents several problems. Handling and blood collection are stressful to many animals and can alter GC levels, as well as other physiological and behavioral measures, over the short-term and potentially the long-term, which complicates characterization of basal GC levels. Collection of repeated blood samples is compromised by the small body size of many animals and the limited volume of blood that can be removed without adverse effects. To avoid these problems, many researchers are turning to fecal hormone assays, especially in small and/or free-living animals. Fecal hormone levels are not as sensitive to minor disturbances as are hormone concentrations measured in the circulation, and reflect circulating hormone levels over a period of hours due to the processes of metabolism and excretion (Touma et al., 2003, 2004; Touma and Palme, 2005). Fecal measures can thus provide more time-integrated hormone data, thereby yielding a more comprehensive measure of hormone levels over a period of time (see Mostl and Palme, 2002). Glucocorticoids or their metabolites have been measured from fecal samples in numerous mammals, birds and reptiles (Case et al., 2005; Good et al., 2003; Keay et al., 2006; Mostl and Palme, 2002; Touma and Palme, 2005, Wasser et al., 2000); however, proper and extensive assay validation for each species is essential to confirm that biologically relevant GCs or GC metabolites can be measured accurately (Mostl and Palme, 2002; Palme, 2005; Touma et al., 2003, 2004; Touma and Palme, 2005). Although fecal GC measures in California mice have been reported previously

(Bardi et al., 2011), a fecal GC assay has not been validated for this species, and GC or GC metabolite excretion patterns have not been characterized.

The primary objectives of this study were to characterize plasma corticosterone (CORT) concentrations in *P. californicus* (1) under baseline conditions across the diurnal cycle, (2) in response to pharmacological manipulation of the HPA axis, and (3) in response to a variety of stressors at different times of day. In addition, we explored the use of fecal samples as a means to monitor adrenocortical activity non-invasively in this species.

METHODS

Animals

We used California mice that were born and maintained in our breeding colony at the University of California, Riverside. They were descended from individuals purchased from the Peromyscus Genetic Stock Center, University of South Carolina (Columbia, SC) in 2007. The colony was kept on a 14:10 light:dark cycle, with lights-on at 0500h and lights-off at 1900h. Ambient temperature was approximately 23°C with humidity of about 65%. Mice were housed in standard shoe-box-style, polycarbonate cages (44 x 24 x 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. Mice either remained in same-sex groups or were paired with an individual of the opposite sex after 90 days of age.

We used a total of 147 mice (69 males and 78 females) from either same-sex (virgin) or male-female pairs. We were very interested at obtaining basic information about the HPA axis in a representative sample of California mice as this species is becoming increasingly popular animal model. Additionally, data from our lab show that baseline CORT concentrations do not differ between males and females (see results), and that baseline and stress-induced CORT levels do not vary across differing reproductive conditions (Chauke et al., 2011; unpub. data) or adult ages (unpub. data). Therefore, mice of both sexes and of various ages and reproductive conditions were used for our HPA-characterization experiments; whenever possible, however, we avoided the use of pregnant and possibly pregnant females (6 out of 78 females were housed with a reproductively mature adult male, and might have been pregnant during testing). Additionally, some mice were used for more than one data set (diurnal rhythms, stress tests, pharmacological manipulation, or fecal collection); the mean ± SE number of data sets to which each mouse contributed data was 1.02 ± 0.05, range 1-3. Different experiments on the same animal were separated by at least 1 week to allow recovery. The sex, number, and housing condition of mice used for each experiment are listed in the description of each experiment, below. 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*.

Blood Collection and Analysis

Mice were anesthetized with isoflurane, and blood (70-140 µl) was collected from the retro-orbital sinus using heparinized microhematocrit tubes. Time from disturbance or end of the preceding test to collection of the blood sample was always less than 4.6 minutes (mean ± SE: 89 ± 2.61 s; range 22-279 s); 97% of samples were collected in under 3 minutes and 84% in 2 minutes or less. Blood samples were centrifuged for 12 min (13,300 rpm, 4°C), and plasma was removed and stored at -80°C until assay.

Plasma was assayed in duplicate for CORT using an ^{125}I double-antibody radioimmunoassay (RIA) kit (#07-120102, MP Biomedicals, Costa Mesa, CA) previously validated for this species (Chauke et al., 2011). Samples from each experiment were analyzed in the same assay if possible, or balanced evenly across multiple assays; however, an individual mouse's samples from a given experiment were always analyzed in a single assay run. The standard curve ranged from 12.5 ng/ml (91% bound) to 1000 ng/ml (20% bound), and plasma samples were assayed using dilutions ranging from 1:50 to 1:1600 depending on anticipated CORT concentrations. Inter- and intra-assay coefficients of variation (CVs) were 11.2% and 4.7%, respectively (N = 45 assays).

Characterization of Diurnal Rhythm

A total of 6 plasma samples (1200h, 1600h, 2000h, 2400h, 0400h, 0800h) were collected from 8 virgin male and 7 virgin female California mice (all housed in same-sex pairs) under undisturbed conditions to characterize the diurnal pattern of circulating

CORT concentrations. At least 7 days elapsed between collection of successive samples from the same animal, and the order of sampling time points was approximately balanced across individuals within each sex.

Pharmacological Stimulation of Adrenal Activity

We used adrenocorticotropic hormone (ACTH) to stimulate the production of CORT. Synthetic ACTH (Cosyntropin, Penn Veterinary, Lancaster, PA) was diluted to 25µg/ml with sterile saline (0.9%) immediately prior to use. Injection volumes ranged from 0.13 to 0.35 ml. The volume of saline injections (vehicle control) was adjusted for body mass in the same manner as ACTH. All injections were administered i.p. using a 27G needle.

In the first ACTH challenge, male mice (n= 6 per injection condition), either housed in same-sex or male-female pairs, were injected with either 100 µg/kg ACTH or an equivalent volume of sterile saline at 0900h, and a blood sample was collected 1 h later. These mice were not used in any other experiments. The relatively high dose of ACTH was chosen to maximally or near-maximally stimulate the adrenal gland and was based off previous studies in rats (Bamber et al., 2011; Lepschy et la., 2007). We conducted a second ACTH challenge to elucidate temporal differences in the adrenocortical response to exogenous stimulation at times near either endogenous peak (evening) or nadir (morning) HPA activity. Based on response from the 100 µg/kg dose (see results), mice in this experiment received two consecutive injections of either saline or ACTH (150 µg/kg). We chose this higher dose and double-injection protocol because

we aimed to maximally stimulate the adrenal glands, and visual comparison with our stressor data (see below) the 100 µg/kg does was not sufficient to produce maximal response. In order to maintain comparable groups, we repeated the AM injections with the new protocol. All mice (n=48) used in the 150 µg/kg ACTH double-injection protocol were virgin females housed in same-sex pairs and had not been used in any prior experiments; however, 8 of them were later used in the DEX (see below) experiments. The first set of mice was injected with either ACTH (n=8) or saline (n=8) at 0915h and again at 0945h. Half of the mice from each group underwent blood sampling 30 min after the second injection (1015h; n=4 per group), and the others were sampled 60 min after the second injection (1045h; n=4 per group).

The double-injection procedure was repeated in a different set of animals 3 h prior to lights-off to determine if the response to ACTH injection changed throughout the day. A total of 32 animals were injected with ACTH (150 µg/kg) or saline at 1600h and then again at1630h. Half of the mice in each injection group (saline or ACTH; n=8 per group) underwent blood sampling at 1700h (30 min after the second injection) and the remaining animals (n=8 per group) had samples collected at 1730h (60 min after the second injection). Plasma from all ACTH challenge trials was assayed for CORT as described above.

Pharmacological Suppression of Adrenal Activity

Dexamethasone sodium phosphate (DEX, a synthetic glucocorticoid; American Regent, Inc., Shirley, NY) was injected i.p. using a 27G needle at 0.5, 5, or 10 mg/kg.

The stock solution of DEX (4 mg/ml) was diluted with sterile saline prior to injection to concentrations of 0.1 mg/ml or 1 mg/ml, or left undiluted, respectively. A total of 24 mice housed in either same-sex (12 females and 10 males) or male-female pairs (2 males) were used for the DEX injection study; reproductive conditions were balanced across dosages when possible. Sixteen of the 24 animals were used in other experiments. On day 1 all mice underwent blood sampling at 1700h for determination of baseline CORT levels, and were then weighed. On day 2 each animal received a DEX injection at one of the three doses (n=4 males and 4 females per dose; injection volume ranged from 0.09 to 0.33ml) at 0900h. Blood was collected from each animal at 1700h on day 2 (8 h post-injection), day 3 (32 h post-injection) and day 4 (56 h post-injection). Thus, each mouse underwent blood collection at each of the four time points, but was treated with only a single dose of DEX. Equipment failure occurred during processing of one male's 8 h post-injection sample for the 10mg/kg group; therefore, that animal's data were omitted from the analyses.

Physical and Environmental Stimulation of Adrenal Activity

To quantify adrenal activity in response to a variety of challenges, we exposed mice to four commonly used laboratory stressors (Jaggi et al., 2011): predator urine, forced swim, restraint, and shaking. All four stimuli were presented once in the morning at 0755-0945h (lights-on, inactive period) and once in the evening at 1930-2100h (lights-off, active period), in order to determine how the HPA response to stressors changes throughout the day. For forced swim, restraint, and shaking stress data, some mice were

used in two of the three experiments; all mice were given at least 7 days to recover between testing.

Predator-Urine Exposure

Predator urine (fox and bobcat) was purchased from Maine Outdoor Solutions (Hermon, ME). Pilot studies and previous research from our lab suggest that fox and bobcat urine both elicit a rise in plasma CORT and that response magnitude does not differ between odor types (Chauke et al., 2011; unpub. data). Odor testing is described in detail elsewhere (Chauke et al., 2011). Briefly, mice were taken to a procedure room and placed individually in clean cages identical to their home cage but containing no nesting cotton, food or water. A cotton ball soaked with 1ml predator odor was placed in a shallow plastic cup, and the cup was placed in the cage for the allotted time. The cup and urine-soaked cotton ball were then removed from the cage and from the testing room. A time-course study was conducted following 8 min of fox-urine exposure beginning at 0755h (during lights-on). Blood samples were collected from different mice (n=4 per time point; 12 females and 4 males, housed in same-sex (8 females) or male-female pairs (4 females, 4 males)) at 1, 15, 30, and 60 min following the end of urine exposure; 0800h samples from the diurnal-rhythm study (see above) were used as undisturbed control values for comparison. These mice were not used in any other experiments.

A lights-off trial was conducted following 5 min of bobcat-urine exposure beginning at 1930h. A blood sample was collected from each mouse (n = 8; 5 males (all pair-housed with females) and 3 females (pair-housed with a male (n=2) or female (n=1))

immediately following exposure to the urine; 2000h samples from the diurnal-rhythm study (see above) were used as undisturbed control values for comparison. These mice were not used in any other experiments.

Forced Swimming

Containers for swimming (1500 ml Vac-Rite beakers, Baxter, Deerfield, IL) were filled with approximately 850 ml of water (24-25°C); this volume prevented mice both from touching the bottom of the beaker and from climbing out of the top. Mice (n=4 per time point; all males, housed in either same-sex (n= 6) or male-female (n=10) pairs, housing conditions balanced across time points) were transferred to a procedure room and placed in individual swim containers for 5 min, during which they were monitored closely. After the swimming session the mice were towel-dried and either underwent blood collection immediately (1-2 min post-stress) or were placed back in their home cage, which remained in the procedure room for 30 min, after which a blood sample was collected (30 min post-stress). The lights-on session began at 0845h and the lights-off session at 2050h; different groups of mice were used for the two times of day.

Restraint

Mice (n=4 per time point; all males, housed in either same-sex (n= 5) or male-female (n=11) pairs, with housing conditions balanced across time points) were taken to a procedure room and placed in a plastic DecapiCone (MDC-200, Braintree Scientific, Braintree, MA) for 5 min. The back portion of the DecapiCone was folded around the tail

and secured with a binder clip, or was held in place manually, in order to prevent movement. Upon termination of the procedure mice either underwent blood sampling immediately (1-2 min post-stress) or were placed back in their home cage, which remained in the procedure room, for 30 min and then sampled (30 min post-stress). The restraint stressor was performed during both lights-on (beginning at 0840h) and lights-off (beginning at 2050h), and different groups of mice were used for each time of day.

Shaking

Mice (n=4 per time point; all males, housed in either same-sex (n=10) or male-female (n=6) pairs, with housing conditions balanced across time points) were placed individually in small plastic containers (approximate volume 700ml), transferred to a procedure room and shaken on a lab rotator (Unico, Dayton, NJ) at a speed of 200 rpm for 5 min. Mice were then immediately taken for blood sampling (1-2 min post-stress) or placed back in their home cage, which remained in the procedure room, until blood collection (30 min post-stress). The stressor was administered both during lights-on, beginning at 0825h, and during lights-off, beginning at 2030h; different groups of mice were used for each time of day.

Corticosterone Metabolism and Excretion

^{3}H-Corticosterone Injections

To examine the metabolism and time course of excretion of CORT in *P. californicus*, we followed the methods previously reported by Touma and colleagues (2003) for house mice (*Mus musculus domesticus*). Eight mice (4 males and 4 females; all virgin animals housed in same-sex pairs that had not been part of any other experimental procedure) were injected i.p. with ^{3}H-CORT (NET399250UC, Perkin Elmer, Waltham, MA). Each mouse received 100 µl of ^{3}H-CORT in saline, yielding a dose of 40 µCi (~37,000,000 cpm per injection). Beginning 3 h prior to injection, mice were individually housed in polycarbonate cages (44 x 24 x 20 cm) designed to facilitate collection of feces and urine. The cages did not contain bedding or cotton, and the cage bottom was lined with filter paper to absorb urine. A wire mesh grate (23G galvanized steel, 0.64 cm mesh) raised 2.5 cm above the bottom of the cage was placed on the filter paper so that fecal pellets would fall to the bottom of the cage. In order to determine if the time course of CORT metabolism and/or excretion changes over the course of the activity cycle, the 8 mice were split into two groups (n=2 males and 2 females per group): one group was injected with ^{3}H-CORT at 0600h (1 h after lights-on) and the second group was injected at 2000h (1 h after lights-off); the two groups were injected on separate days.

For both groups, all fecal pellets and the filter paper that absorbed urine were collected every 2 h for the first 24 h following injection, and then every 12 h until 84 h post-injection. Collections that occurred during the dark phase were done using a red-light headlamp in order to minimize disturbance. During sample collection each mouse was placed individually into a plastic container while all fecal pellets were gathered from the cage and filter paper was replaced. Following the return of each mouse to its cage, fecal pellets collected from the cage and the temporary holding container were counted and combined as a single time-point sample. Fecal pellets and urine samples (filter paper) were stored at -20°C until extraction and analysis. At the end of the experiment (84 h post-injection), mice were euthanized by pentobarbital overdose; thus, each mouse was used for only one injection.

Preparation and Extraction of ^{3}H-corticosterone samples

All urinary and fecal steroid extractions, high-performance liquid chromatography, and radioimmunoassays were performed at the San Diego Zoo Institute for Conservation Research (Escondido, CA). Individual sheets of filter paper containing absorbed urine samples were placed on a UV light table to allow visualization of urine spots. Spots were traced with a pen, cut from the sheet, placed into scintillation vials along with 5 ml of scintillation cocktail, and then counted in a LS 6500 liquid scintillation counter (Beckman Coulter, Brea, CA). The percentage of ^{3}H-CORT and ^{3}H-CORT metabolites excreted in urine at each time point was calculated as the total amount

of radioactivity recovered from the filter paper at each time point divided by the total radioactivity recovered in the urine across the entire 84-h collection period.

All fecal pellets collected from each mouse at a single time point were extracted and analyzed together in order to determine the total amount of ^{3}H-CORT and ^{3}H-CORT metabolites excreted in feces at each collection time point. Fecal samples were transferred to 150 x 16 mm glass tubes and extracted in 20 ml of 80% methanol by shaking for 60 min. After allowing the fecal pellets to settle to the bottom of the tube, the extract was drawn off and stored at -20°C until further analysis. Total radioactivity at each time point was determined by transferring 1 ml of fecal extract to a scintillation vial along with 5 ml of scintillation cocktail, counting on a liquid scintillation counter, and multiplying the counts per minute by 20. The percentage of ^{3}H-CORT and ^{3}H-CORT metabolites excreted in feces at each time point was calculated as the total amount of radioactivity recovered at each time point divided by the total radioactivity recovered in the feces across the entire 84-h collection period.

High-Performance Liquid Chromatography (HPLC)

In an effort to characterize the major glucocorticoid metabolites present in *P. californicus* feces, fecal extracts from the ^{3}H-CORT-injected mice were analyzed by HPLC. A total of 8 fecal extract samples were chosen that corresponded to the time points that yielded the greatest amount of recovered radioactivity: the 4-h and 14-h post-injection samples from the 2000h and 0600h injections, respectively. Fecal extracts (1 ml) were dried down, reconstituted in water, and then passed through C18 cartridges

(WAT051910 Sep-Pak, Waters, Milford, MA) that had been conditioned with 10 ml each of 100% methanol and then water. The samples were then washed with 10 ml of water, eluted from the cartridges with 5 ml of 100% methanol, dried down under vacuum, and reconstituted in 0.2 ml of 100% methanol.

Following solid-phase extraction, 20 µl of each fecal extract sample was injected into a Beckman System Gold 3-piece unit (Programmable Solvent Modules 125/406 and Diode Array Detector Module 186, Beckman Coulter) and separated on a Nova Pak C18 column (WAT086344, 3.9 x 150 mm, Waters). Samples were separated along an acetonitrile gradient beginning with 2:98 (acetonitrile:water, v/v) and increasing to 75:25 over 75 min at a flow rate of 1 ml per min. Fractions were collected at 1 min intervals; 250 µl of each fraction was added to 5 ml of scintillation cocktail and counted to determine the radioactivity of each fraction. The elution times of ^{3}H-labeled fractions were compared to the elution times of several commercially available (Steraloids, Newport, RI) steroids (corticosterone, cortisol, testosterone, estrone sulfate, and progesterone) and steroid metabolites (5β-androstane-3α-ol-11-17-dione and 5α-pregnane-3β,11β,21-triol-20-one) similarly prepared and fractionated by the same HPLC protocol.

Fecal Glucocorticoid Immunoreactivity

To determine the feasibility of noninvasive adrenal activity monitoring, we tested a fecal glucocorticoid RIA based on a commonly used (e.g., Behie et al., 2010; Cavigelli et al., 2005; Good et al., 2003; Harper and Austad, 2000; Wasser et al., 2000) and

commercially available polyclonal antibody (rabbit anti-corticosterone, 07120113, MP Biomedicals, Costa Mesa, CA). Fecal samples were collected from two female mice at 1600h by gently holding the animals by the scruff until fecal pellets were produced. The fecal samples were immediately place in 1.5 ml vials and stored at -20°C until extraction and assay. Non-radioactive fecal pellets were extracted as previously described with the following exceptions. Individual fecal pellets were lyophilized overnight and extracted in 1 ml of 80% methanol in 12 x 75 mm borosilicate tubes. Following 60 min of shaking, 0.9 ml of the extract was transferred to a clean tube, dried down under vacuum, and reconstituted in 1 ml of phosphate-buffered saline (PBS, pH 7.0). Duplicate aliquots of 100 µl were set aside from each sample for direct analysis by RIA, while the remaining extracts were combined and prepared for HPLC by solid-phase extraction as described above. Duplicate aliquots of the combined and concentrated extract were fractionated by HPLC, resulting in 75 duplicate 1 ml fractions. Duplicate HPLC fractions were dried down and reconstituted in 1 ml PBS prior to analysis by RIA.

Serially diluted standards of 0.19 – 24 ng/ml corticosterone were prepared in PBS and run in duplicate in each RIA, along with quality control samples of approximately 0.4 and 4.0 ng/ml. To reduce nonspecific binding, 0.4 ml of 0.4% bovine serum albumin (Fraction V; Fisher Scientific, Pittsburgh, PA) in PBS was added to each tube. Tritiated CORT (1,2,6,7-H^3; Perkin Elmer, Waltham, MA) was added at 10,000 cpm per 0.1 ml. Finally, CORT antiserum was added in 0.1 ml of PBS at a dilution of 1:1200 to bring the final volume to 0.7 ml. Following an overnight incubation at 4°C, bound-free separation was performed by adding 250 ml of 5% charcoal/0.5% dextran in PBS, incubating at 4°C

for 30 min, and centrifugating at 2000 x g for 15 min. The supernatant was combined with 3.5 ml of scintillation cocktail and counted on a Beckman LSC6500 scintillation counter. The concentrations of corticosterone standards were plotted against the log-logit transformation of the % ^{3}H-CORT bound. Immunoreactivity was expressed as mean pg/fraction.

Statistical Analysis

All analyses were performed using SPSS 17.0 for Windows (IBM Corporation, Armonk, NY). Data were tested for normality and were visually inspected to ensure that results were not being driven by a small number of extreme values. All plasma CORT data were $\log_{10}$-transformed to meet normality assumptions; however, data are presented as non-transformed values, in the text and the figures, for ease of interpretation. Plasma CORT data were analyzed via independent-samples t-test, Pearson's correlation, ANOVA, or repeated-measures ANOVA. Post-hoc tests following ANOVA were either Fisher's LSD (for resolution of simple main effects, and for diurnal rhythm data), or were Sidak-corrected comparisons (all other post-hoc analyses). All tests were two-tailed, and $P \leq 0.05$ was considered significant. ^{3}H-CORT results were analyzed using a mixed-model ANOVA so that animals with missing data (no fecal samples produced) could be included in the analysis. Akaike Information Criterion was used to determine which covariance structure provided the best fit (Gueorguieva and Krystall, 2004; Wang and Goonewardene, 2004); compound symmetry, correlative compound symmetry, and the first autoregressive covariance structures were tested.

RESULTS

Diurnal Rhythm in Plasma CORT Concentrations

CORT data were analyzed via repeated-measures ANOVA, and Fisher's LSD post-hoc tests were used to determine which time points differed from one another, due to our prediction that CORT would follow a daily cycle with highest levels occurring just prior to the onset of activity. Plasma CORT concentration (FIG. 1.1) varied dramatically throughout the day ($F_{5,65}$=75.840, $P<0.001$) but did not differ between the sexes ($F_{1,13}$=0.025, P=0.876), nor was there a time*sex interaction ($F_{5,65}$=0.527, P=0.755). The highest plasma CORT levels occurred shortly after lights-off (1818.01 ± 159.63 ng/ml at 2000h) and were significantly elevated compared to all other time points ($P\leq0.007$ for each post-hoc comparison), whereas the lowest CORT levels occurred at the beginning of the inactive period (39.94 ng/ml at 0400h and 41.69 ng/ml at 0800h). Plasma CORT concentration did not differ between the 0400h and 0800h samples (t_{14}=0.19, P=0.853), or between the 1600h and 2400h samples (t_{14}=1.04, P=0.320); as with 2000h samples, the 1200h time point (207.31 ± 65.18 ng/ml) was different from all other time points ($P\leq0.001$ for each post-hoc comparison).

To determine whether time elapsed from cage disturbance until sample collection had an effect on plasma CORT levels, Pearson's correlations were performed using seconds until sample collection and $\log_{10}$-transformed CORT concentration, for both the nadir (0800h) and peak (2000h) time points. Time from disturbance of cage until collection of blood sample (range: 60-168) was not significantly correlated with plasma

CORT concentration at either time point (0800h: r=0.036, P=0.898, n=15; 2000h: r=0.198, P=0.480, n=15).

Plasma CORT Response to ACTH Challenge

100ug/kg vs. saline

Injection of 100 µg/kg ACTH i.p. at 0900h significantly increased plasma CORT levels 1 h later when compared to saline injection (1255.57 ± 217.24 vs. 26.92 ± 7.03 ng/ml CORT, respectively; t_{10}=12.597, P<0.001; independent-samples t-test). Additionally, post-saline-injection CORT levels were lower than 0800h baseline values from the diurnal-rhythm study (26.92 ± 7.03 vs. 41.69 ± 3.84 ng/ml, respectively; t_{19}=2.146, P=0.045).

150ug/kg vs. saline

CORT data (FIG. 1.2A&B) were analyzed by two 2x2 ANOVAs, one for each time of day, with time post-injection (30 or 60 min) and treatment (ACTH or saline) as fixed factors.

Morning (0900h) injections

Plasma CORT levels were significantly higher at 30 min than at 60 min following the second ACTH injection ($F_{1,12}$=5.632, P=0.035). Additionally, ACTH-injected

animals had higher plasma CORT levels than did saline-injected animals ($F_{1,12}$=74.541, $P<0.001$). There was no interaction between treatment and time ($P=0.695$).

Afternoon (1600h) injections

In contrast to morning CORT levels, afternoon CORT levels did not differ significantly between ACTH- and saline-treated animals ($P=0.234$) or between the 30- and 60-minute time points post-injection ($P=0.514$). Additionally, there was no interaction between treatment and time of sampling ($P=0.877$).

Plasma CORT Response to DEX Challenge

Data were analyzed using repeated-measures ANOVA with day of sample (day 1, 2, 3, and 4) as a within-subjects factor and dose (0.5, 5, and 10 mg/kg) as a between-subjects factor (FIG. 1.3). Day of sample had a significant effect on plasma CORT ($F_{3,60}$=7.941, $P<0.001$), as did dose of DEX ($F_{2,20}$=10.554, $P=0.001$), and a day*dose interaction ($F_{6,60}$=4.279, $P=0.001$) was observed.

Within treatment groups, 0.5 mg/kg DEX did not suppress plasma CORT levels at any time point measured, as plasma CORT levels did not differ across time ($P>0.794$ for all post-hoc comparisons). A dose of 5 mg/kg DEX caused a decrease in CORT 8 h post-injection compared to baseline levels ($t=3.672$, $P=0.009$); however, CORT concentrations at 32 h and 56 h post-DEX did not differ from baseline levels ($P=0.929$ and $P=0.105$, respectively). The 10 mg/kg DEX dose suppressed CORT at 8 h post-injection when

compared to baseline (t=4.864, P=0.001), but, as seen with the 5 mg/kg dose, neither 32 h (P=0.992) nor 56 h post-DEX values differed from baseline (P=0.999).

On day 2 (8 h post-DEX) the 0.5mg/kg DEX group had higher plasma CORT compared to the 5 mg/kg DEX group (t=3.778, P=0.004) and the 10 mg/kg DEX group (t=5.303, P<0.001); CORT levels did not differ between the 5 mg/kg and 10 mg/kg DEX groups (t=1.656, P=0.303). CORT concentrations did not differ significantly among the groups on day 1 (baseline; all pairwise P values >0.8), day 3 (32 h post-DEX; P>0.6), or day 4 (56 h post-DEX; P≥0.25).

Plasma CORT Response to Predator Odor

Plasma CORT levels over time following exposure to predator odor during lights-on were analyzed using a one-way ANOVA (FIG. 1.4). Time of sample post-exposure (baseline, 1, 15, 30, or 60 min) affected plasma CORT levels ($F_{4,26}$=17.883, P<0.001). CORT concentrations were significantly elevated above baseline at each time point following predator-odor exposure (1 min: t=4.570, P=0.001; 15 min: t=7.282, P<0.001; 30 min: t=4.361, P=0.002; 60 min: t=4.175, P=0.003). Plasma CORT levels peaked at 15 min post-stress and then declined at 30 and 60 min post-stress; however, CORT levels did not differ significantly between any of the post-stress samples (P>0.186 for all post-hoc comparisons).

A different set of animals was exposed to predator odor during lights-off (~2000h). Data were analyzed via independent-samples t-test. Exposure to 1 ml of bobcat urine did not significantly elevate plasma CORT, 1-2 min post-stress, above

baseline values collected from control mice at the same time of day (1818.01 ± 159.63 vs. 2281.14 ± 271.38 ng/ml CORT, respectively; t_{21}=1.36, P=0.189). Evening post-stress CORT levels were significantly elevated compared to morning (0800h) baseline levels (independent-samples t-test; t_{21}=22.142, P<0.001) and, samples collected 1 min following evening predator-urine exposure contained higher levels of CORT than samples collected 1 min following morning predator-urine exposure (2281.14 ± 271.38 vs. 365.123 ± 177.17 ng/ml; $t_{3.32}$=4.096, P=0.022). Additionally, CORT concentrations following morning urine exposure (all time points combined, n=16) were lower than evening (2000h) baseline concentrations (504.36 ± 140.35 vs. 1818.02 ± 159.63 ng/ml; t_{29}=5.916, P<0.001). These data show that predator odor elicits a significant rise above baseline CORT concentrations in the morning, when endogenous CORT is low, but does not elevate CORT above baseline levels in the evening, when baseline levels are high. Additionally, post-exposure values in the evening were significantly higher than post-exposure values in the morning, and morning post-exposure levels did not reach evening baseline concentrations.

Plasma CORT Responses to Stressors During Lights-on and Lights-off

Plasma CORT responses to each stressor (restraint, shaker, forced swim) were compared between lights-on and lights-off using a 2 (time of day; 0800h or 2000h) x 3 (blood sample time; baseline, 1-2 min or 30 min) ANOVA; baseline values from the diurnal-rhythm data set (see section 3.1) were used for comparison (FIG. 1.5). Analysis for each stressor type revealed significant effects of blood sample time ($F_{2,40}$= 63.658,

restraint; $F_{2,40}$=56.369, shaker; $F_{2,40}$=104.306, forced swim; $P<0.001$ for each analysis), time of day ($F_{1,40}$ =24.865, restraint; $F_{1,40}$ =54.789, shaker; $F_{1,40}$ =76.297, forced swim; $P<0.001$ for each analysis), as well as an interaction between the two ($F_{2,40}$ =126.925, restraint; $F_{2,40}$ =83.562, shaker; $F_{2,40}$ =63.187, forced swim; $P<0.001$ for each analysis). Samples collected in the evening contained higher CORT concentrations than those collected in the morning ($P<0.001$ for each analysis). The results of the interaction between time of day and blood sample time for each stressor are presented below.

Forced Swim

Morning post-stress CORT levels were higher than morning baseline values (1-2 min vs. baseline, t=12.48, $P<0.001$; 30 min vs. baseline, t=15.45, $P<0.001$). CORT levels did not differ significantly between the two morning post-stress time points, but tended to be higher 30 min post–stress than 1-2 min post-stress (P=0.068). In the evening, CORT levels were significantly elevated above baseline at 1-2 min (t=2.83, P=0.022) but not at 30 min post-stress (P=0.666); CORT concentrations did not differ between the two post-stress time points (P=0.412). Samples taken 1-2 min following forced swim in the evening contained more CORT than those taken 1-2 min following forced swim in the morning (t=3.09, P=0.004); however, there was no difference between plasma CORT levels 30 min following forced swim in the morning and evening (P=0.491).

To determine whether CORT levels following evening post-swim were indicative of maximal CORT output, we compared CORT concentrations from 1-2 min post-evening swim to those obtained 30 min following the second AM 150μg/kg ACTH

injection, as this was a very high dose of ACTH and maximal adrenal output may have been achieved. CORT levels 1-2 min post-swim in the evening were higher than those obtained by pharmacological manipulation (3729.27 ± 370.51 vs. 2625.62 ± 193.63, respectively; independent-samples t-test; t_6=2.859, P=0.029).

Restraint

In the morning, CORT was elevated above baseline at both 1-2 (t=15.28, P<0.001) and 30 min post-restraint (t=13.70, P<0.001), but did not differ between the two post-stress times (t=1.25, P=0.521). In the evening, CORT levels 1-2 min post-restraint did not differ from baseline (P=0.999). Unexpectedly, CORT levels 30 min after restraint were lower than CORT levels both at the evening baseline (t=5.84, P<0.001) and at 1-2 min post-stress (t=4.65, P<0.001). CORT levels 30 min following restraint were lower in the evening than in the morning (t=3.79, P<0.001), whereas plasma CORT levels 1-2 min following restraint did not differ between the morning and evening (P=0.693).

Shaking

In the morning, plasma CORT concentrations differed at all three time points (baseline, 1-2 min- and 30 min-post stress), with post-stress values being higher than baseline (baseline vs. 1-2 min: t=15.01, P<0.001; baseline vs. 30 min: t=8.52, P<0.001) and 1-2 min post-stress levels being higher than 30 min post-stress (1-2 min vs. 30 min: t=5.16, P<0.001). In the evening, CORT levels 1-2 min post-stress did not differ from baseline (P=0.999), but CORT levels 30 min post-stress were significantly lower than

both baseline ($t=4.84$, $P<0.001$) and 1-2 min post-stress levels ($t=3.84$, $P=0.001$). CORT levels 1-2 min ($P=0.484$) and 30 min ($P=0.546$) following shaking did not differ between the morning and evening.

Route of ^{3}H-labelled Corticosterone Excretion

Route-of-excretion data were analyzed using a multivariate ANOVA with sex and time of injection (0600h or 2000h) as fixed factors, and total proportion of injected radioactivity excreted, proportion of excreted radioactivity present in fecal samples, and proportion of excreted radioactivity present in urine samples as dependent variables. Sex did not affect any of these measures ($P>0.276$ for all measures), so data from males and females were pooled. Animals injected at 0600h excreted a lower proportion of total injected radioactivity than did mice injected at 2000h (0.160 ± 0.005 vs. 0.211 ± 0.008; $F_{1,6}=28.955$, $P=0.002$). The proportion of injected radiation excreted in feces (0600h vs 2000h: 0.599 ± 0.064 vs. 0.557 ± 0.053; $F_{1,6}=0.254$, $P=0.632$) or urine (0600h vs 2000h: 0.401 ± 0.064 vs. 0.443 ± 0.053; $F_{1,6}=0.254$, $P=0.632$) did not differ based on injection time. Paired-samples t-tests revealed that the proportion of excreted radioactivity contained in feces and in urine did not differ following ^{3}H-CORT injection at either 0600h (0.599 vs. 0.401, respectively; $t_3=1.536$, $P=0.222$) or 2000h (0.526 vs. 0.443, respectively; $t_3=1.075$, $P=0.361$).

^{3}H-labelled CORT Excretion Time Course

The proportion of total excreted radiation in either feces or urine was calculated for each time point and was analyzed using two separate mixed-model ANOVAs (one for each form of excreta) to account for missing data points (which occurred when no fecal or urine samples were excreted in a given collection period). The covariance structure used for the analysis of fecal CORT was compound symmetry, and the first-order autoregressive structure was used for urine data. Time of injection (0600h or 2000h) did not affect the total proportion of radioactivity excreted in urine ($F_{1,57.55}$<0.01, P=0.999) or feces ($F_{1,2.89}$=0.944, P=0.405). Time of collection post-injection affected urinary radioactivity (Supplemental Table A; $F_{16,27.17}$=52.512, P<0.001) as well as fecal radioactivity (Supplemental Table B; $F_{16,77.47}$=6.747, P<0.001). When injection was administered at 2000h, a greater amount of urinary radioactivity was excreted at 2 h post-injection than at any other time point; when injection occurred at 0600h, however, urinary radioactivity did not differ between the 2 and 4 h post-injection time points, but radioactivity at both of these times did differ from all other time points (Supplemental Table A). Excretion of fecal radioactivity was most prominent in the first 8 h following injection at 2000h, but showed a more bimodal pattern of excretion when injection of radioactivity occurred at 0600h, with peaks at both 2-4 h and 14-16 h post-injection (Supplemental Table B). Additionally, the interaction between time of injection and time of collection was significant for both fecal ($F_{16,77.474}$=5.00, P<0.001) and urine ($F_{16, 27.174}$=2.895, P=0.007) samples; those results are discussed below.

Animals injected with ^{3}H-CORT at 2000h excreted a higher proportion of fecal radioactivity in the first 2 h post-injection ($t=4.81$, $P<0.001$), but excreted a lower proportion of fecal radioactivity at 14 h ($t=6.06$, $P<0.001$) and 16 h ($t=2.86$, $P=0.005$) post-injection, when compared to animals injected at 0600h (FIG. 1.6). Proportion of fecal radioactivity excreted did not differ between the two groups at any other time point.

For urine samples, animals injected at 2000h excreted a higher proportion of urinary radioactivity in the first 2 h post-injection ($t=5.45$, $P<0.001$), but a lower proportion of urinary radioactivity 4 h post-injection ($t=2.62$, $P=0.014$; FIG. 1.7), when compared to animals injected at 0600h. Proportion of urinary radioactivity did not differ between the two groups at any other time points.

HPLC Results

Elution Time of Known Hormones and Radioactive Fractions

Fecal extract containing the highest proportion of radioactivity from mice injected with ^{3}H-CORT at 0600h (14 h post-injection) and 2000h (4 h post-injection) were separated by HPLC. The resulting 75 HPLC fractions were analyzed for presence of radioactivity; radioactivity in each HPLC fraction was divided by the total radioactivity per sample, and results are displayed as average percentage of radioactivity per fraction (FIG. 1.8). Five of the most radioactive fractions from the 4 h post-injection samples eluted during or after fraction 18, whereas the 5 most radioactive fractions from the 14 h post-injection samples eluted prior to or during fraction 18 (Table 1.1). The majority of

radioactivity from both injection times (67% and 78% for the 4 h and 14 h, respectively) eluted prior to fraction 31 which is the elution time for pure CORT (FIG. 1.8). No two mice displayed the exact same ranking of the top five radioactivity containing fractions, and no single fraction fell in the top five for all mice (data not shown).

Presence of Immunoreactive Fractions

HPLC-partitioned fecal extract from unmanipulated California mice contained several fractions that reacted with the MP Biomedicals antibody; however, none of the major immunoreactive peaks corresponded to the 5 most radioactive fractions following ^{3}H-CORT injections (FIG. 1.8, Table 1.1). The two highest values for immunoreactivity from unmanipulated fecal extract fractions corresponded to elution times of known hormones (estrone sulfate and progesterone), but the concentration and cross-reactivity or the MP antibody for these steroids was not investigated.

DISCUSSION

Over the course of this study we characterized plasma CORT concentrations in *P. californicus* 1) under baseline conditions across the diurnal cycle, 2) in response to pharmacological manipulation of the HPA axis (ACTH and dexamethasone challenges), and 3) in response to four different stressors at different times of day. In addition, we described steroid hormone metabolism and excretion time course in these mice. Our results suggest that the HPA axis of California mice exhibits several unusual features, including 1) extremely pronounced changes in circulating CORT levels from trough to

peak of the diurnal cycle, 2) high diurnal peak CORT levels that were only surpassed following one of the applied stressors (but several stressors produced CORT levels higher than diurnal trough), 3) reduced DEX-induced negative feedback (as compared to most other rodents that have been studied), 4) decreased response to exogenous ACTH near the diurnal peak of the endogenous CORT rhythm, and 5) a decrease, as opposed to an increase, in circulating CORT concentrations following several stressors near the time of the diurnal peak.

Diurnal Rhythm in Plasma Corticosterone Levels

One of our most striking findings was the high concentrations of baseline CORT experienced throughout the day by California mice. The range of basal CORT values observed in this study, ~40-1800 ng/ml, is markedly higher than those in many other species, including several laboratory rodents and other organisms (e.g., house mice ~4-65 ng/ml, Malisch et al., 2008; Sprague-Dawley rats ~50-360 ng/ml, Thanos et al., 2009; Gambel's white-crowned sparrows ~6-18 ng/ml, Breuner et al., 1999; marine iguanas ~2-8 ng/ml, Woodley et al., 2003; for review on birds see Bókony et al., 2009). It should be noted, however, that prairie voles also have high circulating baseline glucocorticoid levels (~475-1100 ng/ml; Taymans et al., 1997), as do guinea pigs (~100-400 ng/ml; Kapoor and Matthews, 2005) and some New World primates and human lineages (Chrousos et al., 1982, 1986, 1993; Coe et al., 1992; Saltzman et al., 1998; Yamamoto et al., 1977), so California mice are not entirely unique in this regard. Baseline CORT values from other studies of California mice (Davis and Marler, 2003; Glasper and

DeVries, 2005; Oyegbile and Marler, 2006) are consistent with values obtained here, and the assay that we used has been validated specifically for use in this species (Chauke et al., 2011), indicating that the high CORT values observed in this study are not artifacts of our methodology.

California mice also show a very dynamic diurnal CORT profile, with plasma CORT concentrations increasing approximately 45-fold from trough to peak (inactive to active periods). For comparison, Sprague Dawley rats experience diurnal changes in CORT concentrations on the order of 8.5-fold (Thanos et al., 2009), while Wistar rats experience a change of approximately 20-fold in males and 7.3-fold in estrous females (Atkinson and Waddell, 1997). House mice show about a 5-fold change from trough to peak (Dalm et al., 2005), marine iguanas show an approximate 4-fold change (Woodley et al., 2003) and White-crowned sparrows experience about a 3-fold change over the course of the day (Breuner et al., 1999). How differences in fold increase over the diurnal cycle relate to differences, if any, in glucocorticoid function is not known.

In our study, peak plasma CORT levels occurred around the onset of the active period (2000h, 1 h after lights-off), and the lowest values were obtained during the inactive period, consistent with HPA function in other species (Dallman et al., 1987; for an exception see Breuner et al., 1999). Surprisingly, we found no differences in CORT levels between the sexes; in other species, females have generally been found to have elevated CORT concentrations compared to males, due to interactions between sex hormones and the HPA axis (Atkinson and Waddell, 1997; Handa et al., 1994; Kudielka and Kirschbaum, 2005; Seale et al., 2004; Viau, 2002).

Plasma CORT Responses to Adrenocorticotropic Hormone (ACTH) Injection

In an attempt to maximize adrenal response we chose a dose of ACTH that has been used in rats (100 μg/kg; Bamberg et al., 2001; Lepschy et al., 2007) but is typically higher than doses used in other species (2 μg/kg in guinea pigs, Kapoor and Matthews, 2005; Lui and Matthews, 1999; 0.0075 or 0.015 μg (total) in rats, Dallman et al., 1987; 10 μg/kg in marmosets, Saltzman et al., 2000). ACTH at a dose of 100 μg/kg produced a marked increase in plasma CORT 1 h following a morning (0900h) injection, when compared to saline injection. A CORT increase within this time frame post-injection is consistent with reports from other species. Additionally, we found that CORT levels 1 h following saline injection were significantly lower than those obtained under baseline conditions, suggesting that our handling and injection procedures elicit, at most, a very transient CORT elevation.

As with a single dose of ACTH, two injections, separated by 30 min, of a higher ACTH dose (150 μg/kg), in the morning (0900h) elicited a robust CORT elevation at both 30 and 60 min following the second injection. Notably, even two consecutive injections of 150 μg/kg ACTH did not produce maximal CORT output, as CORT values following evening forced-swim were significantly higher than those following morning ACTH injection.

Surprisingly, when the double-injection paradigm was repeated in the afternoon (injections at 1600h and 1630h), plasma CORT concentrations did not differ between ACTH-injected and saline-injected animals at either 30 or 60 min after the second injection. This does not seem to be a result of the adrenals being maximally stimulated, as

both morning ACTH injections and other stressors resulted in higher CORT values than those achieved following afternoon ACTH injection (visual comparison of the data). These results suggest that adrenal responsiveness to ACTH changes across the diurnal cycle in California mice. One interpretation is that when baseline CORT concentrations are increasing around the time of the diurnal peak (evening), the adrenal gland cannot increase CORT output when stimulated with exogenous ACTH, whereas when basal CORT concentrations are low (morning) ACTH injection stimulates the adrenals and increases CORT concentration.

Differential responses of the adrenal glands based on time of day have also been noted in other species (reviewed by Bornstein et al., 2008; Kalsbeek et al., 2012). Two studies found that rats showed greater CORT responses to ACTH administered near the peak of endogenous CORT production (evening) as compared to the trough (morning; Dallman et al,. 1978; Kaneko et al., 1981). Splanchnic innervation of the adrenal gland is thought to partly mediate the diurnal rhythm of CORT secretion in rats by increasing adrenal sensitivity to ACTH around the time of the diurnal peak (Ulrich-Lai et al., 2003) and/or by decreasing adrenal sensitivity to ACTH during the diurnal trough (Jasper and Engeland, 1997). In contrast, California mice are more responsive to ACTH near the trough, not the peak, of the diurnal CORT rhythm. The mechanism underlying the change in adrenal responsiveness to ACTH across the diurnal cycle in California mice is unknown, but further studies investigating splanchnic innervation, adrenal ACTH-receptor binding, adrenal ACTH receptor density, and intra-adrenal CORT synthesis would be illuminating.

Plasma CORT Responses to Dexamethasone (DEX) Injection

DEX is commonly administered to test the responsiveness of the HPA axis to negative feedback: the longer CORT remains suppressed following DEX treatment, or the lower the DEX dose that suppresses CORT secretion, the more sensitive the axis. DEX acts primarily at the anterior pituitary, as it does not readily cross the blood-brain barrier (Cole et al., 2000); therefore effects are expected to be driven primarily by a decreased synthesis or secretion of ACTH, leading to reduced CORT production. Due to the high levels of endogenous circulating CORT in this species, we tested the response to a range of DEX doses (0.5, 5 and 10 mg/kg). Both the 5 and 10 mg/kg doses suppressed CORT 8 h following injection, whereas the 0.5 mg/kg dose did not; no dose suppressed CORT until the 32 h mark. In comparison, 0.1 mg/kg is sufficient to suppress CORT for 12 h in rats (Taymans et al., 1997), and roughly 0.02 mg/kg suppresses cortisol for at least 9 h in humans (reviewed by Seeman and Robbins, 1994). The finding that 5 mg/kg of DEX was needed to suppress baseline CORT activity 8 h post-injection, and that even a 10 mg/kg dose could not suppress CORT to 32 h post-injection, suggests that California mice might be somewhat glucocorticoid-resistant at the level of the pituitary. Alternatively, it is possible that DEX did suppress ACTH release in our animals, but that decreased ACTH levels did not translate to an observable decrease in plasma CORT due a non-linear relationship between ACTH and CORT (as may be suggested from the ACTH-injection data, see 3.2). Another possibility is that California mice might clear DEX very rapidly, thus preventing long-term CORT changes. We did not analyze

pituitary GC receptor affinity or binding dynamics, nor did we measure plasma ACTH levels or DEX clearance in this study, but characterizing these factors would be an important future step to further elucidating negative feedback in the HPA axis of California mice.

Effects of Experimental Stressors on Plasma CORT

Predator Odor

Exposure to predator urine in the morning resulted in a significant elevation of plasma CORT when compared to time-matched baseline samples, consistent with previous data from our lab (Chauke et al., 2011). When comparing CORT concentrations at different times post-exposure (1, 15, 30 or 60 min), all values were elevated above baseline but no one time point was significantly higher than any other. These results suggest that CORT increased rapidly and then was slow to clear from the system, or that the stressor elicited a prolonged increase in CORT secretion, even beyond the period of acute exposure. Additionally, morning post-stress CORT concentrations were not significantly higher than evening baseline values, so despite the post-urine-exposure rise, achieved levels were not higher than concentrations that occur on a daily basis.

When mice were exposed to predator odor in the evening and blood sampled 1 min later, no difference between post-stress and time-matched baseline CORT concentrations was observed. However, post-urine-exposure CORT levels in the evening were significantly higher than post-urine levels in the morning, and were also

significantly higher than morning baseline levels. The differential responses seen across the day in our mice could be mediated by inputs to the HPA axis, as it is possible that mice do not perceive the odor stimulus in the same manner. In rats, for example, olfactory sensitivity to both fox urine and mineral oil, measured by fos activation in several olfactory-related brain regions (e.g. main olfactory bulb, primary olfactory cortex) is higher in the evening than in the morning (Funk and Amir, 2000). On the other hand, it is possible that predator odor is perceived identically at both time points, but that the high levels of circulating CORT already present in the blood stream during the evening result in mechanistic constraints at some level of the HPA axis. In addition to plasma CORT, other HPA hormones (e.g. corticotropin-releasing hormone) are important in behavioral and physiological responses to stressors, and measurement of these hormones would therefore be valuable to determine if the upstream, intracerebral response to stress differs throughout the day. Unfortunately, we cannot be certain that using a different predator odor in the morning vs. evening (bobcat vs. fox, respectively) did not account for the differential CORT response, although data from our lab suggest that California mice show similar CORT responses to urine from several different predators (Chauke et al., 2011, unpub. data).

Morning vs. Evening Stress Tests

It is clear from the double ACTH-injection studies and the predator-odor trials that time of day, at least broadly defined as morning vs. evening, markedly affects the plasma CORT response to both pharmacological and psychological stimulation in

California mice. The difference in CORT response to acute stress based on time of day is not new, as several studies on rats have reported similar findings (Retana-Márquez et al., 2003; Verma et al., 2010). To investigate this phenomenon further in California mice, we used three common lab stressors to determine if CORT responses to these stressors differ from baseline levels in the morning and in the evening, and if any of these stressors elevate plasma CORT above peak baseline concentrations found across the diurnal cycle.

When mice were stressed in the morning (0800h), each of the three stressors significantly elevated plasma CORT levels above morning baseline at both 1-2 min and 30-min post-stressor, as expected. When stressors were presented in the evening (2000h), only forced swimming elevated CORT above the corresponding evening baseline value, and only at 1-2 min post-stress. Thus, plasma CORT concentrations can be elevated above baseline levels occurring at the peak of the diurnal rhythm in response to at least this one stressor, indicating that the diurnal peak is not maximal CORT output for this species. Therefore, forced swimming, which is both a psychological and a physiological stressor with a thermoregulatory component (water was 24-25° C), appears to affect the HPA axis differently than restraint or shaking, which are predominantly psychological stressors. Similar results have been found in rats, in that stressors with a thermoregulatory component – i.e., forced-swim (Verma et al., 2010) or immersion in cold water (Retana-Márquez et al., 2003) – result in elevated CORT near both the peak and trough of the diurnal CORT rhythm, whereas open-field testing (Verma et al., 2010) and foot-shock or restraint (Retana-Márquez et al., 2003) produced CORT responses only near the diurnal trough.

In our study, CORT concentrations in evening plasma samples collected 1-2-min after restraint or shaking were not different from baseline at that time of day, and surprisingly, values 30 min after stress were lower than evening baseline levels. Thus, two stimuli that elicit a conventional stress response – i.e., an elevation in CORT concentrations – in the morning elicit a decrease in CORT levels in the evening. The reason for the rapid decrease following either restraint or shaking is unknown, but these findings could suggest an increase in CORT catabolism following certain stressors during the active period. Studies in rats have reported half-lives of injected CORT between 10 and 35 min (White et al., 1989; Windle et al., 1998; Woodward et al., 1991). Therefore, increased metabolism might account for the reduced plasma CORT levels observed 30 min post-stress, but probably cannot explain the absence of difference from baseline in the 1-2 min post-stress samples. Data from our ^{3}H-CORT injections show that CORT is cleared more rapidly during the active period when compared to the inactive period, which is in line with the above prediction. Additional data on CORT metabolism and clearance from the plasma would be informative.

Metabolism and Excretion of ^{3}H-CORT

Unfortunately, we were not able to successfully validate a fecal CORT assay for California mice. When comparing the five most prevalent immunoreactive fractions to the 5 most common radioactive fractions, no overlap between the two was observed. These data indicate that the MP Biomedicals antibody was not able to bind the most prevalent metabolites of CORT present in feces and therefore could not provide reliable,

biologically relevant data for this species. These results highlight the importance of thorough validation prior to use of a fecal assay. Our data do, however, provide valuable information on the time course and mode of excretion of CORT and its metabolites. Circulating CORT is excreted in urine and feces in almost equal proportions, which suggests that measuring CORT or its metabolites in feces is a plausible method for detection in this species. Additionally, the time course of excretion is dependent on when the elevation of plasma CORT occurred. CORT excretion in feces occurred rapidly (within approximately 2-6 h) when an injection was given during the active phase (lights-off), but occurred more slowly (4-6 and 14-16 h), and did not peak until the 14-16 h mark, following injection during the inactive phase (lights-on). These results are consistent with CORT excretion times from laboratory house mice, as time frames of 4-6 h vs. 8-12 h after radiolabelled CORT injection during lights-off or lights-on, respectively, were observed (Touma et al., 2003). However, other species have been shown to differ greatly in their temporal patterns of CORT excretion (see Wasser et al., 2000). The activity cycle appeared to be at least partially driving a portion of CORT metabolism in our mice, as mice expelled large amounts of radiation in feces around a specific time of day (2200h), regardless of injection time. However, this activity-related pattern was not observed in urinary excretion of radioactivity. Knowing the time course of hormone excretion is critical for collection of accurate post-stress fecal hormone data.

In conclusion, understanding differences in HPA dynamics – including changes in baseline circulating CORT concentrations, stress reactivity, adrenal responsiveness to ACTH, as well as metabolism, excretion, and clearance of CORT – over the diurnal cycle

will contribute to interpretation of post-stress hormone values in this species. Specifically, having thorough baseline and post-stress data from different times of day will allow us to better understand the biological consequences of elevated CORT in different scenarios. Obtaining species-specific data on HPA function is imperative for proper interpretation of results as we have shown that California mice differ markedly from rats and house mice in several aspects of HPA function, despite the fact they are all rodents. We hope the data presented in this paper will aid in interpretation of CORT concentrations in this species and will allow for more thorough interpretation of interactions between the HPA axis and reproductive behavior.

Table 1.1. The HPLC elution times of the 5 most prevalent ^{3}H-CORT metabolites in California mouse fecal extracts and the elution times of the five most immunoreactive fractions as determined by CORT radioimmunoassay (RIA). Percentages of total radioactivity or immunoreactivity for each fraction are given in parentheses. Fecal extracts were prepared from samples collected either 4 h or 14 h after ^{3}H-CORT injections at 2000 h or 0600 h, respectively.

	Average rank in order of radioactivity (4h and 14 h) or immunoreactivity (RIA)				
	1	**2**	**3**	**4**	**5**
4h	33 (6.50%)	24 (5.55%)	18 (4.75%)	21 (4.53%)	20 (4.37%)
14h	18 (5.17%)	17 (5.13%)	15 (5.06%)	2 (4.95%)	14 (4.89%)
RIA	50 (3.66%)	40 (3.36%)	41 (2.79%)	34 (2.70%)	56 (2.67%)

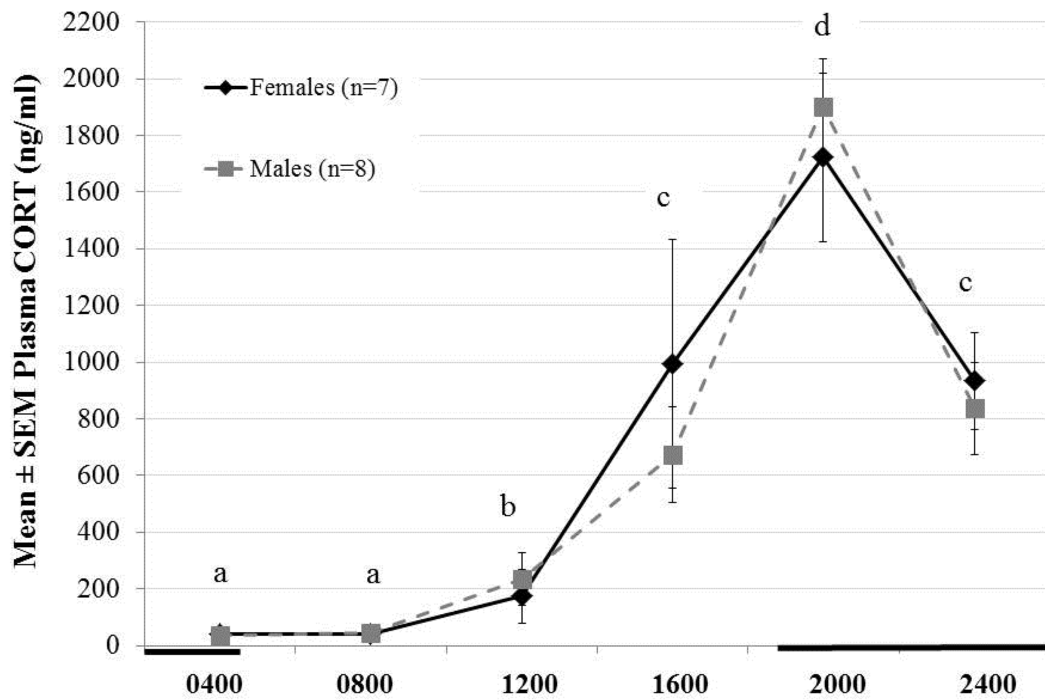

Figure 1.1: Diurnal rhythm of plasma CORT concentrations in adult, same-sex-housed, virgin male (n=8) and virgin female (n=7) California mice. Data were log_{10}-transformed for analysis but are presented as non-transformed values for ease of interpretation. CORT levels changed across time but did not differ between the sexes; main effect of time for both sexes combined is displayed on the graph. Points with different letters differ significantly from one another (P<0.007). Horizontal black bars correspond to lights-off.

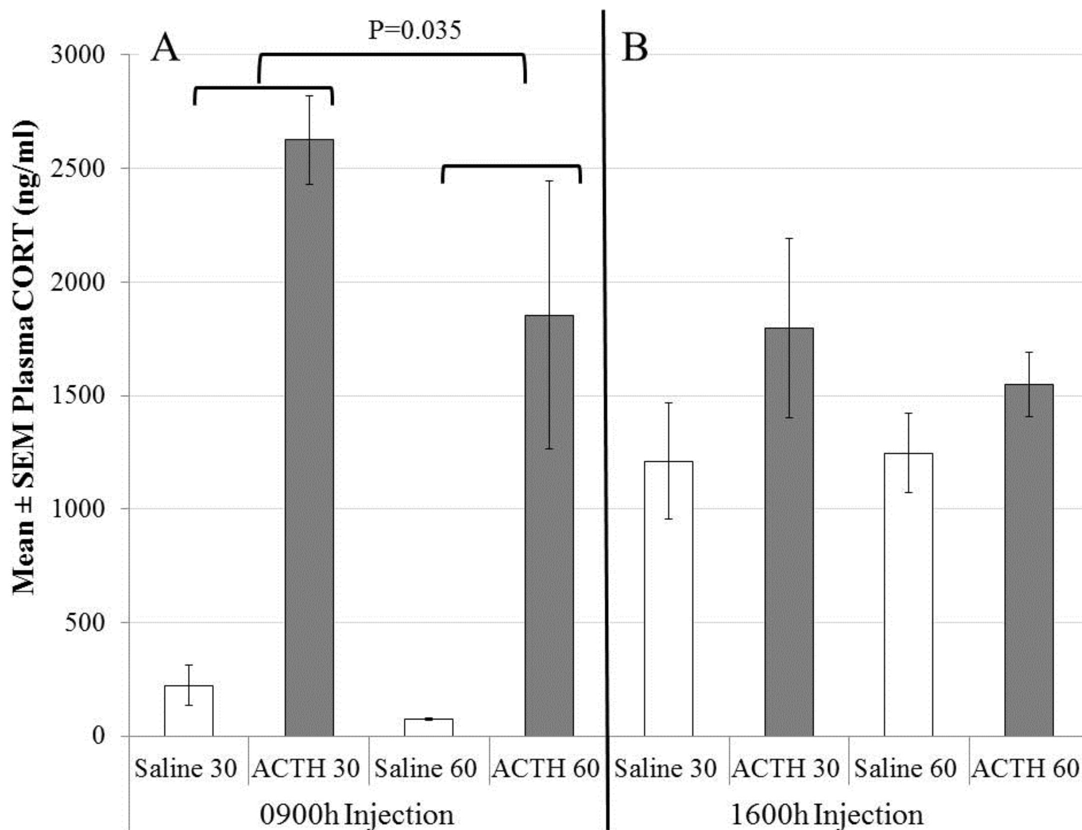

Figure 1.2A&B: Plasma CORT concentrations of adult, virgin, same-sex-housed female California mice at 30 or 60 min following the second of two successive injections of ACTH (150ug/kg) or saline administered at either 0900h and 0930h (A; n=4 per time point) or 1600h and 1630h (B; n=8 per time point) (lights-on: 0500-1900h). Data were $\log_{10}$-transformed prior to analysis, but non-transformed data are presented for ease of interpretation. Injection of ACTH in the morning (A) significantly elevated plasma CORT levels at both 30 and 60 min compared to saline injection ($p<0.001$), and CORT levels 30 min post-injection were higher than those 60 min post-injection ($P=0.035$). ACTH injection did not produce a significant increase in CORT when administered in the afternoon (B).

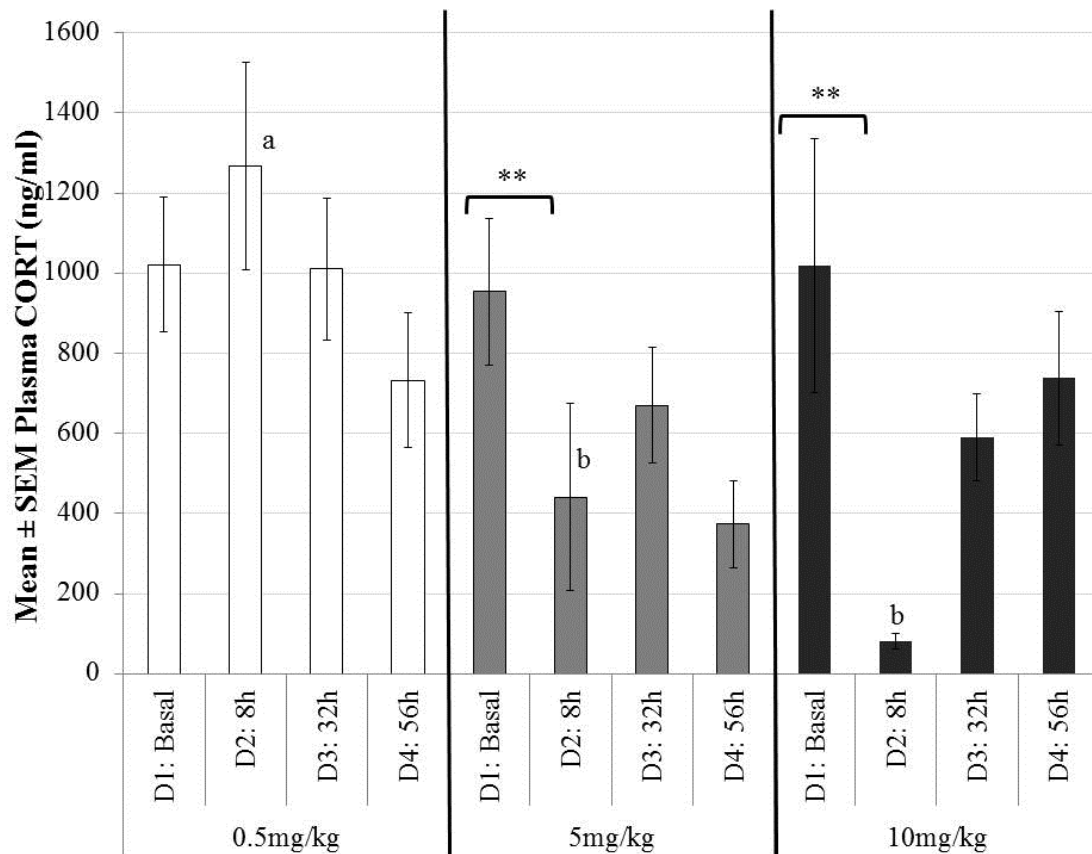

Figure 1.3: Plasma CORT concentrations at 1900h of adult male and female California mice at baseline (D1), and 8 (D2), 32 (D3) and 56 (D4) hours after DEX injection (0.5, 5 or 10 mg/kg) at 0900h on D2. Data were $\log_{10}$-transformed for analysis, but non-transformed values are presented for ease of interpretation; CORT data from males and females are pooled (4 males, 4 females per dose except for the 10 mg/kg which had 3 males and 4 females). Both the 5 mg/kg and 10mg/kg doses of DEX suppressed plasma CORT at 8h after injection as compared to baseline, but not at 32h or 56h post-injection. CORT levels 8h post-DEX did not differ between the 5 and 10mg/kg doses, but both were significantly lower than CORT values 8h after the 0.5mg/kg dose (a vs. b; $P<0.004$ after Sidak-corrected comparison). CORT concentrations did not differ among the three groups on D1, D3, or D4. **$P<0.01$ following Sidak-corrected comparisons after repeated-measures ANOVA.

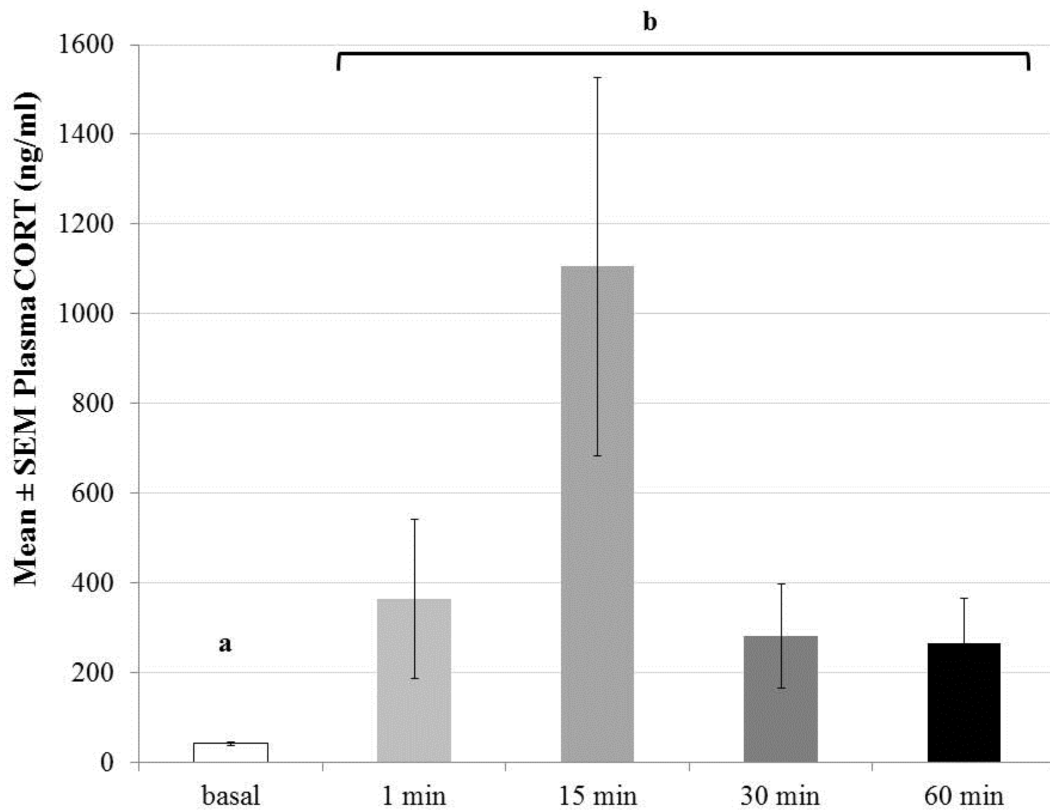

Figure 1.4: Plasma CORT response of adult male and female California mice to predator odor exposure at 0800h. Data for each post-stress time point comprise values from 4 individual mice, and sexes were pooled; basal values are from the diurnal-rhythm data set. Data were $\log_{10}$-transformed prior to analysis, but non-transformed values are presented for ease of interpretation. CORT concentrations did not differ across post-stress time points, but all post-stress values (b) were higher than baseline levels (a). Baseline vs. 1 min, P=0.001; baseline vs. 15, min P<0.001; baseline vs. 30 min, P=0.002; baseline vs. 30 min, P=0.003.

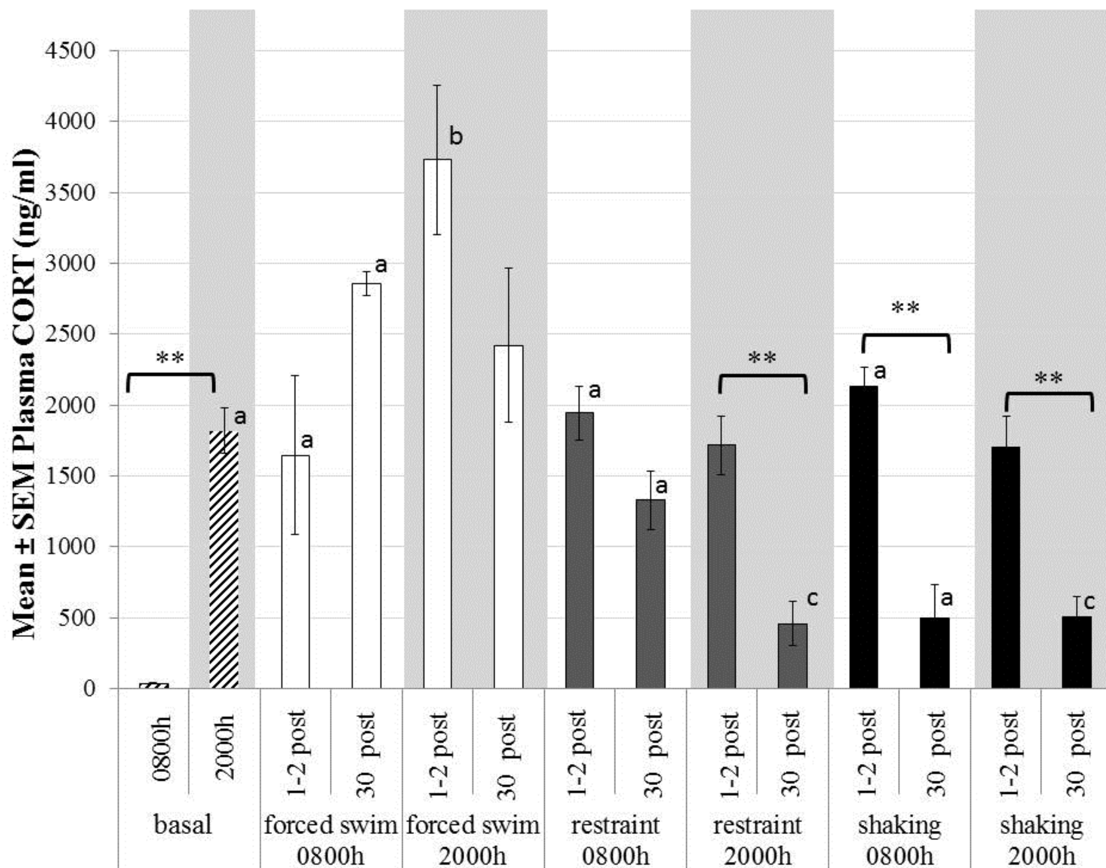

Figure 1.5: Plasma CORT concentrations of adult male California mice (n=4 per sample time) 1-2 and 30 min following exposure to a forced-swim, restraint, or shaking stressor at either 0800h or 2000h. Basal levels from the diurnal-rhythm study were used for comparison. Data were $\log_{10}$-transformed and analyzed via three 2x3 ANOVAs; however, non-transformed data are presented for ease of interpretation. Vertical gray bars signify lights-off time periods. Several post-stress means were significantly higher than 0800h baseline levels (denoted by a), only one post-stress sample time point was higher than 2000h baseline levels (denoted by b), and two post-stress samples were lower than baseline at the same time of day (denoted by c). Baseline CORT levels at 0800h were lower than baseline levels at 2000h; CORT levels decreased rapidly following shaking at both times of day, and following restraint at 2000h. $^{**}P<0.01$ following Sidak-corrected comparisons. In addition to differences shown on the graph, samples taken 1-2 min post forced-swim at 2000h contained more CORT than did samples collected 1-2 min post forced-swim at 0800h; and samples collected 30 min post-restraint at 0800h had higher corticosterone than did samples from 2000h.

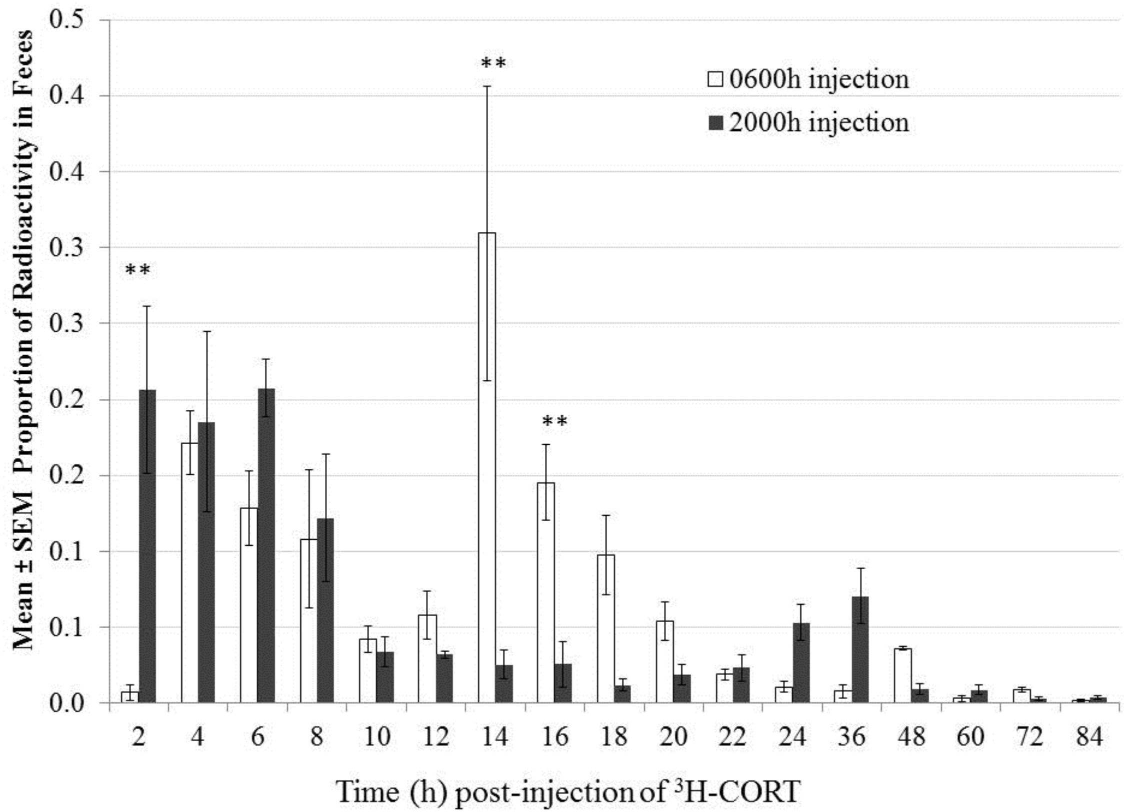

Figure 1.6: Mean proportion of total fecal radioactivity excreted by adult male and female California mice (4 male, 4 female; half of which were injected at 2000h and the other half at 0600h) per time point; data from the sexes were pooled. Animals injected at 2000h excreted more radioactivity 2h post-injection but less radioactivity at 14 h and 16 h post-injection, compared to animals injected at 0600h. **P<0.01, *P<0.05

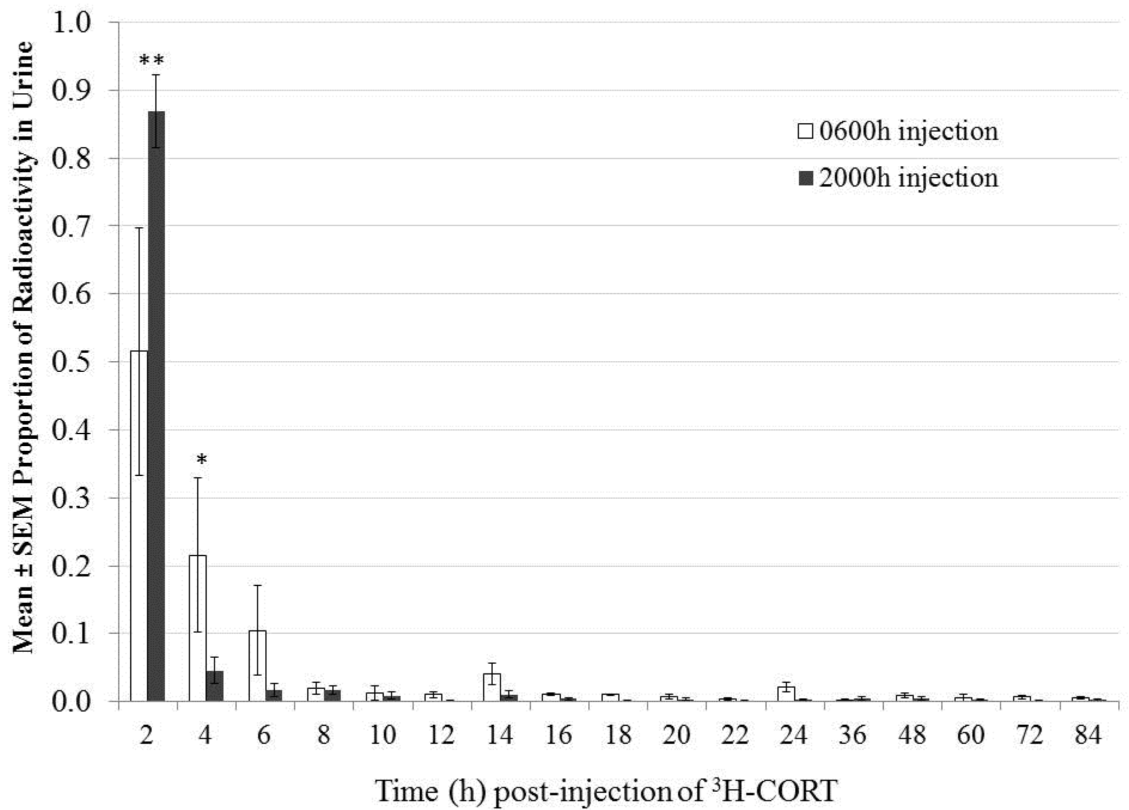

Figure 1.7: Mean proportion of total urinary radioactivity excreted by adult male and female California mice (4 male and 4 female; half of which were injected at 2000h and the other half at 0600h) per time point; data from the sexes were pooled. Animals injected at 2000h excreted more radioactivity 2h post-injection but less radioactivity at 4 h post-injection, compared to animals injected at 0600h. **P<0.01, *P<0.05

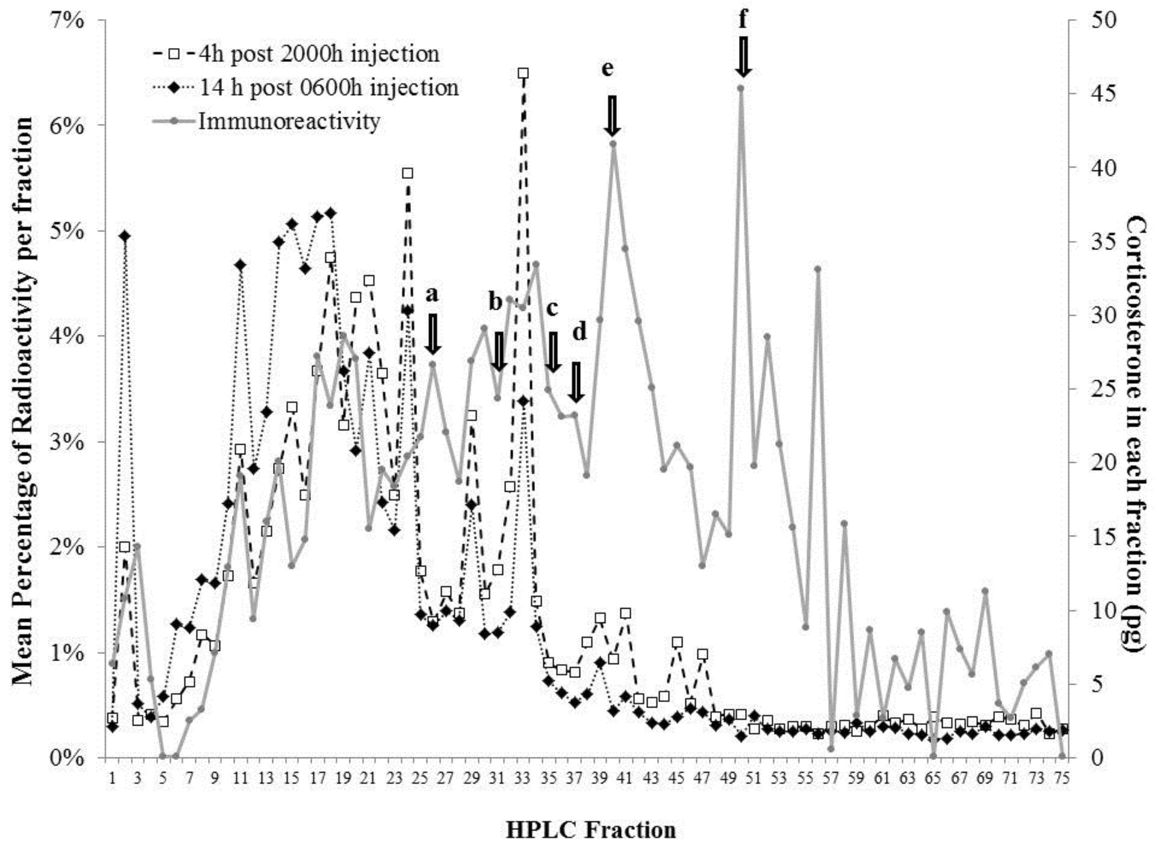

Figure 1.8: Immunochromatogram displaying percent of radioactivity contained in each post-HPLC fecal extract, and the immunoreactivity of each fraction (displayed as amount of CORT), following injection of ^{3}H-CORT. The identity of the most radioactive fractions differed between the 4h and 14h post-injection samples. Additionally, none of the most radioactive fractions were the same as the most immunoreactive. Arrows represent elution times for known hormones (cortisol, a; corticosterone, b; 5α-pregnane-3β,11β,21-triol-20-one, b; 5β-androstane-3α-ol-11-17-dione, c; testosterone, d; estrone sulfate, e; progesterone, f).

Chapter 2

Effects of aging on hypothalamic-pituitary-adrenal (HPA) axis activity and reactivity in virgin male and female California mice (*Peromyscus californicus*).

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ABSTRACT

Life history theory posits that organisms face a trade-off between current and future reproductive attempts. The physiological mechanisms mediating such trade-offs are still largely unknown, but glucocorticoid hormones are likely candidates as elevated, post-stress glucocorticoid levels have been shown to suppress both reproductive physiology and reproductive behavior. Aged individuals have a decreasing window in which to reproduce, and are thus predicted to invest more heavily in current as opposed to future reproduction. Therefore, if glucocorticoids are important in mediating the trade-off between current and future reproduction, aged animals are expected to show decreased hypothalamic-pituitary-adrenal (HPA) axis responses to stressors and to stimulation by corticotropin-releasing hormone (CRH), and enhanced responses to glucocorticoid negative feedback, as compared to younger animals. We tested this hypothesis in the monogamous, biparental California mouse by comparing baseline and post-stress corticosterone levels, as well as corticosterone responses to dexamethasone (DEX) and CRH injections, between old (~18-20 months) and young (~4 months) virgin adults of both sexes. We also measured gonadal and uterine masses as a proxy for investment in potential current reproductive effort. Adrenal glands were weighed to determine if older animal had decreased adrenal mass. Old male mice had lower plasma corticosterone levels 8 h after DEX injection than did young male mice, suggesting that the anterior pituitary of older males is more sensitive to DEX-induced negative feedback. Old female mice had higher body-mass-corrected uterine mass than did young females. No other differences in corticosterone levels or organ masses were found between age groups

within either sex. In conclusion, we did not find strong evidence for age-related change in HPA activity or reactivity in virgin adult male or female California mice; however, future studies investigating HPA activity and reproductive outcomes in young and old breeding adults would be illuminating.

INTRODUCTION

Life history theory posits that organisms face a trade-off between current and future reproductive attempts (Roff, 1992, 2002; Stearns, 1992). The physiological mechanisms mediating such trade-offs are largely unknown; however, hormones have been viewed as probable candidates (Stearns, 1989, 2000; Ketterson and Nolan, 1999; Zera and Harshman, 2001). Specifically, the glucocorticoids, end products of the hypothalamic-pituitary-adrenal (HPA) axis, are likely to play a role (Ricklefs and Wikelski, 2002; Moore and Hopkins, 2009). Glucocorticoids are best known for their role in the stress response, as plasma concentrations rise ~3-5 minutes following the onset of a stressor (Dallman and Bhatnagar, 2001; Romero and Reed, 2005); however, basal levels are also important for organismal functioning, and basal glucocorticoid release displays a predictable diurnal pattern (Sapolsky et al., 2000). Glucocorticoids, at both basal and post-stress concentrations are important for the response to and recovery from stressors and are critical for maintenance of homeostasis (Sapolsky et al., 2000; Landys et al., 2006; Wingfield et al., 1998). These hormones influence glucose regulation (glucocorticoids can suppress insulin secretion as well as stimulate gluconeogenesis, lipolysis, glycogenolysis, and proteolysis), energy partitioning, and reproduction

(Dallman et al., 1993; Sapolsky et al, 2000; Sapolsky, 2002; Wingfield and Sapolsky, 2003; Boonstra, 2005; Reeder and Kramer, 2005; Ferin, 2006), as well as myriad other physiological and behavioral functions.

Across vertebrate taxa, the HPA axis plays a role in regulating reproduction in the face of stress (Greenberg and Wingfield, 1987; Wingfield et al., 1998; Ricklefs and Wikelski, 2002): while basal levels of glucocorticoids can facilitate physiological and behavioral aspects of reproduction, elevated glucocorticoid hormones have been shown to suppress both reproductive physiology and reproductive behavior (Lerman et al., 1997; Wingfield and Sapolsky, 2003). Reproductive suppression by stress (and glucocorticoids) is thought to be adaptive in the short term, as it promotes individual survival (Sapolsky et al., 2000), but may be detrimental when the value of current reproduction is high compared to future reproductive prospects (Wingfield and Sapolsky, 2003). For example, aged individuals have a decreasing window in which to reproduce, and are thus predicted to invest more heavily in current as opposed to future reproduction (Meddle et al., 2003; Wingfield and Sapolsky, 2003). Consequently, it has been hypothesized that older individuals should display decreased glucocorticoid responses to stressors, as compared to young conspecifics, to buffer short-term reproductive efforts from the detrimental effects of elevated glucocorticoids (Wingfield and Sapolsky, 2003; Ricklefs and Wikelski, 2002).

Evidence supporting this prediction has been documented in a handful of species. For example, older terns (*Sterna hirundo*; Heidinger et al., 2006, 2008) and Leach's storm-petrels (*Oceanodroma leucorhoa*; O'Reilly et al., 1999) of both sexes showed a

smaller CORT (and in terns, adrenocorticotropic hormone (ACTH)) response to handling or capture stress during the breeding season than did young adult conspecifics. In addition, old, non-breeding green sea turtles (*Chelonia mydas*) of both sexes exhibited lower concentrations of CORT following capture stress than did juvenile turtles (Jessop and Hamann, 2005; adult turtles were reproductively capable, but adults breed less than annually so non-breeding animals of that year were used). Aged male Sprague-Dawley rats produced less corticosterone in response to an injection of corticotropin-releasing hormone (CRH) than did young adult rats (Hylka et al., 1984; Scaccianoce et al., 1995), suggesting that pituitary responsiveness to CRH and/or adrenocortical responsiveness to ACTH is blunted in old individuals. Additionally, old male F344/BN hybrid rats showed greater suppression of ACTH and CORT in response to the synthetic glucocorticoid dexamethasone (DEX) than did younger adult rats, suggesting that sensitivity to glucocorticoid negative feedback increases with advancing age (Kasckow et al., 2005).

In the present study we compared HPA activity and reactivity, as well as adrenal and reproductive organ masses, in young adult and aged California mice (*Peromyscus californicus*) of both sexes to test the hypothesis that aged animals display dampened HPA reactivity as compared to young animals, possibly as a means to protect current reproductive potential. California mice are monogamous and biparental (Ribble and Salvoni, 1990; Ribble 1991,1992), and both males and females invest heavily in each reproductive bout (Gubernick et al., 1993; Cantoni and Brown, 1997a,b; Gubernick and Teferi, 2000). Thus, we expected both sexes to exhibit changes in HPA reactivity (measured by circulating concentrations of corticosterone, CORT) with age. This species

has an average lifespan of 9 to 18 months in the wild (Merritt, 1999), but can live up to 4 years in the lab (C.A. Marler, pers. comm.). Females become reproductively mature around 40 days of age (Gubernick, 1988), and males begin to breed successfully at 60-90 days of age (J. Crossland, pers. comm.; unpub. obs.). In captivity, both males and females breed successfully until 3 year of age or later, with no age-related decrease in number of pups born per litter (unpub. obs.).

We used virgin animals to avoid potential confounds of reproductive behavior, reproductive hormones (and in females, lactation), parental experience, and any reproduction-related effects on organ mass. We measured plasma CORT concentrations under basal conditions, in response to an acute stressor (bobcat urine), and in response to pharmacological suppression (DEX) and stimulation (CRH) in young and old adult mice of both sexes. We also compared gonadal and adrenal gland masses between the age classes. In line with the hypothesis that glucocorticoids mediate the trade-off between current and future reproduction, we predicted that aged individuals of both sexes would show a decreased CORT response to the predator-odor stressor, enhanced CORT suppression in response to simulated glucocorticoid negative feedback (DEX injection), and a diminished CORT response to CRH injection, when compared to young animals. Baseline levels of CORT were not predicted to differ between the age groups, as post-stress CORT concentrations are implicated in disruption of reproductive physiology and behavior (Wingfield and Sapolsky, 2003), whereas comparatively small changes in circulating basal CORT would not be predicted to alter reproductive investment. In terms of organ mass, we predicted that older animals would have heavier gonads and, in the

case of females, heavier uteri, as an index of increased preparedness for investment in current reproduction when future reproductive potential is low. Additionally, we predicted that older individuals might have lower adrenal mass than young individuals (if CORT modulation is a function of adrenal capacity), or that adrenal mass might not differ between age groups (if CORT modulation occurs via adrenal or pituitary sensitivity).

METHODS

Animals

We used California mice that were bred and housed at the University of California, Riverside (UCR), and that were descended from mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC) in 2007. All mice had *ad libitum* access to food (Purina rodent chow 5001) and water, and were housed in polycarbonate cages (44 x 24 x 20 cm) lined with aspen shavings; cotton wool was provided for nesting material. Lights were on from 0500-1700h (14:10 L:D cycle), and ambient temperature was maintained at approximately 23^{0}C with humidity around 65%. Animals were weaned from their birth cage at 27-32 days of age (prior to the birth of any younger siblings), ear punched for identification and housed in same-sex groups of two or four until just before the experiment began. From the time of weaning, animals were never housed with an individual of the opposite sex and were thus virgins at the time of testing.

We used a total of 19 virgin females, 10 young (123 ± 1 days at the beginning of data collection; range: 120-124 days; ~ 4 months) and 9 old (546 ± 4 days; range: 525-566 days; ~18 months), and a total of 43 virgin males, 22 young (135 ± 2 days at the beginning of data collection; range: 120-146 days; ~4.5 months) and 21 old (624 ± 10 days; range: 497-688 days; ~20 months). The ages chosen (range: 4 -20 months) are within the age range of California mice found in the wild (Merritt, 1999) and are thus ecologically relevant. In addition, the ages chosen are in line with ages used in several other rodent aging studies (e.g., rats: 3-30 months, Hylka et al., 1984; Keck et al., 2000; Kaskcow et al., 2005; Mizoguchi et al., 2009; mice: 3-16 months, Dalm et al., 2005). 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*.

Experimental Design

At least seven days prior to the start of data collection, groups of four mice were split into same-sex pairs. Hormonal data collection occurred over a period of 14-15 days (females) or 21-22 days (males). During the first week of data collection two baseline blood samples were collected from each mouse, one at 0800h and another at 2000h, to capture both the nadir and peak, respectively, of CORT levels across the diurnal cycle (Harris et al., in press). Consecutive samples were separated by two days, and all animals were weighed 1 day prior to initial blood collection. One week (females) or two weeks (males) after collection of the first baseline blood sample, mice were exposed to bobcat

urine (see section 2.5) for 5 min and a blood sample was collected immediately following exposure. Seven to eight days after predator-urine exposure, mice were injected with dexamethasone (DEX; see section 2.6). Eight hours after the DEX injection, a blood sample was collected, and mice were then immediately injected with CRH. Two additional blood samples were collected, one at 45 min and one at 90 min post-CRH. All mice were euthanized after the 90-min blood sample. For females, a vaginal lavage was performed (see section 2.2.1), and then right- and left-side adrenals and ovaries, as well as the uterus, were removed, placed in physiological saline, blotted dry 3x, and weighted to the nearest 0.00001g. For males right-side adrenal glands and testes were dissected out (left-side organs were frozen for possible future analysis), placed in physiological saline, blotted dry 3x, and weighed to the nearest 0.0001 g.

Vaginal Lavage

In addition to the procedures described above, we performed vaginal lavage on each female mouse 6 times over the course of the experiment in an attempt to assess estrous cycles. Cycle stage can influence baseline CORT levels in female rats and house mice (females in proestrus have higher CORT concentrations; Atkinson and Waddell, 1997; Lo et al., 2006; Nichols and Chevins, 1981); however, previous data from California mice show that the estrous cycle does not affect CORT concentration (Davis and Marler, 2003; Karelina et al., 2010), and male and female California mice do not differ in basal or post-stress CORT concentrations (Harris et al., 2012). We refrained

from lavaging animals every day as lavage itself can be stressful to rodents (Sharp et al., 2003) and can alter experimental outcomes (see Walker et al., 2002).

The first lavage occurred two days prior to the first baseline blood sample. Then, starting two days after predator-odor exposure (11 days from first the lavage), lavages were conducted on 4 consecutive days (ending 2 days prior to the final day of the experiment). A final lavage was conducted post-mortem on the last day of data collection. Lavages were performed by gently holding the female by the scruff of the neck and inserting a glass Pasteur pipette filled with approximately 100-150µl of sterile saline into the vagina. The saline was squirted into the vaginal canal and then removed, placed onto a slide, stained with methyl blue, and immediately placed under a compound microscope for analysis. Cell types and proportions were determined, and mice were classified in one of five categories: diestrus, proestrus, estrus, metestrus, or no sample (vagina closed; Caligioni, 2009; Gubernick, 1988; Mettus and Rane, 2003).

Blood Sample Collection

Mice were anesthetized with isoflurane, and blood samples (70-210 µl) were collected from the retro-orbital sinus using heparinized glass microhematocrit tubes. Time from disturbance of the cage or end of the test to collection of the blood sample was less than 3 minutes for all but 6 out of 352 blood samples (males: 78.9 ± 2.0 s, range 40-275 s; females: 73.8 ± 4.2 s, range: 39-235 s). Blood was centrifuged for 12 min (13,300 rpm, 4°C), and plasma was collected and stored at -80°C until assay.

Final blood collection from females was performed using cardiac puncture. After euthanasia via CO_2 inhalation, blood was collected from the heart with a 1ml heparinized syringe fitted with a 27G sterile needle. Average time from initial administration of CO_2 to collection of blood was 284.8 ± 20.1 s (range: 189-500 s). Blood was processed and stored as described above.

Corticosterone Assay

Plasma was assayed in duplicate for corticosterone using an ^{125}I double-antibody radioimmunoassay kit (#07-120102, MP Biomedicals, Costa Mesa, CA) that has been validated for this species (Chauke et al., 2011). Inter- and intra-assay coefficients of variation (CVs) were 10.7% and 4.1%, respectively (N = 45 assays). Plasma samples from each age group were balanced across assays, and samples from males and females were run in separate assays. Therefore, hormone data from males and females were not directly compared to each other.

Predator-urine Exposure

Same-sex pairmates were exposed to predator urine together. Between 0800 and 0845h, animals were placed in a fresh cage that contained clean bedding but no food, water or cotton, and taken to a testing chamber. A cotton ball wetted with 1ml of bobcat *(Lynx rufus)* urine (Maine Outdoor Solutions, Hermon, ME) was immediately placed in a corner of the test cage for 5 min. As soon as exposure ended, a blood sample was

collected from each mouse and mice were returned to their home cage. We have previously found that exposure to predator urine, including bobcat urine, produces a pronounced CORT response in California mice at this time of day (Chauke et al., 2011; Harris et al., 2012).

Dexamethasone and Corticotropin-Releasing Hormone Injections

Mice were weighed one day prior to injection to permit calculation of body-mass-corrected hormone doses. Dexamethasone sodium phosphate (DEX; 4mg/ml, American Regent, Shirley, NY) was diluted with sterile saline to a concentration of 10 mg/kg and injected i.p.; this dose has previously been shown to suppress plasma CORT levels in California mice 8 h following injection (Harris et al., 2012). Corticotropin-releasing hormone (CRH; C3042, Sigma Aldrich, St. Louis, MO) was diluted in sterile water to a 1 µg/ml solution, and mice were injected i.p. with 2 µg/kg CRH; this dose has been shown to increase DEX-suppressed CORT levels in this species (unpub. data).

Animals were injected with DEX at 0730-0830h on the last day of testing, and then placed back in their home cage. Eight h following DEX injection, at 1530-1630h, each animal was blood sampled, injected with CRH, and then returned to its home cage. A second blood sample was collected via the retro-orbital sinus from each animal 45 min following CRH injection, and the animal was again returned to its home cage. Finally, 90 min after CRH injection, a third blood sample was collected from either the heart via

cardiac puncture following CO_2 inhalation (females) or from the retro-orbital sinus (males).

Analysis

Data were checked for normality using the Shapiro-Wilk test and transformed if necessary. All CORT values were $\log_{10}$-transformed prior to analysis, but non-transformed values are presented for ease of interpretation. Each mouse's plasma CORT concentration from the 0800h sample was used as a baseline for within-subjects analyses of the response to bobcat urine. CORT data were analyzed via repeated-measures ANOVA. Additionally, area under the curve (AUC) was calculated on post-injection CORT concentrations using two formulas (see Pruessner et al., 2003) to quantify total CORT release over time following CRH treatment. AUCg corresponds to the integrated amount of hormone produced over time with respect to a starting value of zero (not taking post-DEX CORT concentration into account). AUCi is calculated using a baseline value (here, post-DEX CORT levels) and measures CORT increase over time from each individual animal's starting value. One young male's 2000h plasma sample was lost during processing, leaving 21 samples for young males at that time point. Additionally, due to problems with sample processing, three post-CRH-injection plasma samples from old males were lost, leaving 20 45 min post-CRH and 19 90 min post-CRH samples from old males for analysis. Hormone concentrations were correlated using Pearson's correlation.

Body mass was analyzed by ANOVA. Organ masses (gonads, uteri, and adrenal glands) were analyzed using an ANCOVA with final body mass as a covariate, following the methods of Tomkins and Simmons (2002), except that organ mass was not subtracted from body mass due to differences in the number of significant figures (body mass was measured to the nearest 0.01 g and organs to the 0.0001 or 0.00001 g). For females, associations between right and left organ masses were evaluated using Pearson's correlation. The left ovary of one old female was damaged during dissection and thus that animal was not included in analysis of ovarian mass.

RESULTS

Females

Basal CORT

Plasma CORT concentrations in female California mice were markedly higher at 2000h than at 0800h (1466.44 ± 169.27 vs. 42.48 ± 5.67 ng/ml; n=19; $F_{1,17}$= 353.72, P <0.001; Table 2.1). However, CORT levels did not differ between young and old adult females ($F_{1,17}$= 0.46, P=0.505), nor was there a time*group interaction ($F_{1,17}$=0.01, P=0.914). Across all females, plasma CORT level at 0800h was not correlated with plasma CORT level at 2000h (r=0.090, n=19, P=0.715).

CORT Response to Predator-urine Exposure

Exposure to predator urine increased plasma CORT above baseline levels measured at the same time of day (878.74 ± 123.06 vs. 42.48 ± 5.67 ng/ml; n=19; $F_{1,17}$= 175.77, P <0.001; Table 2.1), but there was no effect of age group ($F_{1,17}$= 0.90, P=0.356), nor was there a time*group interaction ($F_{1,17}$ =0.14, P=0.714). For both age groups combined, post-urine-exposure CORT levels were not correlated with time-matched baseline concentrations (r=0.182, n=19, P=0.445).

CORT Response to DEX and CRH Injection

Injection of DEX followed by CRH elicited an increase in plasma CORT (8 h post-DEX vs. 45 min post-CRH vs. 90 min post-CRH: 80.62 ± 9.80 vs. 124.01 ± 39.18 vs. 821.42 ± 186.08 ng/ml; main effect of time: $F_{2,34}$= 61.78, P <0.001; Fig. 2.1), but CORT response did not differ between age groups ($F_{1,17}$= 0.20, P=0.665), nor was there a time*group interaction ($F_{2,34}$ =0.73, P=0.494; Table 2.1). CORT levels increased at each time point measured, as CORT values were higher 90 min post-CRH than 45 min post-CRH (t=8.00, P<0.001; Fisher's LSD), and as CORT values both 45 and 90 min post-CRH were higher than levels 8 h after DEX injection and immediately before CRH treatment (t=2.42, P=0.029; t=8.27, P<0.001, respectively; Fisher's LSD).

In addition to repeated-measures ANOVA, we analyzed time-integrated CORT responses to CRH using two calculations for area under the curve (AUC; Pruessner et al.,

2003). Female age did not influence either AUCg ($F_{1,17}$=0.34, P=0.565) or AUCi ($F_{1,17}$=2.63, P=0.151).

For both age groups combined, post-DEX CORT concentrations were significantly and positively correlated with CORT levels 45 min post-CRH (r=0.852, n=19, P<0.001), but not 90 min post-CRH (r=0.362, n=19, P=0.128). Additionally, 45 min post-CRH CORT levels were significantly and positively correlated with 90 min post-CRH levels (r=0.537, n=19, P=0.018).

Body Mass

Body mass of female California mice was influenced by a main effect of day ($F_{1,17}$ =19.21, P<0.001) and a day*group interaction ($F_{1,17}$=6.33, P=0.022), but not a main effect of age group ($F_{1,17}$= 1.79, P=0.199). Old females lost body mass from the start to the end of the experiment (approximately 2 weeks; t=4.75, P<0.001) whereas young females did not (t=1.36, P=0.193; Sidak-corrected post-hoc tests following repeated-measures ANOVA). Females did not differ in body mass at the start of the experiment (P=0.117) or on the day prior to dissection (P=0.323; Table 2.2).

Organ Masses

Body mass on the day prior to dissection was used as a covariate for organ-mass analyses. Uterine mass was log_{10}-transformed prior to analysis to meet normality assumptions. Across all females, right and left adrenal gland masses were highly

correlated ($r=0.892$, $n=19$, $P<0.001$), as were right and left ovary masses ($r=0.615$, $n=18$, $P=0.007$). Therefore, only total (left + right) organ masses were used in the remaining analyses.

Initially, ANCOVAs were computed using group, body mass, and the group*body mass interaction. For all organs, the interaction term was not significant and was dropped from the model. Body mass remained in the model as a covariate and was significant for $\log_{10}$-transformed uterine mass ($F_{1,16}=8.27$, $P=0.011$; Table 2.1), total ovarian mass ($F_{1,15}=11.32$, $P=0.004$), and total adrenal gland mass ($F_{1,16}=5.56$, $P=0.031$). After accounting for body mass, old females had significantly higher $\log_{10}$-transformed uterine mass ($F_{1,16} = 7.10$, $P=0.017$), but neither total adrenal gland mass ($F_{1,16}=0.479$, $P=0.499$) nor total ovarian mass ($F_{1,15}=2.23$, $P=0.156$) differed by group.

Vaginal Lavage

Lavages were carried out in an attempt to characterize cycle lengths of females. We were not able to discern a reliable pattern of cyclicity in vaginal smears in either age group. However, we did observe that 9 females (5 young and 4 old) had vaginal cytology typical of estrus in at least one lavage, suggesting that these animals were undergoing estrous cycles.

Males

Basal CORT

Plasma CORT levels were dramatically higher at 2000h when compared to 0800h levels, regardless of age group (1761.27 ± 105.05 vs. 53.79 ± 7.42 ng/ml; n=42; $F_{1,40}$=881.27, P<0.001). However, there was neither a main effect of age group ($F_{1,40}$ <0.001, P=0.999; Table 2.1) nor a time*group interaction ($F_{1,40}$=0.221, P=0.641). Across both age groups, basal CORT concentrations at 0800h and 2000h were not correlated with one another (r=-0.021, n=42, P=0.897).

CORT Response to Predator-urine Exposure

Exposure to bobcat urine significantly increased plasma CORT concentrations in male California mice when compared to time-matched baseline values ($F_{1,41}$ =283.79, P<0.001; Table 2.1), but there was no effect of age group ($F_{1,41}$= 0.01, P = 0.978), nor was there a group*time interaction ($F_{1,41}$=0.40, P=0.531). Post-urine-exposure CORT levels were not correlated with 0800h basal CORT concentrations (r=0.082, n=43, P=0.602).

CORT Response to DEX and CRH Injection

DEX and CRH treatment affected CORT levels over the three time points measured, as there was a main effect of time (8 h post-DEX vs. 45 min post-CRH vs. 90 min post-CRH: 89.51 ± 7.09 vs. 335.04 ± 84.54 vs. 1038. 56 ± 156.45 ng/ml; $F_{2,78}$ =

82.90, $P<0.001$; Fig. 2.2). Irrespective of age group, CORT levels 90 min post-CRH were higher than those post-DEX ($t=11.39$, $P<0.001$; Table 2.1) and 45 min post-CRH ($t=9.78$, $P<0.001$; Fisher's LSD). Additionally, CORT levels 45 min post-CRH were higher than those obtained 8 h post-DEX injection ($t=3.94$, $P=0.001$; Fisher's LSD). We did not find a significant main effect of age group on CORT levels ($F_{1,39}=0.31$, $P=0.582$), but there was a time*group interaction ($F_{2,78}=4.33$, $P=0.016$). Specifically, young males had higher CORT levels than did old males 8 h after DEX injection ($t=2.74$, $P=0.009$; Sidak-corrected post-hoc test; Fig. 2.2 inset), but CORT concentrations did not differ between the two age groups either 45 min ($P=0.212$) or 90 min ($P=0.155$) following CRH injection. Neither AUCg ($F_{1,39}= 0.76$, $P=0.785$) nor AUCi ($F_{1,36}=0.12$, $P=0.734$) differed between young and old males.

Across all males, as in females, post-DEX CORT levels were significantly and positively correlated with 45 min post-CRH CORT levels ($r=0.412$, $n=42$, $P=0.007$) but not with 90 min post-CRH CORT levels ($r=0.059$, $n=41$, $P=0.713$). Plasma CORT concentrations at 45 min post-CRH were significantly and positively correlated with concentrations at 90-min post-CRH ($r=0.606$, $n=41$, $P<0.001$), again mirroring results found in females.

Body Mass

Body mass of male California mice increased across the 3-week period of data collection (main effect of day: $F_{1,41}=5.41$, $P=0.025$; Table 2.2). Overall, old males were

heavier than young males ($F_{1,41}$ =33.80, P<0.001), but the change in mass over time did not differ between the two age groups (day*group interaction: $F_{1,41}$ =2.940, P=0.094).

Organ Masses

Ending body mass differed between young and old males (t_{41}=5.02; P<0.001; Table 2.2) and was used as a covariate for analyses of organ masses. Adrenal gland mass was $\log_{10}$-transformed to fit normality assumptions. Initially the interaction term of group*body mass was included in the model. The term was nearly significant ($F_{1,39}$=3.85, P=0.057) for analysis of right testis mass and therefore was retained in this ANCOVA, but it was not significant for right adrenal gland mass ($F_{1,39}$=0.13, P=0.720) and was thus removed. The effect of body mass was significant for right adrenal ($F_{1,40}$=5.50, P=0.024) and right testis ($F_{1,39}$=4.51, P=0.040). Age did not affect body-mass-corrected right adrenal ($F_{1,40}$=1.50, P=0.229; Table 2.2) or right testis ($F_{1,39}$=2.35, P=0.133; Table 2.2) mass.

DISCUSSION

We used virgin male and female California mice to test the hypothesis that aged animals show reduced glucocorticoid responses to stress and CRH injection, and enhanced responses to negative feedback, when compared to young adults, possibly as a means to maximize current reproductive potential. We predicted that old animals would have lower circulating CORT levels in response to predator-odor exposure, following

DEX injection, and at two time points following CRH injection. Sustained elevation of glucocorticoids can be detrimental to reproductive efforts (Lerman et al., 1997; Silverin 1986, 1988; Tilbrook et al., 2000); therefore, enhanced negative feedback or reduced CORT output should be beneficial in preserving reproductive function by minimizing the duration or magnitude of CORT elevation. Additionally, we predicted that older animals would have larger reproductive organs and might have smaller adrenal glands as compared to young animals. We found that old male mice were more sensitive to DEX negative feedback than were young male mice, thus providing partial support for the hypothesis; however, we did not find any evidence that HPA activity changes with age in adult females. No other differences in HPA function between old and young adult mice were noted in either sex. Regardless of age group, all mice experienced an increase in CORT concentration above basal levels after exposure to predator urine and in response to CRH injection. Additionally, all animals displayed a pronounced diurnal rhythm in CORT profiles. Old female mice had heavier body-mass-corrected uteri than did young females, but neither adrenal nor gonadal mass differed between age groups in either sex.

Old male California mice displayed lower CORT concentrations 8 h following injection of a standardized dose of DEX than did young males. This finding suggests that the anterior pituitary of older males is more sensitive to DEX-induced negative feedback; it is unclear whether central sensitivity to glucocorticoids also differs between young and old males, as DEX does not readily cross the blood-brain barrier (Cole et al., 2000). This increased pituitary sensitivity with old age supports the hypothesis that decreased future opportunities for breeding should favor a more easily suppressed HPA axis. However,

we did not find any differences in CRH-stimulated CORT concentrations between the age groups within either sex, even when we analyzed total CORT output with post-DEX values taken into consideration (AUCi values). This suggests that HPA axis sensitivity to CRH is not dampened in older animals. Additionally, age did not affect CORT levels following 5-minute exposure to predator urine, suggesting that HPA axis sensitivity to an acute stressor is not altered by age.

Our equivocal findings on effects of age on CORT are consistent with previous research, as several other studies have also noted mixed findings on effects of age on HPA activity and responsiveness to DEX. In hybrid (F344/BN) male rats, 30-month-old males had lower post-DEX CORT concentrations than 3-month-old males, but age groups (3 vs. 15 vs. 30 months) did not differ in their CORT response to restraint stress (Kasckow et al., 2005), similar to our findings in male California mice. Additionally, 24-month-old F344/BN hybrid males had lower post-DEX CORT concentrations as compared to 3-month-old males when the DEX injection was given systemically (Mizoguchi et al., 2009). However, when DEX was administered directly into the brain (prefrontal cortex, hippocampus, or hypothalamus), the suppressive effect of DEX on plasma CORT was abolished in old rats whereas young rats still experienced a decrease (Mizoguchi et al., 2009), suggesting that age differentially affects responsiveness to glucocorticoid negative feedback at both the brain and the pituitary. In contrast, old (15-27 yrs) female rhesus monkeys (*Macaca mulatta*) displayed an earlier release from DEX-induced cortisol suppression than did younger adult (7-8 yrs) females; however, the two age groups did not differ in glucocorticoid concentrations following CRH injection (Gust

et al., 2000). Additionally, post-DEX and post-CRH cortisol levels were higher in older men and women compared to their younger counterparts (Heuser et al., 1994). In sum, these findings suggest that pituitary sensitivity to DEX and/or glucocorticoids increases with old age in male rodents but decreases with old age in primates.

Effects of aging on HPA responses to stress also differ among studies. For example, old (24 months) female Fisher 344 rats had lower CORT levels following 15-min exposure to a novel environment and to a bout of anesthesia with blood collection, as compared to young female rats (3 months; Brett et al., 1983). However, young and old females did not differ in CORT levels following a 3-min novel-environment exposure. Moreover, young and old male Fisher 344 rats did not differ in CORT concentration following any of the three stress paradigms (Brett et al., 1983), suggesting that the effects of age on CORT responses can be modulated by both sex and stressor type. Old (22-24 months) and young (3 months) male Wistar rats did not differ in CORT response following 15 min in a novel environment, but old males displayed higher baseline CORT concentrations than did young males (Keck et al., 2000). Moreover, 33-month-old male F344/BN hybrid rats had higher CORT levels following a 30-min novel environment exposure compared to 15- and 3-month-old males, but the three age groups did not differ in the CORT response to restraint stress or in morning basal CORT levels (Herman et al., 2001), contrary to findings on basal levels in Wistar rats and again suggesting that strain, stressor type, and possibly age categories can alter results. In humans, a meta-analysis indicated that older adults have more active HPA axes (higher post-stress and post-DEX cortisol levels) than do young adults, and that this difference is more pronounced in

women compared to men (Otte et al., 2005), again pointing to a sex difference. The occurrence of mixed results across several species suggests that age effects on HPA function are complex, and that numerous organismal and experimental variables, including sex, strain, stressor type, test paradigm, age classification, and injection route, can influence the interactions between age and HPA responsiveness.

In contrast to our hormonal results, we found no support for our predictions regarding age effects on organ masses in males, but found partial support in females. Despite being virgins, old females had higher body-mass-corrected uterine mass than did young females, possibly suggesting that older females were better prepared for pregnancy as uterine mass has been noted to increase prior to the breeding season (*P. maniculatus*, Demas and Nelson, 1998) or in response to gonadal steroids present around the time of estrus (*Rattus norvegicus*, Lundeen et al., 1997). In contrast, age (onset of adulthood vs. young adult) did not affect body-mass-corrected uterine mass in Mongolian gerbils (*Meriones unguiculatus*; Saltzman et al., 2006), nor did uterine mass differ between juvenile and mature female Pine voles (*Microtus pinetorum*; Solomon et al., 1996).

Despite differences in uterine mass, old and young *P. californicus* females did not differ in total ovarian mass in this study. Unfortunately, we were not able to use estrous-cycle stage as a covariate because we could not accurately monitor estrous cyclicity. However, similar numbers of old (4/9) and young (5/10) females expressed vaginal cytology typical of estrus at least once during the study; therefore, we believe that differences in uterine mass were not due to age group differences in the number of animals undergoing estrous cycles. Previous reports on California mice show that estrous

cycles in this species are longer and more variable in length (range: 5- 20 days, median: 9 days; Gubernick, 1988) than in rats and house mice (mice: 4-5 days; Caligioni, 2009; rats: 4-5 days; Long and Evans, 1922; Goldman et al., 2007), but are consistent with cycle lengths in other *Peromyscus* species (Bradley and Terman, 1979). Although studies on *Rattus* and *Mus* found higher CORT levels during proestrus, estrous cycle stage does not affect CORT levels of female California mice following a 3 h social interaction (Karelina et al., 2010) or 40 min following a resident-intruder test (Davis and Marler, 2003); therefore, estrous cycling should not have confounded our CORT results.

In conclusion, we did not find consistent evidence for age-related changes in HPA activity or reactivity in virgin male or female California mice. This suggests that aged and young adult California mice are not differentially modulating the perception of potential stressors (i.e. stressful or not), nor are they undergoing age-related alterations in HPA responsiveness to stress or exogenous HPA hormones. Nonetheless, it remains possible that the HPA axis plays a role in the trade-off between current and future reproductive investment and in age-related modulation of this trade-off. For instance, aged animals might increase production of corticosteroid-binding globulin (CBG), thus decreasing the amount of free vs. bound CORT and mitigating the effects of elevated glucocorticoids (Malisch and Breuner, 2010; Wingfield and Sapolsky, 2003). Moreover, aged individuals might exhibit reduced responsiveness of the reproductive system or hypothalamic-pituitary-gonadal (HPG) axis to elevated glucocorticoids (Wingfield and Sapolsky, 2003). In semelparous species such as salmon (*Oncorhynchus spp.*) and certain dasyurid marsupials (*Antechinus spp.),* individuals are able to maintain

reproductive function despite near-lethal levels of circulating glucocorticoids (see Wingfield and Sapolsky, 2003 for review). While the mechanism for this ability is unknown, reduced HPG sensitivity has been proposed (Wingfield and Sapolsky, 2003). Future studies in California mice and other species should evaluate the interactions of age and stress effects on CORT concentrations and HPG-level measures, and should investigate the relationship between HPA reactivity and reproductive outcomes in actively breeding, young and old adults.

Table 2.1. Plasma CORT concentrations (ng/ml) in young and old virgin California mice by sex. All concentrations are reported as mean ± SEM.

		Female			Male		
Condition	Time	Young	Old	P	Young	Old	P
Basal	0800h	45.06 ±7.03	39.62 ±9.44	0.505[a]	54.76 ±10.48	52.78 ±10.78	0.999
Basal	2000h	1522.85 ±229.17	1403.75 ±263.65	--[a]	1682.94 ±128.64	1839.60±167.59	--[a]
Post-stress	0800-0900h	966.85 ± 170.34	780.85 ± 182.74	0.356[b]	654.14 ±82.54	709.87 ± 98.31	0.978[b]
Post-DEX	1530-1630h	81.80 ± 13.28	79.30 ± 15.35	0.665[c]	104.55 ±10.80	73.75 ± 7.97	0.582[c,d]
45 min post-CRH	1615-1715h	87.00 ±7.96	165.14 ±82.53	--[c]	357.94 ±101.72	309.85 ± 140.53	--[c]
90 min post-CRH	1700-1800h	637.65 ± 184.76	1025.61 ± 334.53	--[c]	899.96 ±184.72	1199.05 ± 261.93	--[c]

[a]P-values correspond to main effect of age group in a repeated-measures analysis on basal CORT (0800h and 2000h).

[b]P-values correspond to main effect of age group in a repeated-measures analysis (0800h basal vs. post-stress).
[c]P-values correspond to main effect of age group in a repeated-measures analysis of post-injection CORT (post-DEX, 45 min post-CRH, 90 min post-CRH).

[d]Group*time interaction, P=0.016; old males had lower post-DEX CORT concentration than young males P=0.009.

Table 2.2 Body mass and organ masses in young and old virgin California mice by sex. P values are for main effect of age group from one-way ANOVA (body mass) or ANCOVA (organ masses). All values are presented as mean ± SEM and are reported in grams; bolded P-values are <0.05.

Sex	Variable	Young	Old	P	Covariates
Females		(n=10)	(n=9)		
	body mass - start	43.24 ± 2.02	49.69 ± 3.46	0.117	--
	body mass - end	42.36 ± 2.10	46.47 ± 3.56	0.323	--
	total ovary[a]	0.0111 ± 0.0012	0.0138 ± 0.0013	0.156	body mass - end
	total adrenal[b]	0.0171 ± 0.0013	0.0185 ± 0.0014	0.499	body mass - end
	uterus[c]	0.0421 ± 0.0040	0.0635 ± 0.0101	**0.017**	body mass - end
Males		(n=22)	(n=21)		
	body mass - start	39.66 ± 1.54	53.39 ± 1.44	**<0.001**	--
	body mass - end	41.53 ± 1.68	53.67 ± 1.74	**<0.001**	--
	r. testis[d]	0.1977 ± 0.0210	0.1428 ± 0.0202	0.133	body mass - end, group*body mass
	r. adrenal[e]	0.0097 ± 0.0004	0.0087 ± 0.0006	0.229	body mass - end

[a] body-mass-corrected averages at body mass of 44.61 g

[b] body-mass-corrected averages at body mass of 44.31 g

[c] back-transformed body-mass-corrected averages at body mass of 44.31 g

[d] body-mass-corrected averages at body mass of 47.46 g

[e] back-transformed body-mass-corrected averages at body mass of 47.46 g

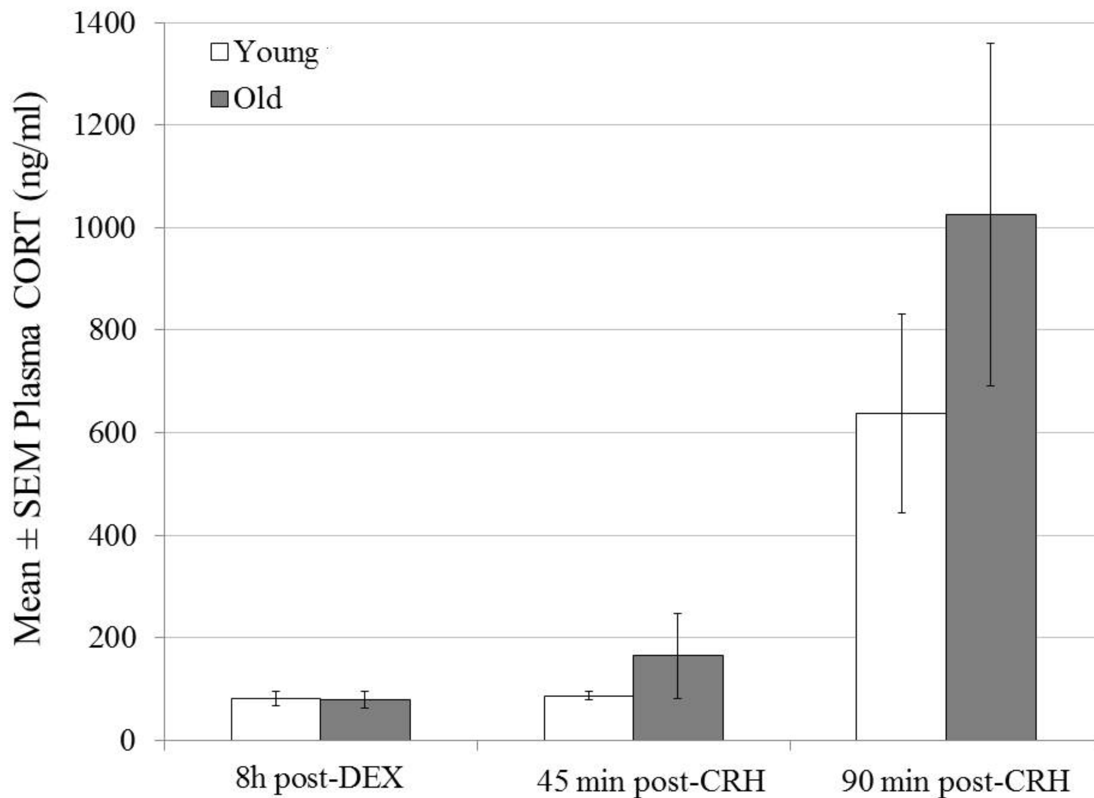

Figure 2.1: Plasma CORT concentrations in young (n=10) and old (n=9) adult, virgin female California mice following an injection of DEX (10mg/kg, i.p.; 0730-0830 h) and a subsequent injection of CRH (2 µg/kg, i.p.; 1530-1630 h). Age group did not affect the response to injection at any time point measured, but CORT levels increased across time (P <0.001) and all time points differed from one another (post-DEX vs. 45 min post-CRH: P=0.029; post-DEX vs. 90 min post-CRH: P<0.001; 45 min post-CRH vs. 90 min post-CRH: P<0.001).

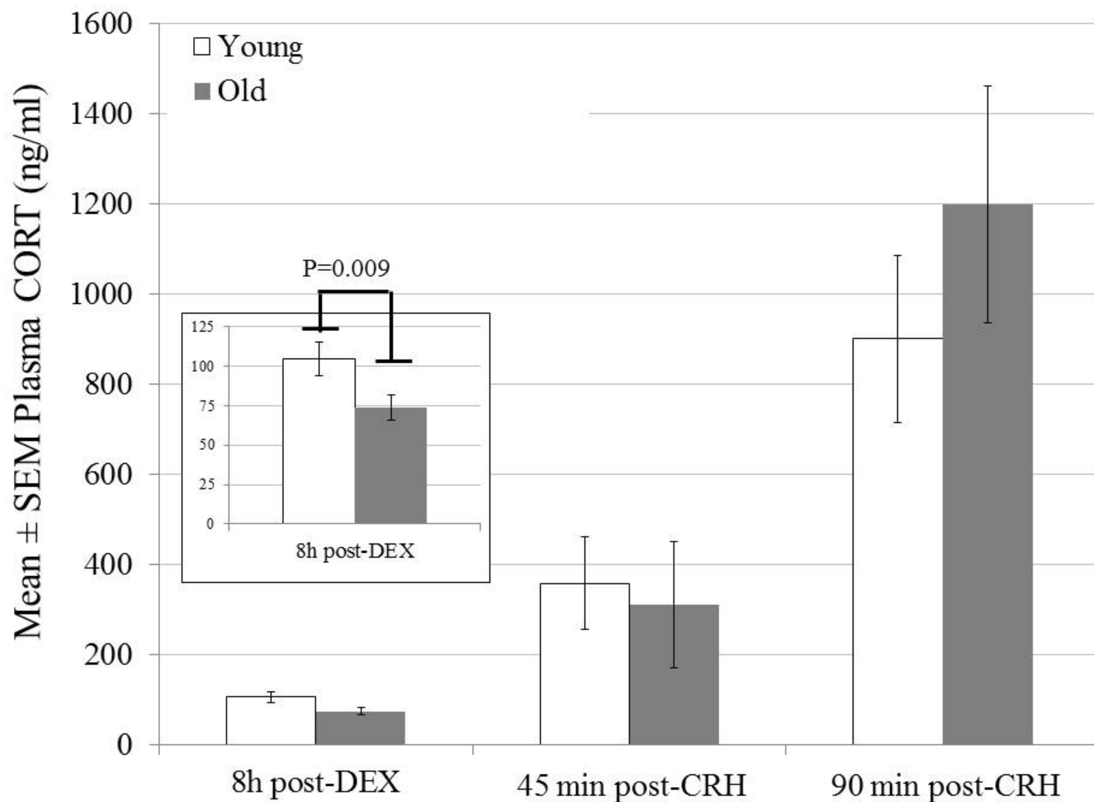

Figure 2.2: Plasma CORT levels in young (n=22) and old (n=21) adult, virgin male California mice following an injection of DEX (10mg/kg, i.p.; 0730-0830 h) and a subsequent injection of CRH (2 µg/kg, i.p.; 1530-1630 h). Regardless of age group, CORT increased over time ($P<0.001$; post-DEX vs. 45 min post-CRH: $P=0.001$; post-DEX vs. 90 min post-CRH: $P<0.001$; 45 min post-CRH vs. 90 min post-CRH: $P<0.001$). Age group significantly affected CORT responses over time (group*time interaction, $P=0.016$). CORT levels were higher in young males than in old males at 8h post-DEX ($P=0.009$; see inset) but not at the remaining time points.

Chapter 3

Effect of reproductive status on hypothalamic-pituitary-adrenal (HPA) activity and reactivity in male California mice (*Peromyscus californicus*)

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ABSTRACT

Previous studies indicate that reproductive condition can alter the stress response and glucocorticoid release. Although the functional significance of hypothalamic-pituitary-adrenal (HPA) axis modulation by breeding condition is not fully understood, one possible explanation is the *behavior hypothesis*, which states that an animal's need to express parental behavior may be driving modulation of the HPA axis. This possibility is consistent with findings of blunted activity and reactivity of the HPA axis in lactating female mammals; however, effects of reproductive status on HPA function have not been well characterized in male mammals that express parental behavior. Therefore, we tested this hypothesis in the monogamous and biparental California mouse. Several aspects of HPA activity were compared in males from three reproductive conditions: virgin males (housed with another male), non-breeding males (housed with a tubally ligated female), and first-time fathers (housed with a female and their first litter of pups). In light of the behavior hypothesis we predicted that new fathers would differ from virgin and non-breeding males in several aspects of HPA function and corticosterone (CORT) output: decreased amplitude of the diurnal rhythm in CORT, a blunted CORT increase following predator-odor stress, increased sensitivity to glucocorticoid negative feedback, and/or a blunted CORT response to pharmacological stimulation. In addition, we predicted that first-time fathers would be more resistant to CORT-induced suppression of testosterone secretion, as testosterone is important for paternal behavior in this species. We found that virgin males, non-breeding males and first-time fathers did not display any CORT

differences in diurnal rhythm, response to a predator-odor stressor, or response to pharmacological suppression or stimulation. Additionally, there were no differences in circulating testosterone concentrations. Adrenal mass was, however, significantly lower in new fathers than in virgin or non-breeding males. These results suggest that the behavior hypothesis does not explain HPA function across reproductive conditions in male California mice.

INTRODUCTION

Stress and reproduction are intimately intertwined, and as such the effects of stress on reproduction have been investigated in a variety of taxa. Stress, both acute and chronic, can dampen reproductive physiology as well as suppress reproductive behavior (McGrady, 1984; Moore and Miller, 1984; Sapolsky, 2002; Tilbrook et al., 2000; Wingfield and Sapolsky, 2003). Conversely, reproductive condition can alter the hypothalamic-pituitary-adrenal (HPA) axis stress response and glucocorticoid (GC) release (Creel, 2001, 2005; Romero, 2002; Saltzman et al. 1994, 1998; Wilson and Wingfield, 1992; Wingfield and Romero, 2001; Wingfield, 2008). For example, changes in HPA axis function (e.g., response to GC negative feedback) and GC release patterns (e.g., amplitude and range of the diurnal rhythm; response to stressors) occur during the breeding season in several species (see Romero, 2002 for review; Moore and Jessop, 2003; Wingfield and Sapolsky, 2003).

The functional significance of HPA-axis modulation during the breeding season has yet to be fully elucidated. One of several posited explanations is the *behavior*

hypothesis (Romero, 2002; Wingfield and Romero, 2001), which states that the need to express parental behavior may be driving seasonal modification of the HPA axis (Romero, 2002). Various types of stress have been shown to disrupt parental behavior, and evidence suggests that GCs are at least partially responsible for this effect (O'Connor et al., 2009; Saltzman and Abbott, 2009; Silverin, 1986, 1998). The disruption of parental investment (e.g., abandonment or cannibalism of offspring) that can occur in response to major stressors (e.g., prolonged severe weather or food shortage) may increase overall lifetime fitness of parents by increasing the parent's survival, but triaging reproductive effort in response to every minor perturbation (e.g., attempted predation or a minor storm) could be detrimental to reproductive success. Lowering both baseline and stress-induced GC concentrations during the time of intensive parental behavior would reduce the chances of GC levels increasing to a point that would disrupt parental care, and therefore could be beneficial both for offspring survival and for parental fitness (Wasser and Barash, 1983; Wingfield, 2008; Wingfield and Sapolsky, 2003).

Due to the universal expression of maternal behavior in mammals, numerous studies have addressed the interactions between *motherhood* and HPA activity (Brunton et al., 2008; Lightman et al., 2001; Numan and Insel, 2003; Tu et al., 2005). However, little is known about the relationship between *fatherhood* and the HPA axis. In 6-10% of mammals, including humans, both parents provide care for their offspring (Kleiman and Malcolm, 1981). In these biparental systems, both mothers and fathers make important contributions to the survival and growth of young (e.g., by providing food, warmth, and protection) and can influence behavioral and neuroendocrine development of offspring

(Gubernick & Teferi, 2000; Schradin & Pillay, 2004; Wright & Brown, 2002). A reasonable hypothesis, therefore, is that in biparental species, both sexes modulate HPA-axis function during periods of parental care in an effort to ensure offspring survival.

Previous data on monogamous, biparental male mammals suggest that reproductive condition, as well as pair bonding, can alter HPA function. For example, in male prairie voles (*Microtus ochrogaster*), loss of a female pairmate, but not a male cagemate, increased circulating GC concentrations and passive stress-coping behavior (Bosch et al., 2009), suggesting that the presence of a pair bond can reduce circulating GC levels in males. Similarly, male California mice (*Peromyscus californicus*) housed with an ovariectomized female had lower basal and stress-induced GC levels than isolated males, suggesting that social living (and presumably pair bonding) can decrease circulating corticosterone (CORT) concentrations (Glasper and DeVries, 2005). Pair bonding in male California mice may also buffer the CORT response to a repeated stressor, as virgin males housed with another male, but not males pair-housed with a female (either with or without pups), showed an increased GC response to predator urine following repeated exposure (Chauke et al., 2011). However, no study to date has systematically investigated baseline, stress-induced, and pharmacologically manipulated HPA activity across reproductive conditions in a biparental male mammal.

The California mouse is a valuable animal model for studying the effects of reproductive condition on the HPA axis, as this rodent is both monogamous and biparental (Ribble 1991, 1992; Ribble and Salvoni, 1990). California mouse fathers are highly attracted to pups, engage in all of the same parental behaviors as mothers (with the

exception of lactation), and can increase offspring survival under both field and laboratory conditions (Cantoni and Brown, 1997a,b; Gubernick and Alberts, 1987; Gubernick and Teferi, 2000; Gubernick et al., 1993). When presented with a newborn pup, virgin males of this species are more variable in their expression of paternal behavior than are new fathers (Chauke et al., 2012; de Jong et al., 2009, 2010; 2012; Gubernick et al., 1994; Trainor et al., 2003). Consistent with this difference in behavior, circulating concentrations of several hormones thought to be associated with the expression of paternal care differ in male California mice as a function of reproductive condition. For example, new fathers have lower plasma progesterone levels than virgin males (Trainor et al., 2003) and fathers have higher systemic levels of prolactin than virgin males or expectant fathers (Gubernick and Nelson, 1989), while expectant fathers have higher systemic levels of oxytocin than do virgin or non-breeding males (Gubernick et al., 1995). Moreover, mated males with pregnant mates have higher testosterone levels than both new fathers and virgins (Trainor et al., 2003); however, fathers have higher levels of aromatase (the enzyme that converts testosterone to estradiol) in the medial preoptic area of the brain (Trainor et al., 2003; a region important for expression of paternal behavior; Lee and Brown, 2002), and testosterone has been shown to increase paternal behavior in California mice via conversion to estradiol (Trainor and Marler, 2001, 2002). Because testosterone is important for paternal care in this species, and because GCs and stress have been shown to decrease circulating testosterone levels (Waite et al., 2009; Wingfield and Sapolsky, 2003), dampening HPA activity around the time of reproduction may help promote and preserve paternal care. Little is known,

however, about how GC (in California mice, CORT) dynamics change with reproductive status in this species.

In this study we evaluated the effects of reproductive status on HPA-axis function in male California mice by characterizing HPA activity and reactivity in virgin males, non-breeding males (pair-housed with tubally ligated females), and first-time fathers. Specifically, we aimed to determine whether fatherhood (or cohabitation with a female) influences 1) baseline HPA activity (diurnal rhythm), 2) HPA response to an acute stressor (predator urine), 3) HPA responsiveness to GC negative feedback (dexamethasone injection), 4) adrenal responsiveness to pharmacological stimulation (corticotropin-releasing hormone [CRH] injection) and, 5) response of the hypothalamic-pituitary-gonadal axis, measured via circulating testosterone, to an acute elevation of CORT. The behavior hypothesis predicts that fathers should show decreased amplitude of the diurnal rhythm in CORT (or an overall reduction in CORT release across the diurnal cycle), a blunted CORT increase following acute stress, increased sensitivity to GC negative feedback, and/or a blunted CORT response to CRH stimulation, as compared to virgins and non-breeding males. In addition, first-time fathers should be more resistant to suppression of testosterone secretion following an increase in CORT, when compared to virgin or non-breeding males, as testosterone is important for paternal behavior in this species.

METHODS

Animals

Animals were bred and housed at the University of California, Riverside (UCR). The UCR colony was started in 2007 with mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC). Mice were housed in polycarbonate cages (44 x 24 x 20 cm) lined with aspen shavings; cotton wool was provided for nesting material. Mice had *ad libitum* access to food (Purina rodent chow 5001) and water. Lights were maintained on a 14:10 light:dark cycle with lights on at 0500h and lights off at 1900h, and ambient temperature was maintained at approximately 23^0C with humidity around 65%. Animals were weaned from their birth cage at 27-32 days of age (prior to the birth of any younger siblings), ear punched for identification and housed in same-sex groups of four until the experiment began.

At the start of the experiment mature male mice were randomly placed into one of three conditions (virgin males, non-breeding males, first-time fathers; n=12 per condition). A power analysis, conducted in G*Power (Faul et al., 2007) using data from a previous study on diurnal rhythms in CORT (Harris et al., in review), indicated that our samples sizes yielded power of >99%.

Virgin males were housed with an unrelated, age-matched male; non-breeding males were housed with a tubally ligated female (see below); and breeding males were housed with an intact female. Non-breeding males were expected to pair-bond (form an emotional attachment) and mate (see Bosch et al., 2009) with the female, but without conception. After being placed in one of the reproductive conditions, all animals were

weighed twice per week in order to monitor body condition and to detect pregnancy in the females from the breeding group. Body mass at the start of the experiment did not differ among the three groups of males (44.48 ± 1.29g, mean ± SEM; range = 30.00-60.02 g). Moreover, male age at the beginning of data collection did not differ significantly among groups (175.0 ± 2.1 days, range = 148-200 days). Data collection on first-time fathers occurred within the first 3 weeks following the birth of the pair's first litter, and data collection in the other groups was time-matched to that in breeding males. 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*.

Experimental design

Data collection spanned 14 days for each mouse. Latency from formation of breeding pairs to birth of each pair's first litter was 36.3 ± 0.7 days (range: 33-41 days), and litters contained an average of two pups (range: 1-3 pups); testing began when pups were 0-5 days old (2.2 ± 0.5 days). During the first week of data collection two blood samples were collected from each male for baseline CORT analysis, one at 0800h and one at 2000h to capture both the nadir and peak of the daily rhythm in baseline CORT levels, respectively (Harris et al., 2012). Consecutive samples from each individual mouse were separated by two days, and the order of sample collection was approximately balanced across animals within each reproductive condition. One week after collection of

the first baseline blood sample, mice were exposed to coyote urine (see below) for 5 min, and a blood sample was collected immediately following exposure. One week after predator-urine exposure (14 days after the initial baseline blood sample), each mouse underwent a combined DEX/CRH challenge (see below), after which it was euthanized by CO_2 inhalation, and additional blood was collected by cardiac puncture for analysis of testosterone concentration. Right-side adrenal glands and testes were dissected out, placed in physiological saline, blotted three times on a paper towel, and weighed to the nearest 0.0001 g.

Tubal-ligation surgery

Female mice were tubally ligated using antiseptic techniques and standard surgical procedure. Briefly, mice were anesthetized with isoflurane gas, a ventral midline incision (approximately ½ cm) was made, the uterus was located and the ends of the right and left uterine horns were tied off using absorbable sutures (Monomend MT, Veterinary Products Laboratories, Phoenix, AZ). The oviducts were then located and severed using microscissors. All reproductive structures were repositioned back in the abdominal cavity, the abdominal incision was closed with absorbable sutures and the skin was sealed using tissue glue. Mice were given an injection of Ketoprofen (5 mg/kg, s.c.) to provide analgesia and allowed to recover in isolation for 7 days, after which time they were paired with a male for formation of non-breeding pairs. Upon termination of the

experiment, tubally ligated females were sacrificed with CO_2 and dissected to check for pregnancy; none of these females had visible embryos or fetuses at the time of sacrifice.

Blood-sample collection

Mice were anesthetized with isoflurane, and blood samples (70-140 µl) were collected from the retro-orbital sinus using heparinized glass microhematocrit tubes. Time from disturbance of the cage or end of the test to collection of the blood sample was less than 3 minutes, with one exception (67.44 ± 1.69 sec; range: 36-210). Blood samples were centrifuged for 12 min (13,300 rpm, 4°C), and plasma was collected 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 has been validated for this species (Chauke et al., 2011). The standard curve ranged from 12.5 ng/ml (91% bound) to 1000 ng/ml (20% bound), and inter- and intra-assay coefficients of variation (CVs) were 10.7% and 4.11%, respectively (N = 45 assays). Samples from each mouse were analyzed in the same assay run, and treatment conditions were balanced across assays to minimize assay-induced variation.

Testosterone enzyme immunoassay

Plasma concentrations of testosterone were measured at the Assay Services Laboratories at the Wisconsin National Primate Research Center (University of Wisconsin – Madison, WI, USA) using procedures validated for *P. californicus* (Trainor and Marler, 2001). Briefly, samples were extracted with ethyl ether, and steroids were separated using celite chromatography. Total testosterone was analyzed in duplicate using an enzyme immunoassay (T antibody R156, University of California, Davis diluted to 1:35,000). Assay sensitivity at 90% binding was 0.9 pg, and inter- and intra-assay coefficients of variation (CVs) were 15.5% and 3.9%, respectively (N = 54 assays).

Predator-odor exposure

Males were stressed alone without their adult cagemate or pups present. We chose to isolate males during predator-urine exposure for two reasons: 1) not all males had pups, and 2) the presence of pups has been shown to increase the response to a psychological stressor in rat dams (Deschamps et al., 2003). Between 0800 and 0930h, males were removed from their home cage, placed into a new cage that contained clean bedding and no food or water, and taken to a testing chamber. A cotton ball soaked with 1ml of coyote *(Canis latrans)* urine (Maine Outdoor Solutions, Hermon, ME) was then placed in the corner of the cage for 5 min. Immediately after exposure, mice were blood sampled and then returned to their home cage. Predator-odor stress has been used in our

lab previously (see Chauke et al., 2011 for details) and produces a robust CORT response in California mice at this time of day (Harris et al., 2012).

Dexamethasone and corticotropin-releasing hormone injections

On the day prior to the DEX/CRH challenge mice were weighed to permit calculation of accurate, body-mass-corrected hormone doses. On the last day of data collection (day 14) males were injected i.p. with 10 mg/kg dexamethasone sodium phosphate (DEX; 4mg/ml, American Regent, Inc., Shirley, NY) at 0730-0830h and then placed back into their home cage. This dose of DEX has previously been shown to suppress plasma CORT levels of male California mice at 8 h following injection (Harris et al., in review).

On day 14, 8 h following DEX injection, each male was blood sampled, injected with CRH (C3042, Sigma Aldrich, St. Louis, MO; 4 ug/kg, i.p.) at 1530-1630h and then placed back into its home cage. Forty-five min after CRH injection each mouse was blood sampled and the animal was again returned to its home cage. Finally, 90 min after CRH injection, another blood sample was collected from the retro-orbital sinus, mice were euthanized by CO_2 inhalation, and additional blood was collected by cardiac puncture for analysis of testosterone concentration. CRH was diluted in sterile water to a 1 ug/ml solution, and injection doses were based on male body mass. Dose was determined via pilot studies in our laboratory (data not shown), which indicated that this dose could successfully elevate CORT 8 h after DEX suppression.

Analysis

Data were checked for normality using the Shapiro-Wilk test and transformed if necessary. All CORT and testosterone values were $\log_{10}$-transformed prior to analysis, but non-transformed values are presented for ease of interpretation. CORT data were analyzed via ANOVA, and area under the curve was calculated in two ways to quantify total CORT release over time following DEX/CRH injection. The first, AUCg, represents the total amount of hormone produced over time with respect to a starting value of zero, thus not accounting for baseline (post-DEX) levels of circulating hormone. The second, AUCi, characterizes the sensitivity of the HPA axis to CRH by evaluating the amount of hormone produced above the starting baseline level (thus taking post-DEX CORT values into consideration) (Pruessner et al., 2003). Associations between CORT and testosterone concentrations were evaluated using Pearson's correlation. Body masses at the start of data collection and on the day prior to dissection were analyzed via repeated-measures ANOVA; age was analyzed via one-way ANOVA. Testis and adrenal masses were analyzed using ANCOVAs with day-13 body mass as a covariate, following the methods of Tomkins and Simmons (2002), except that organ mass was not subtracted from body mass due to differences in the number of significant figures (mass was measured to the nearest 0.01 g and organs to the 0.0001 g).

RESULTS

Basal CORT concentrations

All mice had higher circulating CORT concentrations at 2000h when compared to 0800h (1500.4 ± 81.8 vs. 35.6 ± 4.00 ng/ml, respectively; $F_{1,33}$=1043.02, P<0.001; Fig. 3.1), but CORT levels did not differ significantly among the three groups ($F_{2,33}$=0.07, P=0.993), nor was there a time*group interaction ($F_{2,33}$=2.37, P=0.108). Additionally, a planned comparison between virgin males and fathers did not reveal an effect of reproductive condition on CORT concentrations at either 2000h (t_{22}=1.36, P=0.094) or 0800h (t_{22}=0.89, P=0.253; 1-tailed, independent-samples t-tests). For all animals analyzed together, baseline CORT levels at the two time points were not correlated (r= 0.133, n=36, P=0.439); thus, higher 0800h CORT values were not associated with higher 2000h CORT concentrations.

CORT response to predator urine

Five-minute exposure to coyote urine elicited a significant increase in plasma CORT above time-matched baseline levels (425.80 ± 66.73 vs. 36.60 ± 4.00 ng/ml, respectively; $F_{1,33}$=208.33, P<0.001; Fig. 3.2). However, reproductive status did not affect the CORT response to predator urine ($F_{2,33}$=0.54, P=0.586), nor was there a status*time interaction ($F_{2,33}$=0.59, P=0.560). Baseline CORT values at 0800 h were not correlated with post-stress values (r=0.284, n=36, P=0.093).

CORT response to DEX and CRH

One post-DEX plasma sample was lost during processing. Therefore, data are available from 12 virgin males, 12 non-breeding males, and 11 first-time fathers. CORT levels at 1530-1630h, 8 h following DEX injection, did not differ among reproductive conditions ($F_{2,34}$=0.05, P=0.948; Fig. 3.3). Subsequent CRH injection caused an increase in plasma CORT, as reflected in a significant main effect of time ($F_{2,64}$=67.57, P<0.001), but CORT levels were not influenced by reproductive condition ($F_{2,32}$=0.29, P=0.744), nor was there a time*reproductive condition interaction ($F_{4,64}$=0.35, P=0.842). Sidak-corrected post-hoc tests revealed that CORT concentrations at both 45 and 90 min post-CRH were higher than those 8 h following DEX injection (t=5.79, P<0.001; t=10.47, P<0.001, respectively; Fig. 3.3). Additionally, plasma CORT was higher at 90 min than at 45 min post-CRH injection (t=6.73, P<0.001). Post-DEX CORT concentrations correlated positively and significantly with both post-CRH measures (45 min: r=0.478, n=35, P=0.004; 90 min: r=0.338, n=35, P=0.047); thus, mice that had higher CORT levels before CRH injection also had higher CORT levels after CRH injection. The two post-CRH CORT values were also positively correlated (r=0.749, n=36, P<0.001).

In addition to repeated-measures ANOVA, we analyzed time-integrated CORT responses to CRH using two calculations for area under the curve (AUC), as described above. Reproductive condition did not influence AUCg ($F_{2,32}$=0.42, P=0.664) or AUCi ($F_{2,31}$=0.23, P=0.795).

Testosterone

Data from five animals were omitted due to assay problems, leaving data from 31 males in the analysis (10 virgin males, 10 non-breeding males, 11 first-time fathers). Reproductive condition did not affect plasma testosterone concentrations measured 90 minutes after CRH injection ($F_{2,28}$=0.18, P=0.838). Testosterone concentration averaged 409.06 ± 56.31 pg/ml with a range of 88.21-1203 pg/ml, consistent with testosterone concentrations previously reported for virgin male California mice around the same time of day (Oyegbile and Marler, 2005). Since neither testosterone nor CORT levels differed with reproductive condition, the post-injection $\log_{10}$ hormone concentrations were analyzed for all three conditions together. Testosterone concentrations at 90 min post-CRH were positively correlated with CORT concentrations at both the 45- and 90-min post-CRH time points (r=0.489, n=31, P=0.005; r=0.396, n=31, P=0.028; respectively), but not with post-DEX injection CORT levels prior to CRH injection (r=0.200, n=30, P=0.289).

Body mass

Body mass did not change significantly the day prior to the start of data collection to the day before dissection, 13 days after the first baseline blood sample (44.48 ± 1.29 vs. 44.76 ±1.43 g; $F_{1,32}$=2.01, P=0.166). Additionally, reproductive condition did not

influence body mass ($F_{3,32}$=1.47, P=0.245), and there was no reproductive condition*time interaction ($F_{2,32}$=1.58, P=0.221).

Testis and adrenal mass

Body mass did not differ among groups on the day prior to dissection (44.76 ± 1.43 g, range 31.10-62.99 g; $F_{2,33}$=2.28, P=0.119), and this variable was used as a covariate for organ-mass analyses. Adrenal mass data did not pass the Shapiro-Wilk test and were transformed by taking values to the 0.5 power. Initially, ANCOVAs were computed using organ mass, body mass, and the organ mass*body mass interaction. For both organs of interest the interaction term was not significant and was dropped from the model; body mass remained in the model as a covariate, even though it was not a significant term in either instance.

Adrenal mass differed significantly among reproductive conditions (F=4.70, P=0.016; Fig. 3.4). Pairwise comparisons showed that adrenal mass was lower in fathers than in both virgin (t=2.86, P=0.007) and non-breeding (t=2.50, P=0.024) males but did not differ between the latter two groups (P=0.522; Fisher's LSD pairwise comparisons). Right testis mass did not differ significantly among the three reproductive conditions in a two-tailed test (F=2.92, P=0.068), but non-breeding males tended to have heavier testes than the other two reproductive conditions (non-breeding males: 0.216 ± 0.016; virgin males: 0.163 ± 0.019; first-time fathers: 0.175 ± 0.013 g).

DISCUSSION

This experiment addressed several predictions related to the behavior hypothesis of glucocorticoid regulation. We predicted that first-time California mouse fathers would show decreased amplitude of the CORT diurnal rhythm, a blunted CORT increase following exposure to an acute predator-odor stressor, increased sensitivity to glucocorticoid negative feedback, and/or a blunted CORT response to CRH, as compared to virgin males and non-breeding males. In addition, we predicted that first-time fathers would be more resistant to CORT-induced suppression of testosterone levels when compared to virgin or non-breeding males, as testosterone promotes paternal behavior in this species (Trainor and Marler, 2001, 2002). Contrary to our predictions, first-time fathers did not show any differences in baseline, post-stress, DEX-suppressed, or CRH-stimulated CORT concentrations when compared to non-breeding and virgin males; CORT levels of the three groups were statistically indistinguishable at each time point measured. Additionally, circulating levels of testosterone did not differ among groups, but testosterone values were positively correlated with post-CRH CORT values at both 45 and 90 min post-CRH injection. However, since we were not able to measure baseline testosterone concentrations we cannot be certain that virgin males, non-breeding males and first-time fathers had equivalent resting testosterone values. Therefore, despite the lack of difference in testosterone concentrations between the groups at 90 min post-CRH injection, we cannot determine the magnitude of change in the face of acute CORT elevation.

Although the three reproductive groups did not show differences in circulating hormone levels, differences in adrenal mass were detected: adrenal glands of breeding males weighed significantly less than those of non-breeding and virgin males. Previous studies of other rodents have noted differences in adrenal mass with reproductive condition and season, but results are mixed. Sexually active male mountain voles (*Microtus montanus*) had significantly smaller adrenal glands than sexually inactive males (McKeever, 1959), whereas adrenal mass of male red-backed voles (*Clethrionomys rutilus*) remained relatively consistent throughout the year (Sealander, 1967). Conversely, adrenal glands of male pine voles (*M. pinetorum*) were larger during the reproductive period than during the nonbreeding season, and it was hypothesized that this increase was due to increased social stress during the breeding season (Valentine and Kirkpatrick, 1970). The functional significance of changes in adrenal size is not entirely known, but adrenal size has been shown to correlate with circulating levels of GC in some species (for a brief review see Romero, 2002). However, CORT levels did not differ between reproductive conditions in this study. Without histological analysis to determine which portion(s) of the adrenal gland (medulla and/or one or more of the three cortical layers) differs among reproductive groups, few conclusions can be drawn from adrenal-mass results at this time. Future studies could characterize adrenal histology or measure aldosterone and/or DHEA levels, as both of these hormones are produced in the adrenal cortex and have been suggested to change with parental status in males of biparental species (Bardi et al., 2011; Schradin, 2007).

The findings from our study of California mice suggest that the behavior hypothesis does not explain HPA function in this biparental rodent. Reproductive status does not appear to modulate circulating CORT concentrations or HPA-axis dynamics in males, and changes in CORT levels do not appear to be necessary for breeding or expression of parental behavior. This conclusion is further supported by a previous experiment showing that injecting new California mouse fathers with a supra-physiological dose of CORT does not reduce paternal care or influence pup survivorship (Harris et al., 2011). Additionally, neither morning basal nor post-stressor (predator-urine exposure) CORT levels differed among first-time fathers, vasectomized males housed with a female, and virgin males housed with another male (Chauke et al., 2011). In the latter study, the fathers' pups (and pairmate) were present when the fathers were exposed to predator odor. In other mammals, offspring presence either increases (rats; Deschamps et al., 2003) or decreases (sheep; Tilbrook et al., 2006) the mother's HPA response to an acute stressor. In the current study pups were not present during predator-urine stress, but there was no difference in post-urine-exposure CORT levels between virgin males, non-breeding males and first-time fathers. In sum, these previous and current findings suggest that paternal care in California mice is not likely to be mediated by GCs, and that male reproductive status does not alter basal or stress-induced CORT release or HPA dynamics.

Most of the data in support of the behavior hypothesis have come from a variety of bird species analyzed during the breeding season, and some of the most convincing data come from extreme environments, e.g. the Arctic (Romero, 2002; Wingfield and

Romero, 2001; Wingfield and Sapolsky, 2003). A review of the mammalian literature, with particular focus on males, suggests that the behavior hypothesis does not seem to be as well supported as in birds. In biparental prairie voles (Campbell et al. 2009), no differences in plasma CORT were found among virgin males, paired males, and fathers, mirroring our findings in California mice. Male striped mice (*Rhabdomys pumilio*) switch between three different reproductive tactics –philopatric (alloparental), roamers (not paternal), and breeders (paternal) – and longitudinal data show that baseline plasma CORT levels are higher during life history states that involve care of pups, contrary to the behavior hypothesis (Schradin and Yuen, 2011). In biparental golden lion tamarins (*Leontopithecus rosalia*), fecal GC levels did not differ across male reproductive conditions or between the mating and infant-care seasons (Bales at al., 2006). Similarly, in biparental common marmosets (*Callithrix jacchus*), plasma cortisol levels did not differ among males that were singly housed, pair-housed with a female, or family-housed with a mate and offspring (Ziegler et al., 2005).

These studies are consistent with our findings that reproduction is not associated with decreased CORT levels in California mice. However, a study on human males showed that fathers had lower salivary cortisol levels than did non-fathers (Berg and Wynne-Edwards, 2001). Similarly, parenting-oriented (pairbonded or fathers) men had lower morning and evening salivary cortisol levels than did mating-oriented (non-pairbonded, non-fathers) men (Gettler et al., 2011). The majority of studies on male mammals have been correlational or have focused on changes in hormone levels either before, during, or immediately following a bout of paternal care. Additional studies are

needed in order to provide a more comprehensive data set on the relationship between reproductive status and GC modulation in mammalian fathers.

The absence of detectable differences in CORT levels among male reproductive groups in California mice may be related to features of this species' physiology and/or life history. First, despite the fact that we used a highly controlled experimental design, it is possible that our measure of HPA function was not specific enough. California mice have high levels of circulating GCs as compared to most mammals, and are somewhat resistant to GC negative feedback (Harris et al., 2012). Corticosteroid receptors in this species might have low affinity for GCs, as reported in other species with high GC concentrations (prairie voles; Taymans et al., 1997; New World primates and guinea pigs (*Cavia sp*.); Fuller et al., 2004). Thus, it is possible that corticosteroid receptors, and not plasma levels of CORT, are the main site of modulation of HPA function. Supra-physiological doses of CORT do not disrupt paternal behavior in California mice, and daily peak basal levels of CORT are higher than some post-stress concentrations (Harris et al., 2011; Harris et al., in review), further suggesting that circulating CORT levels may not be the major site of GC-activity regulation. Additionally, corticosteroid-binding globulin (CBG) and/or 11β-hydroxysteroid dehydrogenase, the enzyme that converts CORT to its inactive form, might also play a role in modulating functional levels of CORT (Michael et al., 2003). Analysis of receptor number and density, as well as CBG and 11βHSD activity, might provide illuminating results.

A second possible explanation involves the social organization of California mice. In nature, California mice mate for life, breed almost year-round, and are thus

almost always in a pair-bonded situation with a single partner (Gubernick and Teferi, 2000; Ribble 1991, 1992; Ribble and Salvoni, 1990). Thus, pairing, and not birth of offspring, may trigger modulation of the HPA axis. All mice in our experiment were pair-housed with either a female or another male, but in the wild these animals do not naturally live in male-male pairs, and any dyad of wild mice is likely to be breeding and caring for young. It is possible that using any socially housed animal in the lab mimics the natural living conditions of a reproductive pair, thus making our treatment groups almost indistinguishable. Previous data on California mice lend support to this possibility. CRH and Fos expression in the paraventricular nucleus of the hypothalamus, both under baseline conditions and in response to a predator-odor stressor, did not differ between males that were pair-housed with either another male or a female (with or without pups), but did differ between these groups of pair-housed males and singly housed males (Chauke et al., 2012). Moreover, socially isolated males had higher baseline CORT levels than males paired with a female (Glasper and DeVries, 2005). It could be that laboratory breeding has selected for males that form amicable male-male pairs, and that these males respond to being paired with another male in a similar manner as they would if the pairmate were female, at least with respect to HPA function.

Lastly, California mice are naturally found in areas with mild climates – the mountains of central and southern California – and can (and do) breed almost year-round (Gubernick, 1988; Merritt, 1978). Therefore, it is possible that these mice do not need to fine-tune the HPA response because they rarely experience periods of extreme conditions, at least in comparison to species breeding in very severe (e.g., polar or highly

seasonal) climates. Support for this possibility has been found in birds; HPA modulation is more pronounced in species from harsh or extreme habitats as compared to their more temperately located relatives (see Romero, 2002; Wingfield, 1994; Wingfield and Romero, 2001).

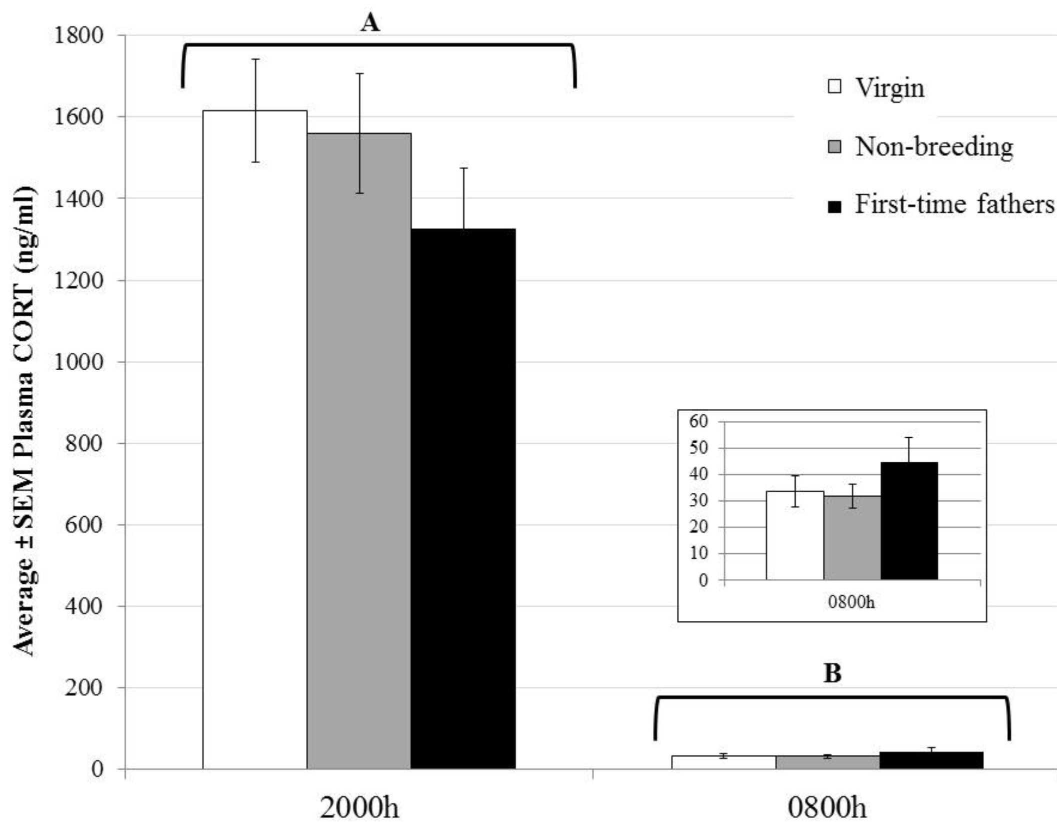

Figure 3.1: Baseline levels of plasma CORT in adult, male California mice at 2000h and 0800h (14:10 L:D cycle; lights on at 0500h). Plasma CORT concentrations did not differ among virgin males, non-breeding males, and first-time fathers (n=12 per group) at either time point, but CORT was higher at 2000h than at 0800h regardless of reproductive condition (A vs. B; $P<0.001$). For a more detailed view of 0800h CORT see inset.

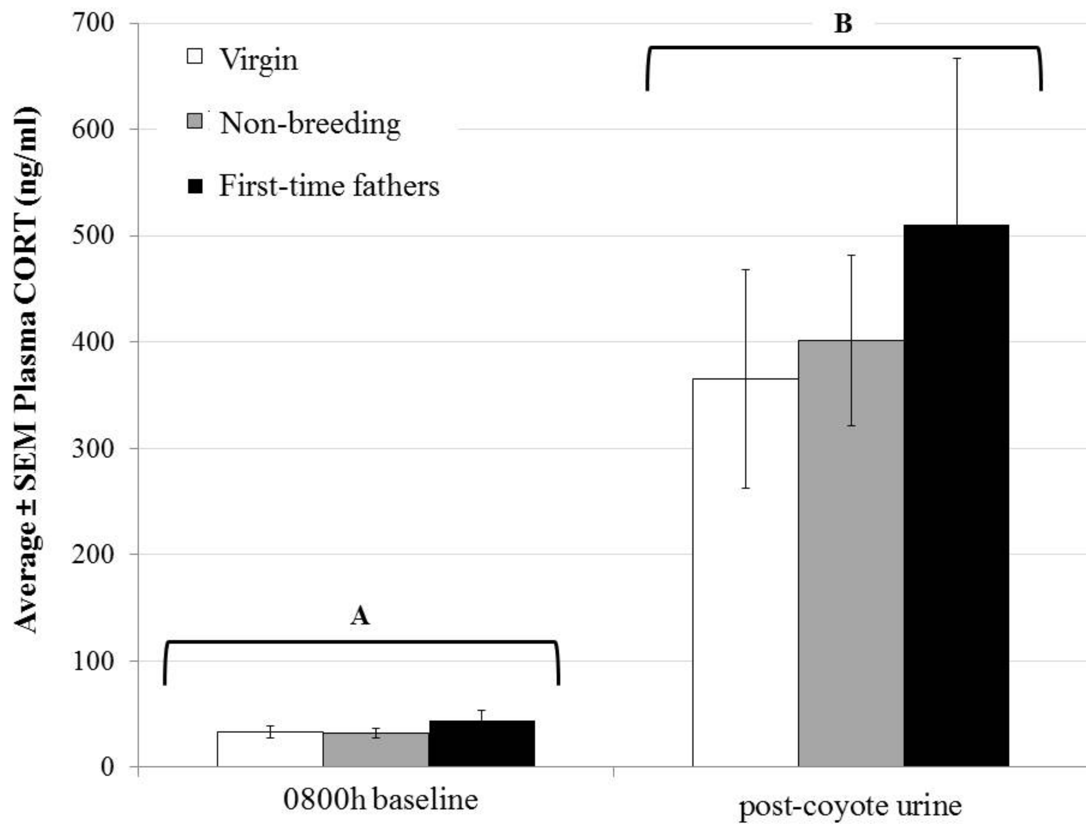

Figure 3.2: Plasma CORT concentrations in adult, male California mice following 5-min exposure to 1 ml coyote urine between 0800 and 0930h. Virgin males, non-breeding males and first-time fathers (n=12 per group) did not differ in their response to predator urine, but predator-urine exposure did elevate CORT in all animals (main effect of time, A vs. B; P<0.001).

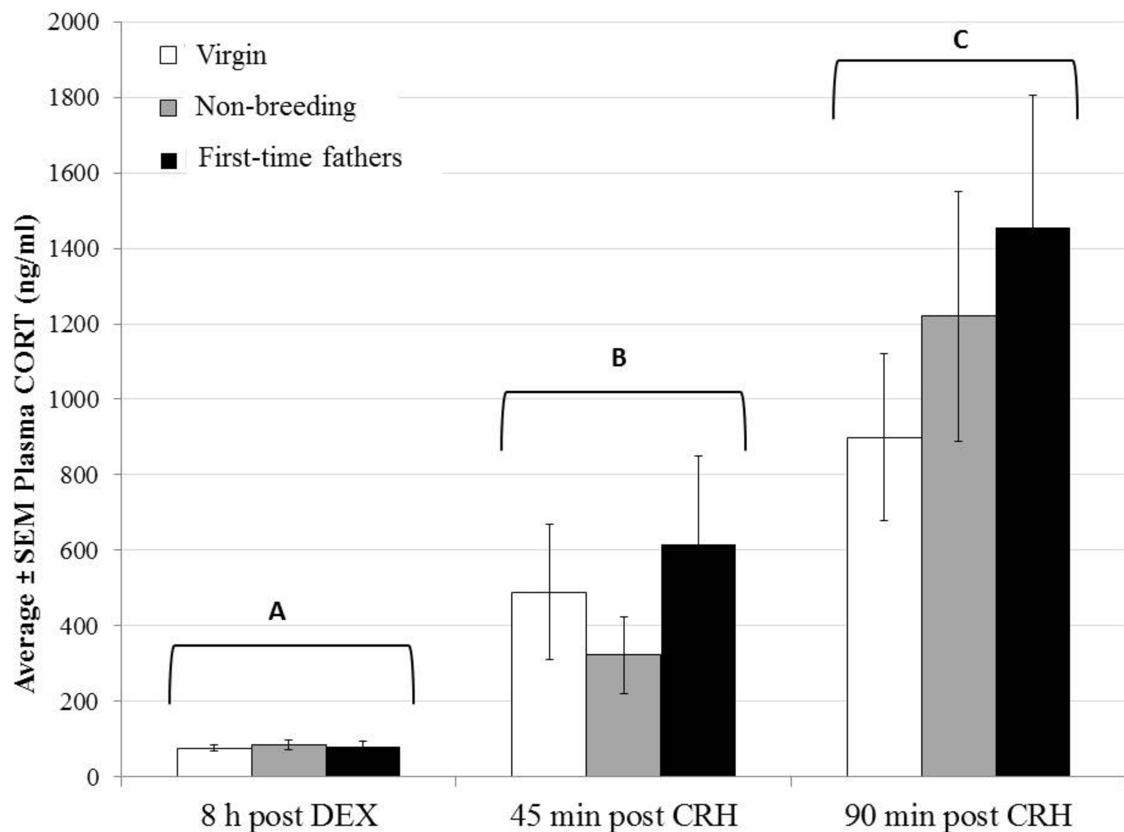

Figure 3.3: Effects of dexamethasone (DEX; injected at 0730-0830 h) and corticotropin-releasing hormone (CRH; injected at 1530-1630 h, 8 h after DEX injection) on plasma CORT concentrations in adult, male California mice. Virgin males (n=12), non-breeding males (n=12) and first-time fathers (n =11) did not differ in circulating CORT concentration within any time point. Irrespective of group, plasma CORT levels changed over time; results for the main effect of time ($P<0.001$) are displayed (time points with different letters differed significantly from one another following Sidak-corrected post-hoc tests, $P<0.001$).

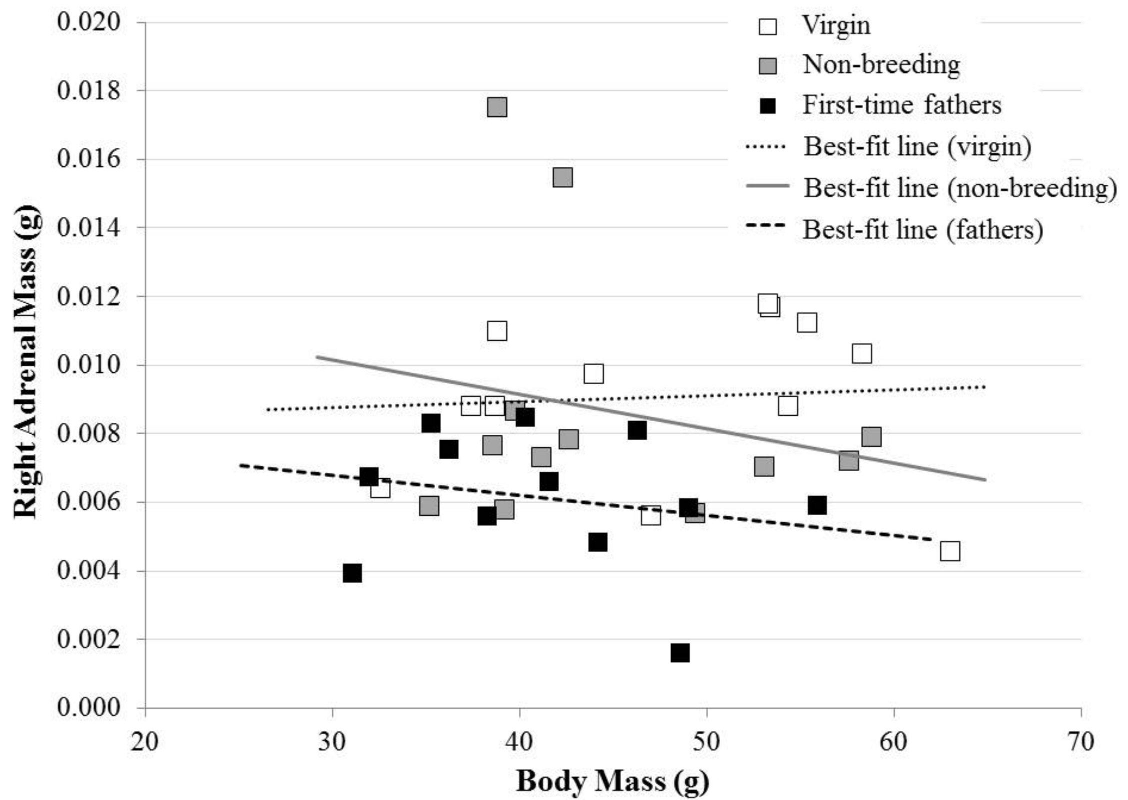

Figure 3.4: Right adrenal mass vs. body mass for adult, male California mice (n=12 per group). Body mass was used as a covariate but was not significant in the analysis model; for this reason the lines graphed above are best-fit lines and not ANCOVA lines. Adrenal glands from new fathers weighed less than did adrenals from non-breeding (P=0.024) and virgin males (P=0.007).

Chapter 4

Effects of chronic variable stress on paternal behavior and pup development in the biparental California mouse (*Peromyscus californicus*).

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ABSTRACT

Stress and chronically elevated glucocorticoid levels have been shown to disrupt parental behavior in mothers; however, almost no studies have investigated corresponding effects in fathers. The present experiment aimed to test the hypothesis that chronic stress would inhibit paternal behavior and consequently alter pup development in the monogamous, biparental California mouse (*Peromyscus californicus*). First-time fathers were assigned to one of three experimental groups: chronic variable stress (n=8), separation control (n=7), or undisturbed control (n=8). The chronic variable stress paradigm (3 stressors per day for 7 days) successfully stressed mice, as evidenced by increased baseline plasma corticosterone concentrations, increased adrenal and decreased thymus mass, and a decrease in body mass over time. We did not observe a differential corticosterone response to a novel stressor at the end of the chronic stress paradigm, nor did spleen or testis mass differ among experimental groups. Chronic stress reduced paternal behavior, but major differences were observed only on day 6 of the 7-day paradigm. At that time point, chronically stressed fathers spent less time with the pairmate and pups, and more time autogrooming, as compared to separation controls and undisturbed controls. Chronic stress in fathers did not appear to alter survival or development of their offspring: pups from the three experimental conditions did not differ in body mass gain over time, in day of eye opening, or in basal or post-stress corticosterone levels. These results demonstrate that chronic stress can disrupt paternal behavior in *P. californicus* fathers, but effects were subtle and did not alter pup development under the present conditions.

INTRODUCTION

Vertebrate animals respond to challenging situations by mounting a highly conserved stress response that includes activation of the hypothalamic-pituitary-adrenal (HPA) axis (Armario, 2006; Sapolsky, 2002;). This response promotes survival under stressful or demanding organismal or environmental conditions (Charmandari et al., 2005; McEwen 2005; Sapolsky, 2002) by inhibiting non-essential activities and processes (e.g., digestion, anabolism, reproduction) in order to promote functions that are immediately necessary (e.g., mobilization of glucose, vigilance, catabolism; Sapolsky, 2002; Sapolsky et al., 2000). In other words, stress has been implicated in mediating a trade-off between current and future reproductive effort, including both sexual and parental behavior (see Breuner et al., 2008; Moore and Hopkins, 2009; Ricklefs and Wikelski, 2002). Accordingly, stress, especially pronounced, prolonged stress, is predicted to result in decreased parental care and lead to decreased current offspring survival (Wasser and Barash, 1983; Wingfield and Sapolsky, 2003; Wingfield et al., 1998). Because glucocorticoids increase rapidly in response to stressors and affect numerous functions and processes, they are predicted to play a major role in the behavioral changes that occur in response to stress (McEwen 2005; Sapolsky et al., 2000; Wingfield and Sapolsky, 2003).

Consistent with these predictions, stress, both acute and chronic, has been shown to disrupt maternal behavior in females, and this disruption seems to be mediated at least in part by glucocorticoids. For example, mouse (*Mus musculus*) mothers showed a

decrease in pup retrievals following 30 min of restraint stress when compared to non-stressed mothers (Yamada et al., 2002). Similarly, rat (*Rattus norvegicus*) mothers retrieved fewer pups and took longer to engage in nursing during a 10-min exposure to predator odor, than did mothers under control conditions (Sukikara et al., 2010). Additionally, rat mothers exposed to the stressor of a novel cage with limited bedding for 5 min were more abusive toward foster pups than were mothers who were given ample bedding and allowed to habituate to the new cage (Roth and Sullivan, 2005). Comparable effects have also been noted in the face of chronic stress. Chronic social stress in rat mothers, implemented by a daily 1-h introduction of an unfamiliar male to the mothers' cage, resulted in decreased maternal care (pup grooming and nursing) and increased latency to initiate nursing as compared to control mothers (Nephew and Bridges, 2011). Similarly, rat mothers exposed to wet bedding and a forced foraging paradigm did not nurse pups as often as control mothers (Léonhardt et al., 2007), and rat mothers given inadequate nesting materials exhibited more fragmented maternal care and spent more time away from pups than did control mothers (Ivy et al., 2008).

These effects of chronic stress may be mediated by glucocorticoids, as chronic corticosterone injection, in the absence of experimentally induced stress, decreased the time spent nursing and time in contact with pups in rat mothers, as compared to vehicle-injected mothers (Brummelte et al., 2006). Similarly, marmoset mothers (*Callithrix jacchus*) that were chronically injected with cortisol spent less time carrying infants when compared to vehicle-injected mothers (Saltzman and Abbott, 2009). Taken together,

these results support the hypothesis that stress, as well as glucocorticoid elevation, can disrupt parental behavior in mothers.

Few studies, however, have addressed the relationship between stress and parental behavior performed by fathers. In the majority of mammalian species the mother is the sole provider of parental care, but in 6-10% of mammals, including humans, males also invest heavily in offspring (Kleiman and Malcolm, 1971). In addition, 90% of bird species are biparental (Lack, 1968), and ~46% of amphibian and ~60% of fish families exhibit some form of paternal care (Gross and Shine, 1981). In species in which it occurs, paternal care greatly enhances offspring survival and development (e.g., Galilaea tilapia, *Sarotherodon galilaeus*, Balshine-Earn, 1997; *Mus musculus*, Barnett and Dickson, 1985; *Peromyscus spp.*, Bester-Meredith and Marler, 2001; *P. californicus*, Bredy et al., 2004; *Homo sapiens*, Lamb, 2010; degu, *Octodon degus*, Ovtscharoff et al., 2006; gerbils, *Meriones unguiculatus,* Piovanotti and Vieira, 2004; striped mice, *Rhabdomys pumilio*, Schradin and Pillay, 2004; termites, *Zootermopsis nevadensis*, Shellman-Reeve, 1997; however, almost nothing is known about how stress can alter paternal investment in offspring.

Only two studies have specifically investigated the effects of stress on paternal care. In California mouse (*P. californicus*) fathers, a 5-min exposure to predator odor did not alter licking/grooming of pups or the father's proximity to pups, when compared to behavior 5 min prior to odor exposure (Chauke et al., 2011), suggesting that a very brief acute stressor was not sufficient to alter care. A second study experimentally investigating the effects of stress on males of a biparental species was conducted in *virgin*

male prairie voles (*Microtus ochrogaster*) and produced conflicting results: following a forced-swim stressor, males' corticosterone levels were negatively correlated with licking and grooming of pups, but positively correlated with pup retrievals (Bales et al., 2006), suggesting that a more potent acute stressor may alter some aspects of pup care by males. Correlational studies of fathers' prepartum baseline corticosterone levels have also produced varying results: studies of humans and hamsters (*Phodopus spp.)* suggested that fathers' baseline glucocorticoid levels naturally increase prior to the birth of offspring (Berg and Wynne-Edwards, 2001; Reburn and Wynne-Edwards, 1999), but a study in biparental Oldfield mice (*Peromyscus polionotus*) indicated that increased corticosterone concentrations in fathers leading up to the birth of pups were associated with decreased pup survival (Good et al., 2005). Similarly, higher baseline cortisol concentrations have been associated with lower rates of infant carrying in marmoset fathers (*Calithrix kuhlii*; Nunes et al., 2001), but with increased male alloparental effort in meerkats (*Suricata suricatta*; Carlson et al., 2006). Thus, no consensus has emerged about how acute stress or baseline levels of corticosterone affect paternal care. Moreover, no study to date has investigated the effects of chronic stress on paternal care in mammalian fathers.

In this study we tested the hypothesis that chronic stress in mammalian fathers can disrupt paternal behavior, leading to decreased rates of growth, development and/or survival in offspring. We tested this hypothesis in the California mouse, a monogamous and biparental rodent (Ribble, 1991, 1992a,b; Ribble and Salvoni, 1990). In this species, infant care by both parents is critical to maximize offspring survival rates, accelerate offspring development, and increase parents' reproductive success, both in the lab and in

the field, especially under energetically challenging conditions (Bredy et al., 2004; Cantoni and Brown, 1997a,b; Dudley, 1974; Gubernick et al., 1993; Gubernick and Teferi, 2000; Wright and Brown, 2002).

To test our hypothesis, we exposed first-time California mouse fathers to chronic variable stress for 7 days and characterized the effects on paternal care and pup development. We predicted that chronically stressed fathers would exhibit elevated circulating corticosterone levels, decreased rates of paternal behavior and possibly increased aggression toward their pups, as compared to non-stressed control fathers, and that these behavioral effects of chronic stress would be associated with reduced survival rates, slower growth, delayed development, and/or altered circulating corticosterone levels in pups.

METHODS

Animals

California mice, descendants of mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC) in 2007, were bred and housed in the University of California, Riverside vivarium. Mice were housed in polycarbonate cages (44 x 24 x 20 cm) lined with aspen shavings, provided with ad libitum food (Purina rodent chow 5001) and water, and given cotton for nesting material. Ambient temperature was held at approximately 23°C with humidity of about 65%; lights were on from 0500-1700 (14:10 L:D cycle). Mice were weaned from their natal cage at

27-32 days of age (prior to the birth of any younger siblings), ear-marked for identification, and placed into same-sex groups of four until experimentation.

At 100-150 days of age, each male was pair-housed with an unrelated adult female (120-160 days of age). We subsequently weighed all animals twice per week in order to monitor health, determine pregnancy, and gather data for calculation of pre-experimental baseline body mass. When a female had gained substantial body mass (6-10 g) the pair was moved to a double-cage system (two regular, polycarbonate housing cages connected by a clear plastic tube approximately 10 cm in length with openings of approximately 5 cm in diameter) to facilitate the experimental manipulations. Hereafter, the double cage will be referred to as the home cage, and each individual cage within the double-cage setup will be referred to as a cage-half. 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*.

This experiment was part of a larger study that included an additional 29 experimental males. A small amount of data from the 23 males used here (fathers' body mass, organ masses, and plasma corticosterone concentrations) is also presented elsewhere (manuscript in prep) as part of the validation of the chronic variable stress paradigm.

Experimental Design

We used a total of 23 first-time fathers, each housed with a female pairmate and the pair's first litter of pups. Each father was randomly assigned to one of three experimental groups: chronic variable stress (CVS; n=8), separation control (SC; n=7), and undisturbed control (UC; n=8). Experimentation began 1-3 days postpartum; the first day of chronic stress or control procedures is referred to as day 1. CVS fathers were subjected to 7 days of chronic variable stress. SC fathers followed the same schedule as CVS fathers, except that they were isolated from their mate and pups each time CVS fathers underwent stressor exposure, but were not otherwise stressed, in order to control for the father's removal in the CVS group. UC fathers were left undisturbed except for data-collection procedures. All males were weighed four times during the period of data collection, and baseline blood samples for analysis of plasma corticosterone concentrations were collected on days 1, 4, and 8 (Table 4.1). On day 8, all fathers were exposed to a novel stressor and subsequently euthanized and dissected. Instantaneous behavioral scans were performed on each male and its cagemates 15 times and 10-minute behavioral observations were performed 4 times during the period of data collection (see below).

Growth, development and plasma corticosterone concentrations of pups were monitored to assess whether pups raised with a stressed father differed from pups raised with a non-stressed father. Pups were weighed and dye-marked for identification on post-natal day (PND) 3 and subsequently weighed on the same schedule as their father until day 8 of the CVS paradigm, after which pups were weighed, dye-marked and checked

daily. On PND19, a blood sample was collected for analysis of basal plasma corticosterone concentrations, and on PND20, pups were exposed to predator odor for 5 minutes, a blood sample was collected, and pups were euthanized.

Chronic Variable Stress Paradigm

Mice in the CVS group were exposed to seven different stressors over a 7-day period, with 2-4 stressor exposures per day (one stressor every 6-10 h, de Jong et al., in prep). We exposed CVS fathers to an average of three stressors per day because pilot studies showed that a paradigm utilizing two stressors per day was not sufficient to chronically elevate corticosterone levels in male California mice (data not shown). Stressors were as follows: 1) **wet bedding**: the mouse was placed for 1 hour in a clean cage containing a mixture of wood shavings and tap water; 2) **shaker**: the mouse was placed in a plastic container on a lab shaker cycling at 3.33 hertz for 15 minutes; 3) **injection of hypertonic saline**: the mouse was injected i.p. with 1.5M NaCl solution (1.5 ml/100g of body mass); 4) **cold exposure**: the mouse was placed in a plastic container in a refrigerator (inside temperature: 4°C) for 15 minutes; 5) **restraint**: the mouse was placed in a "decapicone" (DC M200, Braintree Scientific, Braintree, MA), which was folded tightly, clipped shut, and hung on a suspended wire so that the male was facing downward for 15 minutes; 6) **forced swim**: the mouse was placed in 850 mL tap water (24-26°C) and was forced to swim for 5 minutes; 7) **predator urine**: the mouse was placed in a clean cage containing wood shavings and exposed to a stainless steel, wire-

mesh tea-ball (diameter: 4.5 cm) containing a cotton ball saturated with 1 mL of predator urine (bobcat [*Lynx rufus*], coyote [*Canis latrans*], wolf [*Canis lupus*], or mountain lion [*Puma concolor*]; Maine Outdoor Solutions, Herman, MA) for 8 minutes. Preliminary data indicated that each stressor used in the CVS paradigm resulted in a robust, acute increase in plasma corticosterone concentration (Chauke et al., 2011; Harris et al., 2012; unpub. data).

To control for repeated removal of CVS fathers from their families, SC fathers were isolated from their mate and pups at the same times and for the same durations that CVS fathers were removed from their families for stressor exposures; however, SC fathers were not exposed to experimental stressors. To isolate SC fathers in a manner that involved minimal handling and stress, males were placed alone in one cage-half of the double-cage homecage, and the tube connecting the two halves was plugged for the duration of the CVS males' stressor. UC fathers were left undisturbed.

In order to test stress responsiveness to a novel stressor after the CVS paradigm, all fathers were injected s.c. with 0.2 mL of sesame oil on day 8 at 0900h. A previous study in rats found that animals exposed to a chronic variable stress protocol have an enhanced response to a novel stressor as compared to undisturbed controls, or to animals subjected to chronic homotypic stress (Marin et al., 2007). Blood samples were collected immediately before (baseline), and 10 and 40 min after injection to characterize the corticosterone response to a novel stressor; oil injection has been shown to markedly increase circulating corticosterone levels in male California mice (unpub. data). For the final blood sample, mice were decapitated, and trunk blood was collected into a

heparinized weigh boat. Brains were then harvested, flash-frozen in dry ice and stored for *in situ* hybridization (de Jong et al., in prep), and organs were dissected out and weighed.

Measures of Chronic Stress

Blood Sample Collection

All blood samples, except for the fathers' final one, were collected via the retro-orbital sinus using 70 µl heparinized microhematocrit tubes while mice were under isoflurane anesthesia. For retro-orbital samples, blood was always collected within 3 minutes of disturbance to the animal's cage (mean ± SEM: 60.2 ± 1.4 s; range: 30-124 s; n=88). Following decapitation the final blood sample (trunk blood) was collected in weigh boats primed with 0.1 ml of heparin and was always collected within 90 seconds of disturbance to the cage (mean ± SEM: 44.5 ± 3.7 s; range: 26-90 s; n=21; 0936 -1005 h). Immediately after collection, all blood samples were centrifuged for 12 min (13,300 rpm, 4°C), and plasma was removed and stored at -80°C until assay.

Plasma Corticosterone Assay

Plasma was assayed in duplicate for corticosterone using an ^{125}I double-antibody radioimmunoassay kit (#07-120102, MP Biomedicals, Costa Mesa, CA) previously validated for this species (Chauke et al., 2011). Samples from each experimental group

were balanced evenly across three assays; however, all samples from an individual mouse were always analyzed in a single assay run. The standard curve ranged from 12.5 ng/ml (91% bound) to 1000 ng/ml (20% bound), and plasma samples were assayed using dilutions ranging from 1:100 to 1:800 depending on anticipated CORT concentrations. Inter- and intra-assay coefficients of variation (CVs) were 11.2% and 4.7%, respectively (N = 45 assays).

Body Mass and Organ Masses

During the period of data collection, males were weighed to the nearest 0.01 g four times (days 1, 3, 5 and 7), between 0900 and 1000 h. Baseline body mass was calculated as the average of the last three body mass values prior to the start of data collection. Immediately following decapitation of fathers on day 8, organs (adrenal glands, testes, thymus, spleen) were dissected out, placed in sterile saline, blotted dry 3x and weighed to the nearest 0.00001 g.

Behavior

Instantaneous Scans

An instantaneous scan of each cage was performed immediately prior to each stressor occurring between 0700 and 2300h (15 scans total). The male's and female's

locations with respect to each other and the pups (same cage-half or different cage-half of the double cage) and movement (walking/jumping or other), and the male's behavior (paternal or non-paternal), were recorded. Behavior was considered paternal if the male was actively caring for a pup (i.e., licking, carrying, manipulating with paws or mouth, sniffing) or was passively interacting with the pup (i.e., family huddle, huddling pup, sleeping on pup, sleeping with family in a group). The scan data were organized into four bins: bin 1 (days 2-4, lights-on; n=6 scans), bin 2 (days 2-4, lights-off; n=2 scans), bin 3 (days 5-7, lights-on; n=5 scans), bin 4 (days 5-7, lights-off; n=2 scans). For each bin the proportion of total scans during which a behavior was observed was tabulated for each mouse and used for analysis.

Ten-minute Behavioral Observations

In addition to instantaneous scans, behavior was videotaped for 10 min four times during the experiment (days 2, 3, 4, and 6). Two observations (days 2 and 6) occurred immediately following either exposure to a stressor (CVS fathers) or separation from and reunion with the family (SC fathers), and two (days 3 and 4) were conducted under "baseline" conditions (no stressor or separation within the previous 6 h). Within each observation condition (post-stress or baseline), one observation was performed during lights-on and the other during lights-off conditions (see Table 4.1). For CVS fathers, post-stress observations began when males were returned to the opposite cage-half from their mate and pups, immediately following stressor exposure; for SC fathers, post-stress

observations began when the tube plugs were removed at the end of the separation period.

Videos were scored for paternal and non-paternal behaviors by a single, trained observer using JWatcher software (Blumstein and Daniel, 2007). We recorded durations of several behaviors (autogroom, family huddle [entire family huddling/sleeping together and visible to the observer], family in nest [all animals in cotton and not visible to observer], male groom female, male sniff female, male huddle pup(s), male lick pup(s), male perform kyphosis, male sniff pup(s), male carry pup(s), and male only not in view). Additionally, the numbers of jumps and rears performed by the male were counted, and measures of males' activity (walking/jumping or sitting still/sleeping) and males' and females' locations (within 10 cm of pup(s), touching one or more pups, not in the same cage-half as any pup, male/female in the same cage-half) were recorded every 30 s. Jumps were recorded as a measure of activity, and rears are thought to be associated with exploratory behavior (Espejo, 1997). Location and activity data are presented as proportion of total 30-second scans (out of 20).

Prior to analysis, related behaviors were combined into composites. Paternal behavior was calculated as the total duration of lick pup(s), huddle pup(s), and kyphosis (de Jong et al., 2009, 2010; Harris et al., 2011). Group huddle was calculated as the total duration of visible family huddling and time the family spent under the nest out of view (nests are small, and animals tend to sit together under the small piece of cotton; unpub. obs.). Investigation of the female by the male, a measure of male interest in the female pairmate, was calculated as the total duration of male groom female and male sniff

female. Carry pup(s) and sniff pup(s) did not occur frequently enough to warrant analysis of these behaviors. Additionally, no fighting or aggressive behavior was observed at any time, and mice were rarely observed eating, drinking or performing any other non-scored behaviors.

Pup Development

We collected data from the pups of 8 CVS fathers, 7 SC fathers and 7 UC fathers (39 pups in all; data from the pups of one UC father were not available). Litters contained one (N=6 litters), two (N=15 litters), or three pups (N=1 litter), as is typical for this species (Harris et al., 2011; McCabe and Blanchard, 1950). All pups were weighed and dye-marked on PND3 (regardless of fathers' experimental day) between 0830 and 1230 h. For identification, food color was applied to one limb with a cotton swab.

During the CVS paradigm, pups were weighed on the same schedule as their father to minimize disturbance to the family. Fathers were sacrificed at pup PND8-10 (day 8 of the fathers' experimental protocol). At this time, mothers and their litters were moved to a single cage, and data collection and marking of pups occurred daily thereafter. In addition to body mass, pups were checked for the developmental milestone of eye opening. On PND19, a blood sample was collected from each pup at 1430-1520 h to characterize baseline plasma corticosterone levels; pups were weighed after blood collection on this day to avoid disturbing them prior to sample collection. On the following day (PND20), pups were exposed to predator urine for 5 min (see above for

methods) and a blood sample was collected immediately afterwards, time-matched to the previous baseline sample; pups were then weighed. All blood samples were collected from the retro-orbital sinus under isoflurane anesthesia using heparinized microhematocrit tubes; time between cage disturbance or end of stress test and sample collection exceeded 3 minutes in only one instance (mean ± SEM: 90.8 ± 3.9 s; range: 49-210 s; n=77 samples). Samples were processed and stored as described above. Following the post-stress blood sample, pups were weighed, sex was determined, and pups were then sacrificed. Mothers were also sacrificed and dissected to check for evidence of pregnancy (visible embryos).

Analysis

Fathers

All data were checked for normality using the Shapiro-Wilk test and were transformed if necessary to meet normality assumptions. All corticosterone concentrations were log_{10}-transformed and analyzed via repeated-measures ANOVA. Body mass was calculated as percent change from starting (baseline) value and analyzed via repeated-measures ANOVA with Fisher's LSD post-hoc tests, as we predicted that CVS fathers would lose body mass over time. Organ masses were analyzed as total organ mass (sum of right and left sides, for testes and adrenals) via ANCOVA with day 1 body mass as a covariate; Fisher's LSD post-hoc tests were used due to *a prior* predictions. All

starting parameters (father's age, father's mass, latency to birth of first litter) were analyzed via one-way ANOVA. Behavioral data from instantaneous scans and 10-min observations were analyzed via Kruskal-Wallis tests with appropriate nonparametric post-hoc tests (Siegel and Castellan, 1988). Due to logistical and/or dissection issues, some physiological measures (day 7 body mass, day 8 blood sample, and/or organ mass) are missing for one UC father and one CVS father, leaving n=7 for both groups in certain analyses. Behavioral data from all 23 fathers were used in analysis.

Pups and females

All data were checked for normality using the Shapiro-Wilk test and were transformed if necessary to meet normality assumptions. All corticosterone concentrations were $\log_{10}$-transformed. Due to sex differences in corticosterone levels and growth in some species (McCormick et al., 1995; Weinstock, 2001; Zambrano et al., 2006), we initially performed paired t-tests using opposite-sex littermates to test for effects of sex on starting (PND3) and ending (PND20) body mass, eye opening, basal corticosterone levels and post-stress corticosterone levels. A total of 10 litters (5 CVS, 3 SC, 2 UC) contained one pup of each sex and were therefore included in these analyses. Sex of pups did not significantly affect any of the measures ($P>0.168$ for all cases) and therefore was not factored into any subsequent analyses.

After paired t-tests, pup body mass data, day of eye opening, and $\log_{10}$-transformed CORT data were each analyzed via linear mixed model with father's

treatment, time, and the time*treatment interaction as fixed factors. Family ID was entered as a random factor to control for maternal and natal-cage effects, and time was included as a repeated factor when appropriate (CORT and body mass). One UC pup was excluded from all analyses, because it was extremely small and deformed at birth and did not grow normally.

Data on presence of embryos at the time of dissection was not available for all animals, and the proportion of females with data was not consistent across experimental conditions (UC: 3/7, SC: 6/7, CVS: 7/8). These data were analyzed via Fisher's Exact test.

RESULTS

Starting Parameters

Males in the CVS, SC, and UC groups did not differ in age on day 1 of the experiment (overall mean ± SEM: 159.4 ± 3.5 days), latency from pairing until birth of pups (41.7 ± 1.7 days), number of pups born (mean: 1.9), or age of pups on day 1 of the experiment (mean: 2.0, range: 1-3; see Table 4.2). Groups did, however, differ in length of time that they were housed in the double-cage setup prior to day 1 ($F_{2,20}$=4.118, P=0.032). Fathers in the chronic variable stress condition spent less time in the double cage than undisturbed control fathers (CVS vs. UC: 4.3 ± 0.8 vs. 8.6 ± 1.6 days; t=2.59, P=0.44, Tukey's HSD); neither of these groups differed from separation control fathers (SC: 4.6 ± 1.1 days).

Effects of CVS on Body Mass, Organ Masses, and Corticosterone Levels

Body Mass

Fathers' body mass changed significantly over time (main effect of time: $F_{4,76}$=10.910, P<0.001), differed by experimental group (main effect of group: $F_{2,19}$=23.406, P<0.001), and changed differently across time among groups (time*group interaction: $F_{8,76}$=9.328, P<0.001; Fig. 4.1). Within time points, groups did not differ from one another at baseline or on day 1. By day 3, however, CVS fathers weighed less than both UC fathers (t=5.798, P<0.001) and SC fathers (t=4.024, P=0.001), but UC and SC fathers did not differ from each other. This was also true on day 5 (CVS vs. UC: t=5.780, P<0.001; CVS vs. SC: t=4.977, P<0.001) and day 7 (CVS vs. UC: t=6.191, P<0.001; CVS vs. SC: t=4.653, P<0.001). UC and SC fathers did not show consistent patterns of change in body mass across time: UC fathers weighed more on day 7 than on day 5 (t=2.316, P=0.032). SC fathers were heavier at baseline than on day 3 (t=2.356, P=0.029). In contrast CVS fathers showed a steady decline in body mass over days: they were heavier at baseline than at any other time point (baseline vs. all other time points, P<0.014), and were heavier on day 1 than on all subsequent days (P<0.001 for all comparisons). CVS fathers were also heavier on day 3 than on day 7 (t=2.420, P=0.026).

Organ Mass

For all animals combined, right and left organ masses were highly correlated (testes: r=0.87, n=22, P<0.001; adrenal glands: r=0.79, n=22, P<0.001), and therefore right- and left-side masses were combined to obtain total testis and total adrenal masses. Wet organ masses (total testis, total adrenal, thymus and spleen) were analyzed via ANCOVA with day1 body mass as a covariate (Table 4.2). Initially, body mass, experimental group and the body mass*experimental group interaction terms were included in the ANCOVA. The interaction term was not significant for any organ of interest (P>0.332 in all cases) and was removed from the model. Body mass was significant only for wet thymus mass ($F_{1,18}$=16.431, P<0.001). After we controlled for effects of body mass, wet thymus mass differed among experimental groups ($F_{2,18}$=10.659, P=0.001): thymi of CVS fathers weighed less than those of both UC (t=3.830, P<0.001) and SC fathers (t=2.792, P=0.006); UC and SC fathers did not differ (P=0.277; Fisher's LSD post-hoc tests). No other organs differed in mass among the three experimental groups. Because we had predicted an increase in adrenal mass in the CVS fathers, we performed a planned comparison between CVS and UC fathers on total adrenal mass (ANCOVA result for mass-corrected effect of experimental condition: $F_{2,18}$=2.742, P=0.091). This analysis revealed that CVS fathers had heavier body-mass-corrected adrenal glands than UC fathers (0.019 ± 0.002 vs. 0.014 ± 0.002 g at body mass of 39.60 g; t=2.568, P=0.032; Fisher's LSD post-hoc test).

Basal Corticosterone Concentrations

For analysis of basal corticosterone data, Pillai's Trace multivariate output was used due to lack of sphericity (Berger and Selhorst, 1983). Basal CORT concentrations changed over time (main effect of time: $F_{2,17}=7.214$, $P=0.005$), and this effect differed among experimental groups (time*experimental group interaction: $F_{4,36}=5.015$, $P=0.003$; Fig. 4.2A). On day 4, CVS fathers had higher plasma CORT concentrations than did UC fathers ($t=4.269$, $P<0.001$) or SC fathers ($t=3.244$, $P=0.004$; Fisher's LSD), while UC and SC fathers did not differ ($P=0.320$). Groups did not differ in baseline CORT level at any other time point, and the main effect of experimental group was not significant ($F_{2,18}=2.696$, $P=0.095$).

Corticosterone Response to a Novel Stressor

Plasma corticosterone concentrations changed acutely following application of a novel stressor (oil injection; main effect of time: $F_{2,36}=84.094$, $P<0.001$; Fig. 4.2B). This response did not, however, differ among experimental groups (main effect of experimental group: $F_{2,18}=0.495$, $P=0.618$; time*experimental group interaction: $F_{4,36}=1.697$, $P=0.172$). Oil injection elevated corticosterone levels above baseline concentrations in all groups at both 10 min ($t=15.948$, $P<0.001$; Sidak-corrected post-hoc test) and 40 min post-injection ($t=8.108$, $P<0.001$; Sidak-corrected post-hoc test). Plasma

corticosterone was also higher at 10 min post-injection when compared to 40 min post-injection (t=3.276, P=0.013; Sidak-corrected post-hoc test).

In addition to plasma corticosterone at each time point, area under the curve was analyzed using two different equations (Preussner et al., 2003). The first, AUCg, represents the integrated amount of corticosterone produced over time with respect to a starting value of zero, thus not accounting for baseline (pre-injection) levels of circulating hormone. The second, AUCi, characterizes the response of the HPA axis to oil injection by evaluating the amount of hormone produced above the starting baseline level (thus taking baseline values into consideration) Experimental groups did not differ in either measure of integrated corticosterone production (AUCg, $F_{2,18}$=0.526, P=0.600; AUCi, $F_{2,18}$ =0.787, P=0.470).

Effects of Chronic Variable Stress on Paternal Behavior

Instantaneous scans

For each time bin (see Methods for details) the proportion of scans during which each behavior occurred was calculated for each male, and group medians were compared using Kruskal-Wallis tests with appropriate non-parametric post-hoc analysis (Siegel and Castellan, 1988). Only one difference was found in any of the spot-check measures (Table 4.3). During the dark phase of days 2-4 (bin 2; n=2 scans), CVS fathers spent less time in the cage-half with at least one pup than did fathers from the UC or SC groups

(χ^2=11.375, P=0.003; CVS vs. UC: P<0.05; CVS vs. SC: P<0.05); UC and SC fathers did not differ (P>0.05).

Ten-minute Behavioral Observations

None of the behaviors measured differed among experimental groups on days 2, 3, or 4 (P>0.12 in all cases, Kruskal-Wallis tests; Table 4.4). On day 6 (post-stressor or post-separation, lights-on), the experimental groups differed significantly in almost all of the behaviors measured, including duration of paternal behavior (P<0.001), autogroom (P<0.001), family huddle (P=0.003), male groom/sniff female (P=0.001), rears (P=0.003), proportion of time the male spent walking/jumping (P=0.034) and resting/still (P=0.001). Each measure of male location also differed significantly among groups (P<0.009 in all cases). Female location did not differ (see Table 4.4).

Post-hoc tests revealed that on day 6 of the 7-day CVS protocol, CVS males spent significantly more time autogrooming, and significantly less time in a family huddle, in the same cage-half as the female, in the same cage-half as the pups, and within 10 cm of pup(s) than did UC fathers (P<0.05 for all comparisons) but not SC fathers; UC and SC fathers did not differ in any of these measures. SC fathers spent more time engaging in paternal care and more time sniffing/grooming their mate than did CVS and UC fathers (P<0.05 in all cases); UC and CVS father did not differ in either behavior. Both SC and CVS fathers performed more rears and spent less time sitting still/resting than did UC fathers (P<0.05 in all cases), but SC and CVS fathers did not differ in these behaviors.

Effect of Fathers' Treatment Group on Pups and Subsequent Pregnancy

Pup Body Mass

Pup mass increased significantly over time ($F_{17,496}$=279.80, P<0.0001; FIG. 3), but was not affected by father's treatment group ($F_{2,35}$=0.44, P=0.650; time*father's treatment interaction $F_{34,496}$=0.65, P=0.936). Fisher's LSD post-hoc tests revealed that all time points significantly differed from one another (P<0.05 for all comparisons) except for the following: PND4 vs. PND5 (P=0.114), PND6 vs. PND7 (P=0.059), PND10 vs. PND11 (P=0.092), and PND19 vs. PND20 (P=0.218; see Supplemental Table C).

Day of Eye Opening

Experimental condition of the father did not affect day of eye opening in the pups (UC: 14.62 ± 0.31, SC: 15.55 ± 0.34, CVS: 15.43 ± 0.30 days; $F_{2,35}$=2.622, P=0.087).

Basal and Post-stress Corticosterone Concentrations

After pups were exposed to predator urine for 5 min, plasma corticosterone levels increased above baseline values (back-transformed mean (95% confidence interval): basal 43.55 ng/ml (1.52-1.76) vs. post-stress 384.59 ng/ml (2.53-2.64); $F_{1,35}$=272.058,

P<0.0001), but there was no effect of father's treatment group ($F_{2,35}$=1.826, P=0.176; time*father's treatment interaction, $F_{2,35}$=1.183, P=0.318; FIG. 4.4).

Proportion of Pregnant Females

The proportion of females with detectable pregnancies following dissection did not differ significantly among females that had been paired with an undisturbed control male (1 of 3), with a separation control male (4 of 6), and with a chronically stressed male (3 of 7; P=0.10, Fisher's Exact test).

DISCUSSION

This experiment is the first, to our knowledge, to examine the effects of chronic stress on paternal care in a monogamous, biparental mammal. Since stress is suggested to mediate a trade-off between current and future reproduction (Ketterson and Nolan, 1999; Moore and Hopkins, 2009; Ricklefs and Wikelski, 2002; Zera and Harshman, 2001;), we predicted that chronic stress in California mouse fathers would result in decreased paternal behavior. Consequently, we predicted that pups of stressed fathers would exhibit decreased growth, delayed development, and altered corticosterone release. Compared to control groups, stressed males had increased baseline corticosterone levels on day 4 of the 7-day stress paradigm, and spent less time in the cage-half containing pups during instantaneous scans occurring in the dark phase of days 2-4. On day 6,

behavior was altered in chronically stressed fathers compared to both undisturbed control and separation control males; however, no behavioral differences were evident among groups during 10-min observations on days 2, 3 or 4. These results demonstrate that chronic stress can alter behavior of mammalian fathers, and suggest that duration of the CVS paradigm is important in generating these behavioral changes. Nonetheless, behavioral changes were subtle, and no father was seen displaying direct aggression towards the mate or pups at any time. No pups were harmed, and pup development was not altered under these circumstances. Thus, while the CVS paradigm did alter paternal behavior, this change did not translate into reduction in offspring development or survival under the tested conditions, suggesting that a slight decrease in current reproductive investment does not necessarily result in reduced parental fitness in this biparental rodent.

Following reunion after a brief (15 min) separation from the mate and pups, separation control fathers readily approached and interacted with the family, whereas chronically stressed males did not. Stressed fathers spent significantly more time autogrooming and less time huddling with the family than did control fathers following a stressor on day 6. In contrast, separation control fathers spent more time investigating the female pairmate and more time engaging in paternal care than did stressed or undisturbed control fathers. These results suggest that brief separation from the family can increase paternal care, congruent with a previous finding in California mice (Bredy et al., 2004), whereas separation plus stress can have the opposite effect. These conclusions are consistent with findings in rat mothers: upon return to their pups following a brief separation (15 min), reunited mothers increased licking and grooming of their pups

compared to non-separated control mothers (Boccia and Pedersen, 2001), while both acute and chronic stress decreased maternal care (Ivy et al., 2008; Nephew and Bridges, 2011; Roth and Sullivan, 2005; Sukikara et al., 2010; Yamada et al., 2002).

Our study, as well as those performed on rat mothers, was conducted under laboratory conditions, and therefore the consequences of stress and elevated glucocorticoids for paternal behavior in the wild are unknown. It is possible that chronically stressed fathers would be more likely to abandon the family or to be predated if they were spending more time away from the nest. Fathers' disappearance from the nest would likely have severe fitness consequences, as mother-only families have been shown to wean fewer pups than two-parent families (Cantoni and Brown, 1997a,b; Gubernick and Teferi, 2000). Field studies of birds have found that both stress and chronic elevation of glucocorticoids via implants can decrease parental care and/or increase rates of nest abandonment by both mothers and fathers (Almasi et al., 2008; Angelier et al., 2009; Love et al., 2004; Silverin 1986,1998; Spée et al., 2011; Wingfield and Silverin, 1986; Wingfield and Kitaysky, 2002).

While data on avian parents and mammalian mothers (Brummelte et al., 2006; Saltzman and Abbott, 2009) suggest that chronically elevated glucocorticoid levels can result in abandonment and decreased care, we cannot determine whether corticosterone was responsible for altered paternal behavior observed on day 6 in our study. Glucocorticoids most notably elicit effects by binding to intracellular receptors and altering DNA transcription and protein synthesis, a process taking roughly 1-2 h (Beato, 1989; Strähle et al., 1988). California mouse fathers in our study showed differences in

behavior immediately following a 15-min stressor (or separation), thus likely ruling out genomically mediated effects. Additionally, we previously found that a 90-min elevation of circulating corticosterone concentrations did not alter paternal behavior in California mouse fathers, suggesting that sustained elevation of corticosterone (and presumably glucocorticoid-induced changes in gene expression) is not sufficient to inhibit paternal behavior under otherwise undisturbed conditions (Harris et al., 2011). In addition to genomic effects, however, recent work has suggested the corticosterone may act via membrane-bound receptors to cause rapid (within minutes), second-messenger-mediated changes in behavior (Borski, 2000; Groeneweg et al., 2011; Joels and Baram, 2009; Lösel and Wehling, 2003). A previous study by our lab found that a 5-min stressor did not alter paternal behavior but did increase circulating corticosterone levels (Chauke et al., 2011), suggesting that a single, acute stressor causing elevation of corticosterone was not sufficient to rapidly (non-genomically) alter paternal behavior. We cannot rule out non-genomic actions of corticosterone in the present study, but because we found no behavioral differences following the ~10-min acute stressor or separation procedure on day 2, corticosterone increase following an acute stressor does not appear to be sufficient to cause rapid, non-genomic changes in paternal behavior.

If glucocorticoids are not directly responsible for alterations in paternal care, it may be that they are acting in concert with other stress-reactive hormones, or that they do not mediate behavioral changes. Circulating and central concentrations of several hormones, many of which are implicated in behavioral regulation, can be altered by stress and changes in glucocorticoid levels (e.g., corticotropin-releasing hormone, arginine

vasopressin, prolactin, oxytocin, opioids, testosterone, serotonin; Herman and Cullinan, 1997; Insel and Young, 2000; Sapolsky et al., 2000) and therefore may be important in post-stress modification of behavior. In California mice, the stress-reactive peptide arginine vasopressin (AVP) may be involved. Chronically stressed animals from our larger CVS study (fathers from the present experiment, in addition to males pair-housed with either another male or a tubally ligated female) had elevated AVP, but not CRH, mRNA in the paraventricular nucleus of the hypothalamus (PVN) when compared to undisturbed control males (de Jong et al., in prep). Additionally, a previous study on virgin male California mice found that males with higher AVP mRNA expression in the PVN took significantly longer to contact a foster pup, and that males that approached pups more quickly were less anxious (determined by a urine-marking test; de Jong et al., 2012). In other rodent species, increased AVP expression in the PVN is associated with increased anxiety (Landgraf and Wigger, 2003; Pan et al., 2009; Wigger et al., 2004), suggesting that increased AVP in our chronically stressed fathers may be indicative of increased anxiety in these animals as compared to their non-stressed counterparts. In line with this possibility, chronically stressed fathers in our study spent significantly more time autogrooming than control fathers, and this behavior has been associated with high levels of anxiety in other rodents (Ferre et al., 1995; Kalueff and Tuohimaa, 2005). Moreover, even after 21 days of recovery, male rats exposed to 10 days of chronic immobilization stress displayed enhanced anxiety (measured via an elevated plus maze) when compared to control rats (Vyas et al., 2004), suggesting that chronic stress can result in persistent changes in anxiety; this might explain why more behavioral

differences were noted toward the end of the CVS paradigm in our study. Taken together, these results suggest that anxiety, rather than stress *per se*, might underlie alterations in paternal behavior and pup responsiveness in this species. Contrary to this possibility, a recent study on male California mice found the opposite: non-paternally behaving males, regardless of reproductive status, displayed more entries into the open arms of the elevated plus maze when compared to paternally behaving males, suggesting non-paternal males were less anxious (Chauke et al., 2012); it should be noted, however, that the elevated plus maze has not been validated for use in California mice. Thus, future studies investigating the relationship between AVP, anxiety, and paternal behavior in this species would be revealing, as would studies exploring the roles of other stress-reactive peptides (e.g., CRH, opioids, and prolactin).

In the present study, chronic stress in fathers and the resulting alterations in paternal behavior did not result in any detectable changes in pups, including survival, development (measured as day of eye opening), growth, basal or stress-induced corticosterone secretion during the first 20 postnatal days, suggesting that decreases in current reproductive investment (paternal care) do not necessarily have a measurable consequence on parental fitness. Despite the absences of differences in pup measures in this study, however, it should not be assumed that being reared by a stressed father does not have any consequences for pups. For example, the amount of licking and grooming received by California mouse pups has been shown to influence spatial learning and memory in adulthood, and pups raised by both parents receive more licking/grooming than do pups raised by the mother only (Bredy et al., 2004). Additionally, previous work

has shown that male pups cross-fostered between *P. californicus* and *P. leucopus* families behaved more like their foster parents than their biological parents (Bester-Meredith and Marler, 2001), and more specifically, that *P. californicus* males that were raised by the less-paternal *P. leucopus* males showed decreased paternal behavior when they became fathers (Bester-Meredith and Marler, 2003), thus supporting the idea that paternal behavior can alter offspring behavior later in life. Considering that the stressed males in our study spent less time behaving paternally than control males, future studies investigating more long-term developmental variables, including memory, learning and parental behavior of adult offspring, would be informative, as these variables would likely be important for pup survival and parental fitness.

In conclusion, we found evidence that chronic stress can decrease paternal behavior in a monogamous, biparental mammal, but effects were subtle and did not translate to changes in pup outcomes or parental fitness. Future studies should characterize long-term measures of pup development and behavior, and possibly superimpose chronic stress on more challenging living conditions (e.g., requiring animals to run for food, housing animals at cold ambient temperature; Cantoni and Brown, 1997a,b; Gubernick et al., 1993; Wright and Brown, 2002). Additionally, it may be that a trade-off between current and future reproductive effort could be better characterized by measuring interbirth interval, and mass, size and survival of subsequent litters from pairs with stressed and non-stressed fathers. In this study, we cannot be sure if changes in circulating corticosterone concentration mediated stress-induced changes in behavior, but future work investigating non-genomic corticosterone regulation in this species would be

informative. Additionally, it may be that chronic stress results in increased anxiety and that it is not stress *per se* but anxiety that drives changes in paternal care. Follow-up work on the role of corticosterone and other anxiety-related hormones (e.g., AVP) would be illuminating.

Table 4.1. Experimental timeline for first-time California mouse fathers that underwent a chronic variable stress (CVS) paradigm, underwent separation control procedures (SC), or served as undisturbed controls (UC).

	Manipulation			Data Collection	
Day	**UC**	**SC**	**CVS**	**Fathers**	**Pups**
-62 to -34	--	--	--	(pairs formed)	--
-2 to 0	--	--	--	(pups born)	--
1	--	separation x2	stress x2	blood sample; body mass	--
2	--	separation x3	stress x3	lights-off, post-stress 10 min observation	--
3	--	separation x3	stress x3	lights-on, basal 10 min observation; body mass	body mass
4	--	separation x4	stress x4	blood sample; lights-off, basal 10 min observation	--
5	--	separation x3	stress x3	body mass	body mass
6	--	separation x2	stress x2	lights-on, post-stress 10 min observation	--
7	--	separation x4	stress x4	body mass	body mass
8	oil injection euthanized	oil injection euthanized	oil injection euthanized	blood samples (basal, 10 min and 40 min post-injection); organs harvested	body mass
9-18	--	--	--	--	daily: pups weighed, checked for eye opening
19	--	--	--	--	basal blood sample from all pups
20	--	--	--	--	post-stress blood sample from all pups

Table 4.2. Starting variables and organ mass data from first-time California mouse fathers that underwent a chronic variable stress (CVS) paradigm, underwent separation-control (SC) procedures, or served as undisturbed controls (UC).

Variable	UC	SC	CVS	P- Value[b]
	Mean ± SEM			
age at start (days)	161.5 ± 3.6	160.3 ± 7.7	156.5 ± 7.0	0.835
latency from pairing to parturition (days)	42.5 ± 3.0	41.29 ± 3.1	41.25 ± 3.1	0.948
litter size	2.25 ± 0.3	1.71 ± 0.2	1.75 ± 0.2	0.216
pup age at day 1 (days)	2.0 ± 0.2	2.0 ± 0.3	2.0 ± 0.3	0.999
thymus mass (g)[a]	0.077 ± 0.006	0.066 ± 0.006	0.038 ± 0.006	**0.001**
total adrenal mass (g)[a]	0.014 ± 0.002	0.016 ± 0.002	0.019 ± 0.002	0.091
spleen mass (g)[a]	0.073 ± 0.007	0.072 ± 0.007	0.059 ± 0.007	0.300
total testis mass (g)[a]	0.371 ± 0.029	0.386 ± 0.030	0.364 ± 0.027	0.859

[a]body-mass-corrected means taken at mass of 40.47g

[b]P-value for main effect of experimental group from one-way ANOVA or ANCOVA

Table 4.3. Behavioral data (median, range) from instantaneous scans in chronically stressed (CVS, n=8), separation control (SC, n=7), and undisturbed control (UC, n=8) first-time California mouse fathers. P-values from Kruskal-Wallis tests; values less than 0.05 are bolded.

Behavior		CVS	SC	UC	χ^2	P-value
Locomoting (male)						
	D2-4 Light	0.00 (0.00-0.17)	0.00 (0.00-0.17)	0.00 (0.00-0.00)	1.132	0.568
	D2-4 Dark	0.50 (0.00-1.00)	0.50 (0.00-0.50)	0.50 (0.00-1.00)	2.251	0.325
	D5-7 Light	0.00 (0.00-0.20)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	3.929	0.140
	D5-7 Dark	0.50 (0.00-1.00)	0.50 (0.00-1.00)	0.50 (0.00-1.00)	0.368	0.832
Locomoting (female)						
	D2-4 Light	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	1.207	0.547
	D2-4 Dark	0.00 (0.00-0.50)	0.00 (0.00-1.00)	0.50 (0.00-1.00)	1.785	0.392
	D5-7 Light	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.20)	0.731	0.694
	D5-7 Dark	0.00 (0.00-0.50)	0.50 (0.00-0.50)	0.00 (0.00-0.50)	0.731	0.694
Male in same cage half as pups						
	D2-4 Light	1.00 (0.80-1.00)	1.00 (0.80-1.00)	1.00 (1.00-1.00)	2.457	0.293
	D2-4 Dark	0.50 (0.00-0.50)	1.00 (0.50-1.00)	1.00 (0.00-1.00)	11.375	**0.003**
	D5-7 Light	1.00 (0.60-1.00)	1.00 (0.80-1.00)	1.00 (0.80-1.00)	1.673	0.433
	D5-7 Dark	1.00 (0.50-1.00)	0.50 (0.00-1.00)	0.50 (0.00-1.00)	5.218	0.074
Female in same cage half as pups						
	D2-4 Light	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.284	0.526
	D2-4 Dark	1.00 (0.50-1.00)	1.00 (0.50-1.00)	1.00 (0.00-1.00)	2.286	0.319
	D5-7 Light	1.00 (1.00-1.00)	1.00 (0.80-1.00)	1.00 (1.00-1.00)	0.481	0.786
	D5-7 Dark	1.00 (0.50-1.00)	1.00 (0.50-1.00)	1.00 (0.50-1.00)	0.481	0.786
Male behaving paternally						
	D2-4 Light	0.92 (0.60-1.00)	1.00 (0.67-1.00)	1.00 (0.67-1.00)	0.57	0.752
	D2-4 Dark	0.25 (0.00-0.50)	0.50 (0.00-1.00)	0.25 (0.00-1.00)	2.548	0.280
	D5-7 Light	0.90 (0.40-1.00)	0.80 (0.60-1.00)	0.80 (0.40-1.00)	0.05	0.976
	D5-7 Dark	0.00 (0.00-1.00)	0.50 (0.00-1.00)	0.50 (0.00-1.00)	1.012	0.603

Table 4.4. Behavioral data (median, range) from four 10-min observations in chronically stressed (CVS, n=8), separation control (SC, n=7), and undisturbed control (UC, n =8) first-time California mouse fathers. Conditions during observation were as follows: Day 2 – dark-phase, immediately following predator-urine stressor (CVS), 10-min separation (SC) or no manipulation (UC); Day 3 – light-phase, baseline observation (no stress or separation in the previous 6 h); Day 4 – dark-phase, baseline observation; Day 6 – light-phase, immediately following 15 min of restraint (CVS), 15-min separation (SC) or no manipulation (UC). Kruskal-Wallis P-values less than 0.05 are bolded.

Behavior	UC	SC	CVS	χ^2	P-value
Autogrooming					
Day 2	17.1 (0.0-203.8)	20.0 (5.5-65.2)	36.2 (0.0-189.0)	0.21	0.899
Day 3	0.0 (0.0-11.2)	0.0 (0.0-131.8)	0.0 (0.0-186.2)	0.67	0.717
Day 4	101.2 (0.0-117.8)	38.3 (0.0-115.1)	36.0 (0.0-116.8)	0.00	0.999
Day 6	6.7 (0.0-62.9)	71.4 (24.4-221.9)	271.5 (140.8-547.0)	17.68	**<0.01**
Paternal behavior composite					
Day 2	135.7 (0.0-794.0)	236.0 (0.0-423.2)	43.4 (0.0-193.9)	0.80	0.669
Day 3	0.0 (0.0-2.4)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	1.88	0.392
Day 4	0.0 (0.0-50.7)	3.2 (0.0-516.6)	10.2 (0.0-561.3)	1.56	0.460
Day 6	0.0 (0.0-0.0)	5.1 (0.0-85.9)	0.0 (0.0-0.0)	17.23	**<0.01**
Group huddle					
Day 2	167.9 (0.0-438.3)	0.0 (0.0-256.1)	144.6 (0.0-435.8)	2.48	0.289
Day 3	599.2 (597.6-599.9)	599.0 (213.2-599.5)	599.2 (283.7-599.4)	1.56	0.458
Day 4	50.5 (0.0-484.8)	111.9 (0.0-312.6)	108.3 (4.4-582.9)	0.72	0.697
Day 6	598.6 (0.0-599.5)	322.4 (69.0-593.8)	0.0 (0.0-591.2)	11.58	**0.003**
Male groom/sniff female					
Day 2	0.0 (0.0-92.1)	13.1 (0.0-26.9)	6.6 (0.0-30.6)	4.17	0.124
Day 3	0.0 (0.0-6.6)	0.0 (0.0-5.2)	0.0 (0.0-0.0)	1.12	0.572
Day 4	1.3 (0.0-36.4)	3.3 (0.0-41.6)	2.5 (0.0-24.3)	0.05	0.978
Day 6	0.0 (0.0-30.7)	68.6 (23.7-251.7)	0.0 (0.0-8.2)	14.61	**0.001**
Jump					
Day 2	2.5 (0.0-125)	2 (0.0-141)	2 (0.0-144)	0.08	0.961
Day 3	0 (0-0)	0 (0-1)	0 (0-0)	2.29	0.319
Day 4	25 (0-269)	5 (0-258)	3.5 (0-241)	0.83	0.660
Day 6	0 (0-0)	0 (0-14)	0 (0-45)	4.11	0.128
Rear					
Day 2	11 (0.0-22)	16 (4-46)	19 (4-71)	3.95	0.139
Day 3	0 (0-0)	0 (0-13)	0 (0-0)	2.29	0.319
Day 4	17 (0-61)	15 (0-82)	31.5 (0-69)	0.48	0.786
Day 6	0 (0-5)	8 (1-73)	5.5 (0-29)	11.40	**0.003**

Locomoting					
Day 2	0.125 (0.0-0.45)	0.15 (0.05-0.70)	0.15 (0.05-0.90)	1.13	0.568
Day 3	0.00 (0.00-0.00)	0.00 (0.00-0.10)	0.00 (0.00-0.05)	1.15	0.562
Day 4	0.15(0.00-0.80)	0.10 (0.00-0.70)	0.20 (0.00-0.65)	0.60	0.741
Day 6	0.00 (0.00-0.10)	0.10 (0.00-0.65)	0.05 (0.00-0.30)	6.74	**0.034**
Resting/still					
Day 2	0.55 (0.2-0.75)	0.45 (0.0-0.55)	0.20 (0.0-0.70)	3.20	0.202
Day 3	1.00 (0.95-1.00)	1.00 (0.65-1.00)	1.00 (0.40-1.00)	0.86	0.652
Day 4	0.15 (0.00-0.90)	0.30 (0.00-1.00)	0.45 (0.00-1.00)	1.99	0.371
Day 6	0.95 (0.70-1.00)	0.20 (0.05-0.70)	0.35 (0.00-0.65)	14.97	**0.001**
Male in same cage half as female					
Day 2	0.60 (0.0-1.00)	0.60 (0.25-0.85)	0.80 (0.50-0.95)	1.78	0.410
Day 3	1.00 (1.00-1.00)	1.00 (0.85-1.00)	1.00 (0.90-1.00)	1.15	0.562
Day 4	0.50 (0.0-1.00)	0.65 (0.25-0.90)	0.85 (0.70-1.00)	3.31	0.191
Day 6	1.00 (0.90-1.00)	0.90 (0.35-1.00)	0.25 (0.00-1.00)	12.30	**0.002**
Male w/in 10 cm of pups (touching or not)					
Day 2	0.575 (0.50-1.00)	0.60 (0.0-0.80)	0.70 (0.0-0.90)	0.23	0.890
Day 3	1.00 (1.00-1.00)	1.00 (0.40-1.00)	1.00 (0.60-1.00)	1.15	0.562
Day 4	0.35 (0.00-0.85)	0.50 (0.05-1.00)	0.325 (0.10-1.00)	0.92	0.633
Day 6	1.00 (0.00-1.00)	0.55 (0.25-1.00)	0.00 (0.00-1.00)	9.50	**0.009**
Male not in same cage half as any pup					
Day 2	0.05 (0.00-0.50)	0.20 (0.00-1.00)	0.20 (0.00-0.90)	2.58	0.275
Day 3	0.00 (0.00-0.00)	0.00 (0.00-0.15)	0.00 (0.00-0.10)	1.15	0.562
Day 4	0.05 (0.00-0.95)	0.35 (0.00-0.75)	0.05 (0.00-0.30)	2.34	0.310
Day 6	0.00 (0.00-0.10)	0.10 (0.00-0.65)	0.80 (0.00-1.00)	12.19	**0.002**
Female w/in 10 cm of pups (touching or not)					
Day 2	0.475 (0.0-1.00)	0.25 (0.0-1.00)	0.70 (0.0-1.00)	0.22	0.897
Day 3	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	<0.00	>0.99
Day 4	1.00 (0.00-1.00)	1.00 (0.10-1.00)	0.925 (0.05-1.00)	0.60	0.743
Day 6	1.00 (1.00-1.00)	1.00 (0.85-1.00)	1.00 (0.85-1.00)	1.13	0.568
Female not in same cage half as any pup					
Day 2	0.00 (0.00-1.00)	0.30 (0.0-0.65)	0.20 (0.0-0.70)	0.12	0.943
Day 3	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	<0.00	>0.99
Day 4	0.00 (0.00-1.00)	0.00 (0.00-0.55)	0.00 (0.00-0.30)	0.87	0.649
Day 6	0.00 (0.00-0.00)	0.00 (0.00-.015)	0.00 (0.00-0.15)	1.13	0.568

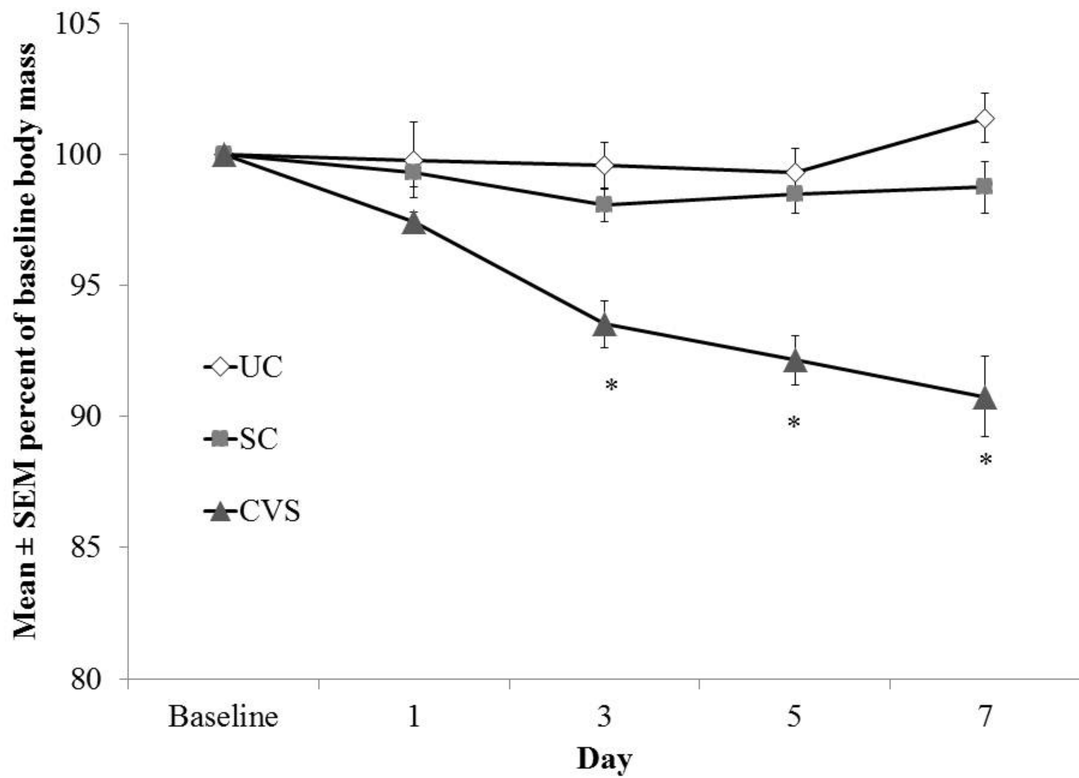

Figure 4.1: Effects of a 7-day chronic variable stress paradigm on the body mass of first-time California mouse fathers. Chronically stressed (CVS, n=8) fathers lost body mass over the course of the experiment whereas separation control (SC, n=7) and undisturbed control (UC, n=8) fathers did not. * P<0.05 comparing CVS to SC and UC; SC and UC did not differ at any time point. See results for additional statistical information.

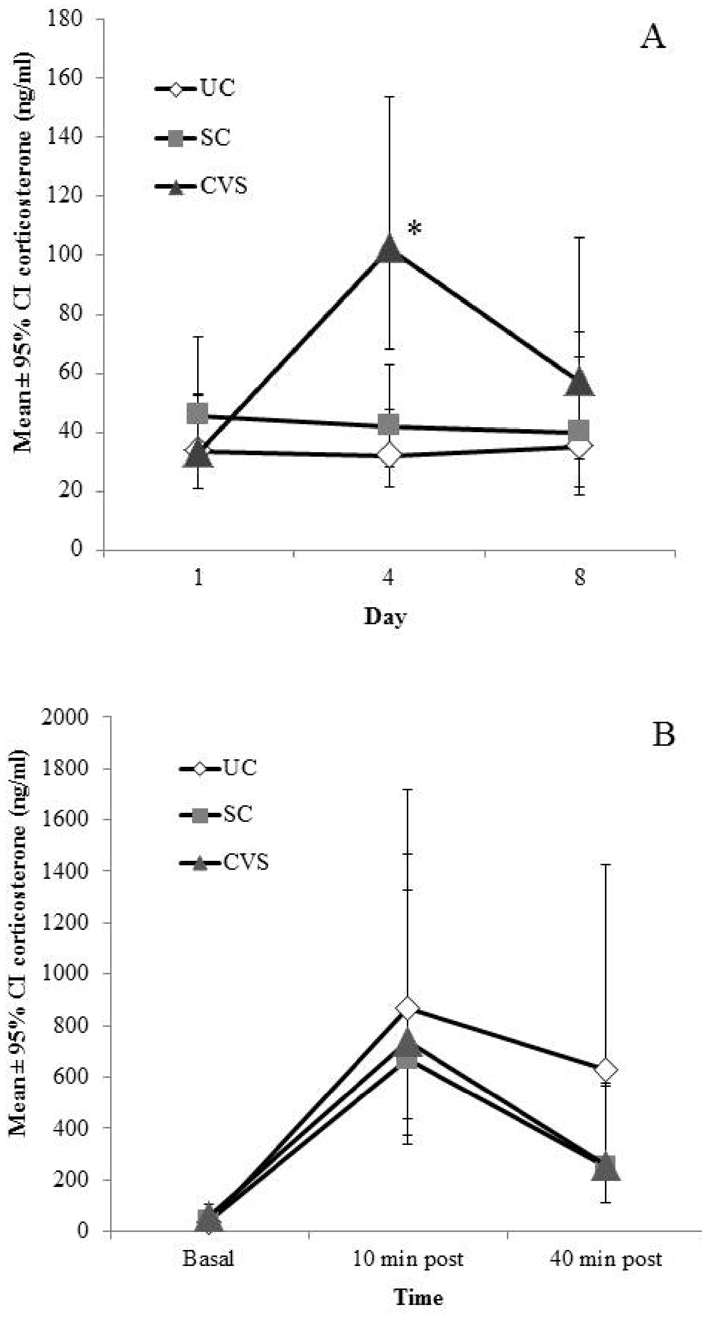

Figure 4.2A&B: A) Back-transformed plasma corticosterone concentration at 0900h over a 7-day chronic variable stress paradigm in first-time California mouse fathers. Chronically stressed (CVS, n=7) fathers had significantly higher corticosterone levels on day 4 when compared to both separation control (SC, n=7) and undisturbed control (UC, n=7) fathers (*P<0.05). B) Back-transformed corticosterone response to s.c. injection of 0.2 ml of sesame oil after 7 days of chronic variable stress. Experimental groups did not

differ in concentration of corticosterone at any point, but each time point differed from all others (see text for details). Note the different y-axis scales on the two graphs.

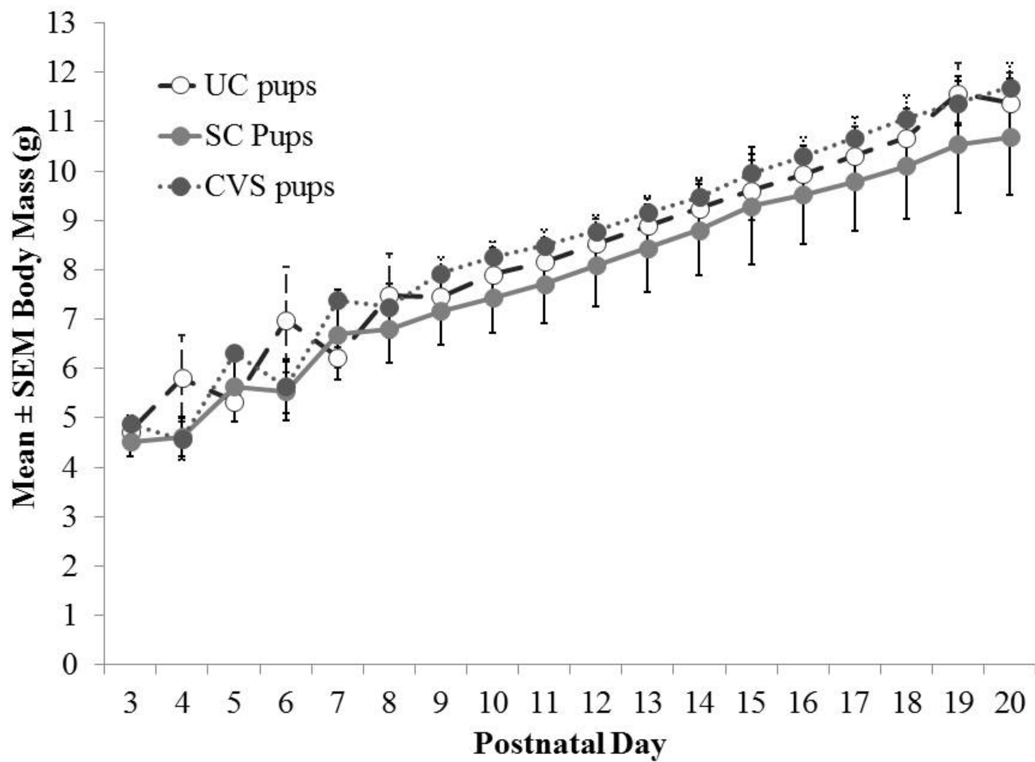

Figure 4.3: Body mass of pups raised for the first 8-10 days post-partum by chronically stressed (CVS; n=14 pups) separation control (SC; n=11), or undisturbed control (UC; n=13) fathers for the first 8-10 days of life. Fathers' treatment condition did not alter body mass of pups, but regardless of fathers' condition body mass changed over time (main effect of time P<0.01; see text for specifics).

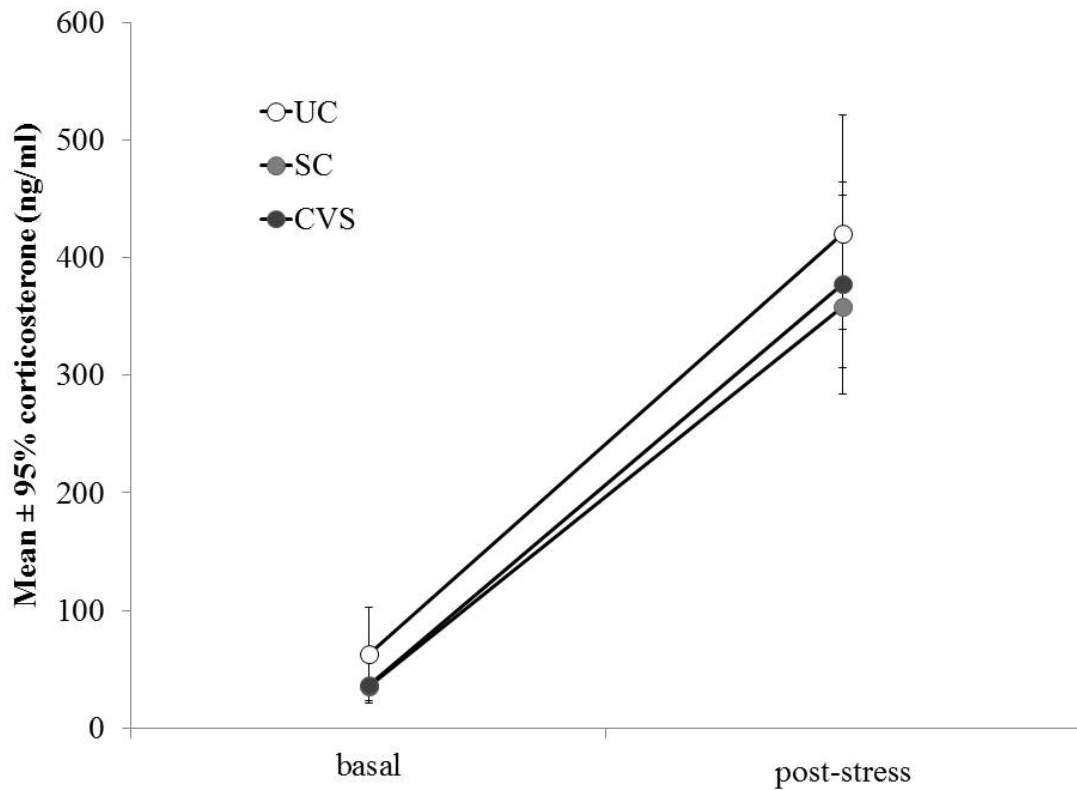

Figure 4.4: Back-transformed plasma corticosterone concentration in pups raised by chronically stressed (CVS; n=14 pups), separation control (SC; n=11 pups) or undisturbed control (UC; n=13 pups) fathers for the first 8-10 days of life. Plasma samples were collected at 1430-1520 h, and predator-odor stress significantly elevated corticosterone levels in pups ($P<0.01$); however, pups' corticosterone concentrations were not affected by fathers' experimental condition.

Chapter 5

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 30mg/kg CORT, 60 mg/kg CORT or vehicle, or left unmanipulated. Interactions between the male and its pup(s) were recorded 1.5-2h after injection and scored for paternal and non-paternal behavior. Treatment groups were combined into control (unmanipulated + vehicle, n=15) and CORT (30mg/kg + 60mg/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.

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., 1997) or decreased nesting material (Ivy et al., 2008), can decrease maternal behavior. As in birds, this effect appears to be mediated, at 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 and colleagues (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., 2007; 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 05:15h and lights off at 19:15h. Ambient temperature was approximately 23°C with a humidity of about 65%. Mice were housed in standard shoe-box style, polycarbonate cages (44 x 24 x 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*.

Experimental Design

The experimental design is summarized in Fig. 5.1. Beginning approximately 1 week prepartum, when the female showed steady weight gain (6-10g), each pair was housed in a double cage consisting of two standard cages connected via clear plastic Crittertrail® tubing forming a z shape (35 x 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 (60mg/kg; n=8), low CORT (30mg/kg; n=8), vehicle (oil; n=8), and unmanipulated controls (n=7). CORT doses were based on a pilot study indicating that these low (30mg/kg) and high (60mg/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 (2200-2700ng/ml) in this species, respectively (Harris et al., 2012). *P. californicus* is nocturnal, so lights-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 08:00h, 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 minutes, and then videotaped for 10 minutes 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 minutes, and then presented with one of its own pups and videotaped for 5 minutes (retrieval test). The pup was always placed in the corner farthest from the father. Immediately following the retrieval test (2h 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 2h 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 (10:00h) 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 (10ml; 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 15mg/ml for the 60mg/kg dose and 7.5mg/ml for the 30mg/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 0.21ml.

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 minutes, 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 minutes. 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 seconds. Location of each parent was categorized as 1) in contact with at least one pup, 2) within 10cm 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 10cm 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 $\geq$ 1.5 seconds), 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 minutes. 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 second 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 seconds engaging in any combination of licking the pup, huddling the pup, or performing nursing posture; non-paternal males spent less than 75 seconds performing these behaviors. The time limits were chosen based on a clear split in the data set between 72 and 100 seconds.

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 minutes, with one exception (range: 63-229 seconds, mean ± SEM: 103.23 ± 32.85 sec). 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 25mm. 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. 5.2A. A two-way repeated-measures ANOVA (day x 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 males on either day E1 (t_{13}=0.45, P =0.65) or day E3 (t_{13}=2.02, P=0.053; Tukey's HSD tests). In addition, the 30 and 60mg/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 HSD tests). On both days E1 and E3, however, unmanipulated and vehicle-treated males had significantly lower CORT levels than the 30 and 60mg/kg CORT groups (P<0.001 in all cases; Tukey's HSD). 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 HSD 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 (30mg/kg: t_7= 0.73, P=0.472; 60mg/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 30mg/kg and 60mg/kg CORT groups, or between the vehicle and unmanipulated groups, the animals were combined into two treatment groups, CORT (30mg/kg + 60mg/kg CORT groups; n=16) and control (unmanipulated + vehicle groups; n=15), for the remaining analyses (Fig. 5.2B). 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 HSD), but not in the CORT group (t_{15}=0.28, P=0.78; Tukey's HSD). 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 HSD).

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 5.1. 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 seconds 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 5.2. 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 seconds 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 seconds 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 seconds, 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 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 5.2).

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. 5.1). 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 HSD for all post-hoc analyses listed above; Fig. 5.3).

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 HSD for all comparisons; Fig. 5.4). 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. 5.4). 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 (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 seconds 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 1.5-2h 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 hours (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 (1500-1800ng/ml, Harris et al., 2012) or post-stressor values during the lights-on phase of the daily cycle (2200-2500ng/ml, Harris et al., 2012) 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 ~4500ng/ml in response to a stressor during lights-off conditions (unpub. data). Therefore, although the achieved levels of ~5900ng/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 24h) 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 and colleagues (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.

Table 5.1. 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) CONTROL	CORT	P[f]	r[g]	q[h]
% Paternally responsive[a]		**46.67%** (7/15)	**31.25%** (5/16)	0.47	--	0.82
Paternal[b]						
	Composite[c]	**0.00** (0.00-578.10)	**0.00** (0.00-196.68)	0.22	0.22	0.73
	Huddle pup	**0.00** (0.00-541.04)	**0.00** (0.00-132.48)	0.28	0.20	0.73
	Lick pup	**0.00** (0.00-151.49)	**0.00** (0.00-54.41)	0.63	0.09	0.83
	Nursing posture	**0.00** (0.00-0.00)	**0.00** (0.00-9.78)	0.33	0.17	0.77
	Sniff pup	**0.00** (0.00-5.51)	**0.00** (0.00-7.89)	0.46	0.13	0.82
Non-pup-related[d]						
	Autogroom	**20.19** (0.00-130.71)	**50.48** (0.00-238.84)	0.23	0.21	0.73
	Dig	**1.64** (0.00-118.35)	**3.43** (0.00-77.34)	0.65	0.08	0.82
	Jump	**7.0** (0.0-195.0)	**1.5** (0.0-26.0)	0.28	0.20	0.73
	Rear	**21.0** (0.0-82.0)	**7.0** (0.0-89.0)	0.58	0.10	0.82
Male location[e]						
	Touch pup(s)	**0.16** (0.00-1.00)	**0.16** (0.00-1.00)	0.90	0.02	0.90
	Within 10 cm but not touching pup(s)	**0.00** (0.00-0.16)	**0.05** (0.00-1.00)	0.10	0.30	0.73
	In same cage but not within 10 cm of pup(s)	**0.11** (0.00-0.79)	**0.21** (0.00-0.84)	0.66	0.08	0.82
Male & female location[d]	Both in same cage	**0.53** (0.00-1.00)	**0.61** (0.21-1.00)	0.58	0.10	0.82
Activity[e]						
	Locomotion	**0.34** (0.00-1.00)	**0.18** (0.00-0.68)	0.27	0.20	0.73
	Rest/sleep	**0.32** (0.00-1.00)	**0.35** (0.00-1.00)	0.95	0.01	0.91
	Stationary	**0.21** (0.00-0.53)	**0.34** (0.00-0.74)	0.25	0.21	0.73

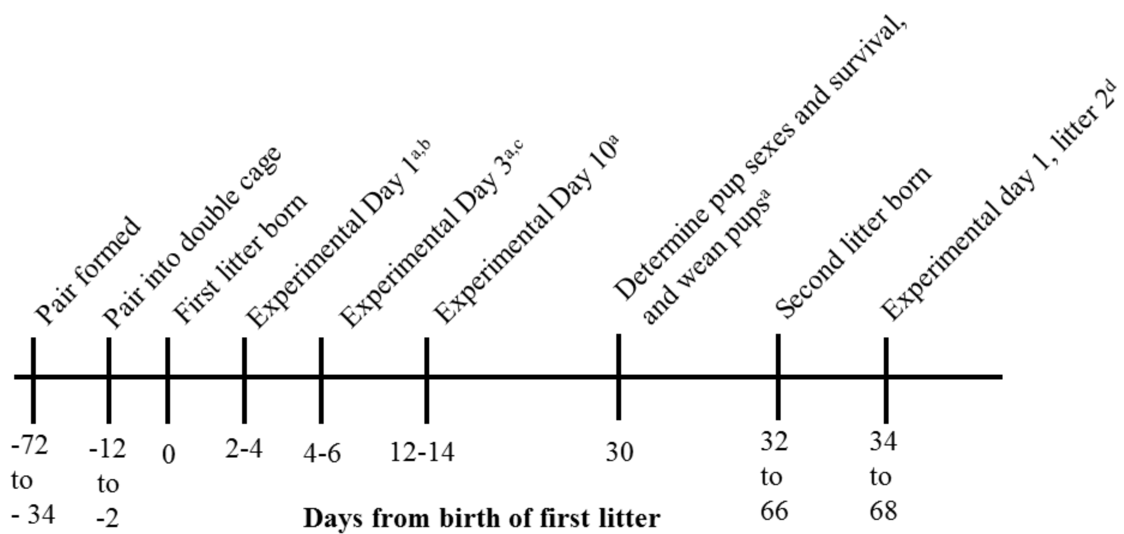
Female location[e]	Touch pup(s)	**1.00** (0.05-1.00)	**1.00** (0.21-1.00)	0.77	0.05	0.90
	Within 10 cm but not touching pup(s)	**0.00** (0.00-0.32)	**0.00** (0.00-0.05)	0.86	0.03	0.90
	In same cage but not within 10 cm of pup(s)	**0.00** (0.00-0.42)	**0.00** (0.00-0.42)	0.89	0.03	0.90
	In different cage than pup(s)	**0.00** (0.00-0.47)	**0.00** (0.00-0.68)	0.20	0.23	0.73
Activity[e]						
	Locomotion	**0.34** (0.00-1.00)	**0.18** (0.00-0.68)	0.27	0.20	0.73
	Rest/sleep	**0.32** (0.00-1.00)	**0.35** (0.00-1.00)	0.95	0.01	0.91
	Stationary	**0.21** (0.00-0.53)	**0.34** (0.00-0.74)	0.25	0.21	0.73

[a]Percentage of males whose composite duration was ≥1.5 seconds. 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 5.2. Behavioral data from the 5-min retrieval test on experimental day 1 in CORT-treated (n=16) and control (n=15) California mouse fathers.

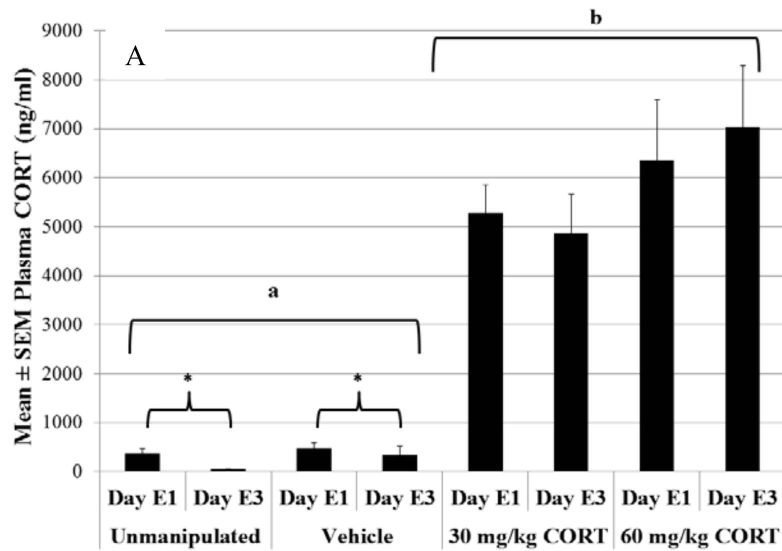
	Median (Range)				
Behavior	**CONTROL**	**CORT**	**P**[e]	**r**[f]	**q**[g]
Latency to contact pup (seconds)[a]	**7.48** (0.37-26.99)	**25.53** (0.80-114.40)	**0.02**	0.89	0.32
% Paternally responsive[b]	**100%** (15/15)	**93.25%** (15/16)	0.99	--	0.99
% Paternal[b]	**80.00%** (12/15)	**56.25%** (9/16)	0.25	--	0.54
Paternal[c]					
Composite	**207.77** (0.95-271.27)	**147.55** (0.00-233.30)	0.08	0.31	0.43
Huddle pup	**50.40** (0.00-222.31)	**41.73** (0.00-193.35)	0.58	0.10	0.74
Lick pup	**68.04** (0.952-230.88)	**37.90** (0.00-177.31)	0.21	0.23	0.52
Nursing posture	**0.00** (0.00-51.38)	**0.00** (0.00-41.27)	0.13	0.27	0.43
Sniff pup	**26.94** (10.32-67.83)	**34.94** (8.65-67.73)	0.69	0.07	0.74
Manipulate pup	**0.00** (0.00-59.79)	**0.00** (0.00-128.61)	0.64	0.08	0.74
Carry pup	**0.00** (0.00-72.60)	**0.00** (0.00-40.17)	0.49	0.12	0.74
Non-pup-related					
Autogroom[c]	**10.23** (0.00-130.96)	**9.36** (0.00-40.17)	0.55	0.11	0.74
Dig[c]	**0.00** (0.00-71.00)	**0.00** (0.00-162.43)	0.66	0.08	0.74
Locomotion[c]	**8.79** (0.99-55.60)	**18.37** (0.00-191.59)	0.14	0.26	0.43
Jump[d]	**0.0** (0.0-9.0)	**0.0** (0.0-44.0)	0.52	0.12	0.74
Rear[d]	**2.0** (0.0-17.0)	**5.0** (0.0-44.0)	0.13	0.27	0.43

[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 seconds of composite behavior (for comparison with family test); paternally behaving males were defined as those with composite scores ≥100 seconds. 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



[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

Figure 5.1: Experimental design



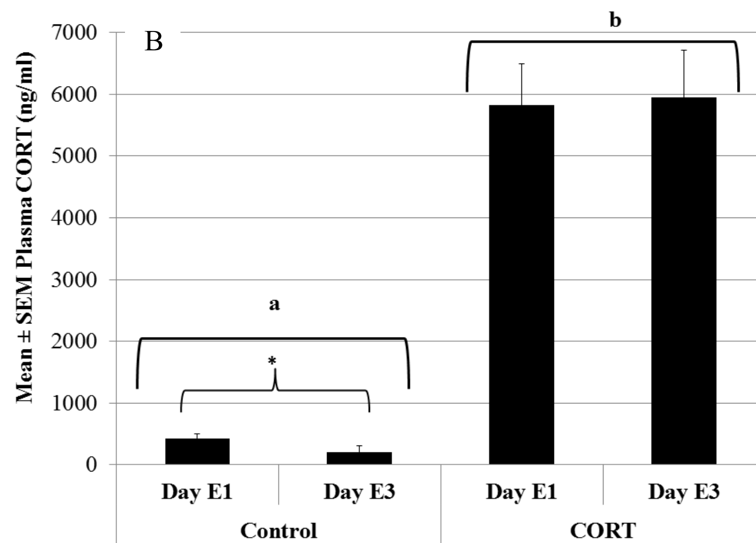


Figure 5.2A&B: Mean plasma corticosterone levels following treatment and behavioral tests (day E1) or treatment alone (day E3) in control and CORT-treated groups. Data were log_{10}-transformed for analysis, but non-transformed values are presented here for ease of interpretation. A: CORT concentrations 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 60mg/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 (30mg/kg and 60mg/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).

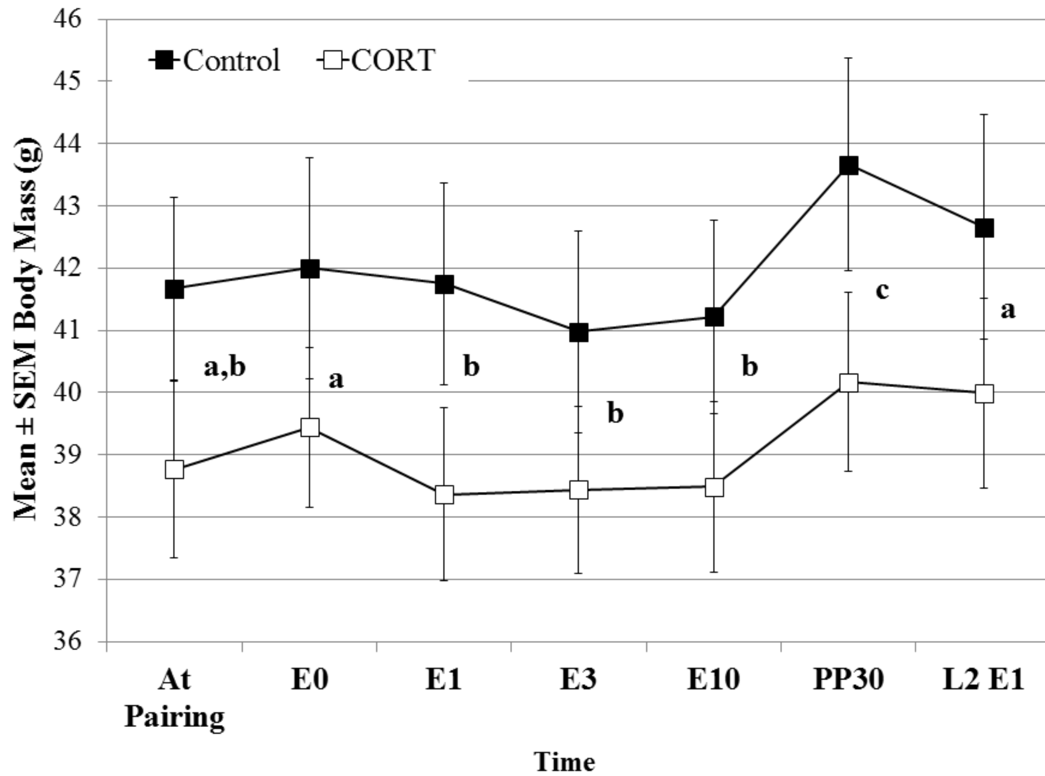


Figure 5.3: Body mass in control (n=15) and CORT (n=16) males over days of the experiment. CORT treatment did not affect body mass at any time point measured, nor was there an interaction between CORT treatment and time. Statistical results presented are for the main effects of time. Time points with different letters are significantly different from one another ($P<0.05$).

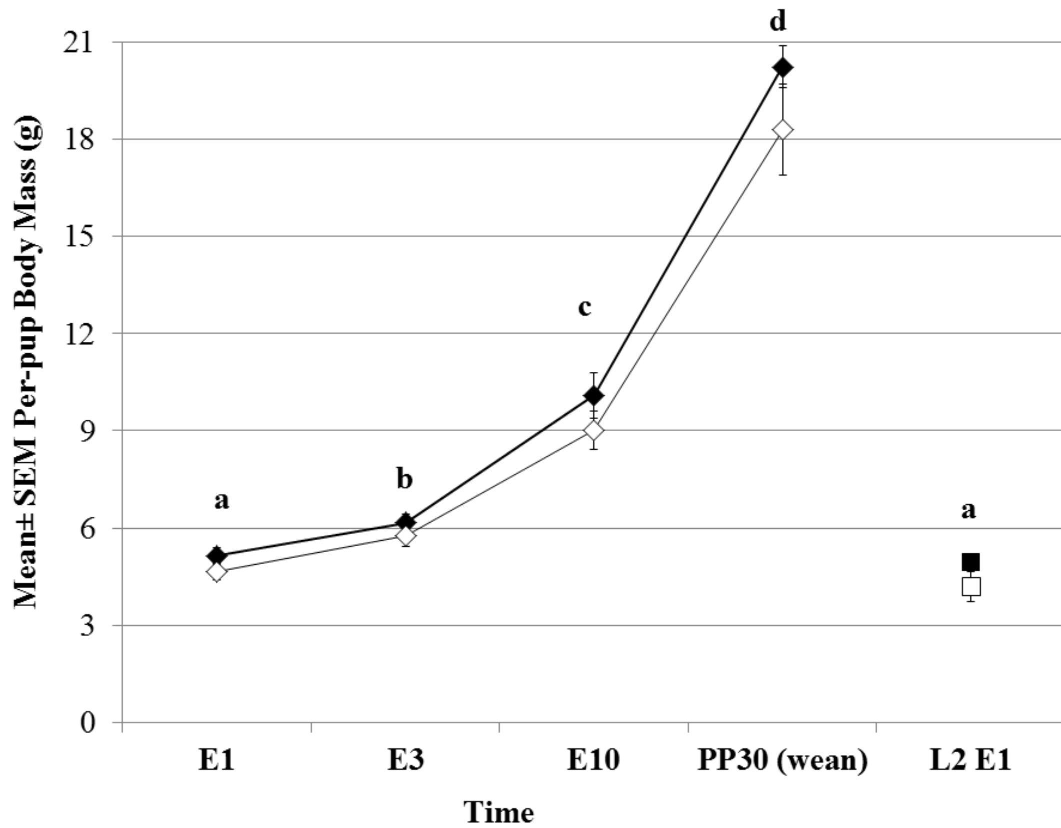


Figure 5.4: Mean per-pup body mass over time of pups of control (filled symbols) and CORT-treated (open symbols) fathers. Data from both the first litter (diamonds) and the second litter (squares) are presented. Per-pup mass was not affected by fathers' treatment group, nor was there an interaction between time and treatment. Statistical results are shown for the main effect of time; time points with different letters are significantly different from one another ($P<0.05$).

DISSERTATION CONCLUSIONS

My dissertation addressed the possible mediating role of stress and glucocorticoids in a trade-off between current vs. future reproduction from two complementary angles: 1) does reproductive status or age alter the HPA response to stress (Aims 2 and 3)? and 2) do stress and elevation of glucocorticoid hormones inhibit parental behavior (Aims 4 and 5)? I hypothesized that fathers would be more resistant to stress than would non-fathers, that older animals would be more stress-resistant than younger animals as they have lower residual reproductive value, and that both chronic stress and exogenous glucocorticoid elevation, outside of external stress, would decrease paternal behavior.

Overall, the data from this dissertation suggest that glucocorticoids do not directly mediate the trade-off between current and future reproduction in male California mice, but that stress can decrease paternal behavior. Results from Aims 2 and 3 suggest that changes in residual reproductive value (age) or reproductive status do not modulate HPA axis activity in these mice. Additionally, Aims 4 and 5 provide evidence that chronic stress can alter paternal behavior, but that these effects are subtle and are not likely mediated solely by changes in circulating corticosterone levels as corticosterone injection did not alter paternal behavior. Moreover, decreased paternal behavior during chronic stress apparently did not translate to altered pup development, growth or pup HPA function, suggesting that no fitness consequences were associated with stress-induced decrease in paternal care.

The predicted mediating effect of glucocorticoids on current vs. future reproduction hinges on the ideas 1) that stress-induced increases in glucocorticoid levels are detrimental to parental behavior, and 2) that measured plasma hormone levels are a good proxy for functional hormone effects in the body. Data from this dissertation suggest that these scenarios may not be met in California mice. Neither a single event of acute-stress-induced elevation nor exogenous increase of corticosterone reduced paternal behavior (Chauke et al., 2011; Harris et al., 2011), and changes in paternal behavior during chronic variable stress did not seem to be mediated by elevated levels of corticosterone, leading us to believe that plasma corticosterone elevation alone does not alter care. Additionally, while plasma hormone levels can be informative, studies in other species have shown that corticosteroid-binding globulin (CBG), glucocorticoid receptor density and type, as well as intracellular enzymes (11βHSD) are important for how plasma levels translate to downstream, tissue-level effects (Lattin et al., 2012; Malisch and Breuner, 2010; Romero and Wingfield, 2001). Investigating the above glucocorticoid-regulatory parameters in California mice would be beneficial as HPA function in California mice differs markedly from many other rodents that have been studied to date (Harris et al., 2012), suggesting that downstream effects may also differ in this species.

While we did not find evidence of glucocorticoids mediating a trade-off between current vs. future reproduction in California mice, evidence does exist in birds, especially in species living in extreme environments (Romero, 2002; Wingfield, 1994; Wingfield and Romero, 2001). Taxonomic differences in the relationship between reproduction and

glucocorticoids may exist, or this mediation might be an ecologically driven phenomenon and have nothing to do with taxonomic differences. Data from a meta-analysis imply that glucocorticoid hormone levels are not phylogenetically conserved traits but rather evolve as species-specific attributes and readily adapt to current conditions (Bókony et al., 2009), suggesting that ecology is important. While few studies on mammals directly address a glucocorticoid-mediated trade-off in reproduction, it should be noted that Arctic ground squirrels (*Spermophilus parryii plesius*, Boonstra et al., 2001) reproduce even in the face of chronically elevated glucocorticoid levels, presumably due to limited future reproductive opportunities, further suggesting that ecology and life-history are important in interpreting the relationship between stress and reproduction. California mice are naturally found in areas with mild climates – the mountains of central and southern California – and can (and do) breed almost year-round (Gubernick, 1988; Merritt, 1978). Therefore, it is possible that these mice do not need to fine-tune the HPA response because they rarely experience periods of extreme conditions, at least in comparison to species breeding in very severe (e.g., polar or highly seasonal) climates.

While life-history theory mainly focuses on the distribution of limited resources (Reznick et al., 1990; Roff, 1992; Stearns, 1992, 2000), trade-offs can also appear when a physiological constraint exists within an organism. It might be true that glucocorticoids influence both the acquisition of resources and the allocation of resources to either current or future reproduction (Sapolsky et al., 2000), but glucocorticoid-mediated resource allocation may not be driving the trade-off directly. Just as physiological and hormonal constraints on behavior are observed in differing life-history states (Jacobs and

Wingfield, 2000), activation of the stress-responsive systems (e.g., SMS and HPA) may produce scenarios that are no longer conducive to the expression of parental behavior. This is an intriguing concept as concentrations of several circulating and central hormones that are thought to play a role in the regulation of paternal behavior are also involved in and/or affected by the stress response (e.g., corticotropin-releasing hormone, arginine vasopressin, prolactin, oxytocin, opioids, testosterone, serotonin; Herman and Cullinan, 1997; Insel and Young, 2000; Sapolsky et al., 2000; Wynne-Edwards and Timonin, 2007; Ziegler, 2000) and therefore may be important in post-stress modification of behavior. Thus stress, and not glucocorticoids *per se*, may cause physiological changes resulting in a system that can no longer support both current investment in offspring care and parental survival. Data from Aim 4 support this idea as it seems that chronic-stress-induced changes in AVP expression (which may or may not be driven directly by elevated glucocorticoids) are associated with decreased paternal behavior in California mice.

The results from this dissertation provide novel information regarding a highly conserved endocrine system. No other study to date has experimentally manipulated glucocorticoids in mammalian *fathers,* and these studies provide important insight into the effects of stress and glucocorticoids on paternal care. In humans, a large number of child abuse and child neglect cases involve fathers and occur in times of great stress or in the face of paternal stress-related pathology (Maestripieri, 1999; Wipple and Webster-Stratton, 1991). Thus, elucidating the role of stress and glucocorticoids in paternal care could have important implications for humans. Moreover, understanding natural

modification of HPA activity could benefit human medicine; several human diseases and psychopathologies present with HPA axis dysregulation (e.g., major depression, post-traumatic stress disorder, Alzheimer's disease, anorexia nervosa, diabetes, metabolic syndrome and cardiovascular disease; Chrousos and Kino, 2005, 2007; de Quervain et al., 2009; McEwen, 1998, 1999, 2000) for unknown reasons and by unknown mechanisms. Furthermore, support from friends or family can aid in recovery or management of stress and mental illness; thus, understanding how social relationships (such as pair bonds) affect the HPA axis may aid in understanding the underpinnings of some psychopathologies (DeVries et al., 2007). Finally, understanding how stress affects paternal care could aid in conservation efforts of paternally behaving endangered species (i.e. some canids, primates, birds, frogs; Monfort, 2003), which are often subjected to chronic stressors (e.g. anthropogenic disturbance, climate change, habitat disruption, pollution).

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Supplemental Table A. Excretion of radioactivity in urine samples over time following injection of ^{3}H-CORT. Fisher's LSD pairwise comparison P values for the main effect of time following mixed-model ANOVA are presented (P values <0.05 are bolded); significant P values mean average proportion of radioactivity recovered during the two time points differed from one another. Results for mice injected at 0600h (n=4) are presented to the right of the grey boxes, and those for mice injected at 2000h (n=4) are displayed to the left of the grey boxes. Following injection at 0600h, the majority of radioactive urine was excreted 2 and 4 h post-injection, whereas following 2000h injection the majority of radiation was excreted 2 h post-injection.

	2	4	6	8	10	12	14	16	18	20	22	24	36	48	60	72	84
2		**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
4	**<0.01**		0.20	**<0.001**	**0.01**	**<0.01**	**0.019**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
6	**<0.01**	0.73		0.32	0.07	0.21	0.29	0.18	0.14	0.15	0.13	0.21	0.12	0.15	0.14	0.14	0.14
8	**<0.01**	0.57	1.00		0.93	0.85	0.78	0.88	0.89	0.84	0.82	0.98	0.79	0.87	0.83	0.84	0.82
10	**<0.01**	0.62	0.88	0.93		0.98	0.59	0.98	0.97	0.94	0.90	0.90	0.87	0.96	0.91	0.93	0.91
12	**<0.01**	0.47	0.84	0.77	0.93		0.72	0.92	1.00	0.97	0.94	0.86	0.90	0.99	0.95	0.96	0.94
14	**<0.01**	0.60	0.91	0.93	0.98	0.92		0.72	0.55	0.65	0.55	0.78	0.55	0.64	0.59	0.61	0.59
16	**<0.01**	0.51	0.85	0.83	0.95	0.97	0.94		1.00	0.95	0.93	0.86	0.90	0.98	0.94	0.95	0.94
18	**<0.01**	0.50	0.80	0.82	0.89	0.99	0.86	0.97		0.97	0.91	0.88	0.89	0.99	0.94	0.96	0.94
20	**<0.01**	0.51	0.84	0.83	0.94	0.98	0.93	0.99	0.98		0.97	0.79	0.94	0.98	0.98	0.99	0.98
22	**<0.01**	0.51	0.82	0.82	0.91	1.00	0.89	0.98	0.99	0.98		0.84	0.96	0.95	0.99	0.98	0.99
24	**<0.01**	0.51	0.83	0.83	0.93	0.99	0.91	0.98	0.98	0.99	0.99		0.82	0.81	0.83	0.81	0.82
36	**<0.01**	0.54	0.86	0.86	0.95	0.96	0.94	0.99	0.94	0.98	0.95	0.98		0.93	0.94	0.95	0.96
48	**<0.01**	0.53	0.85	0.85	0.95	0.97	0.93	0.99	0.96	0.99	0.97	0.97	1.00		0.97	0.96	0.96
60	**<0.01**	0.51	0.83	0.83	0.93	0.99	0.91	0.99	0.97	0.99	0.98	1.00	0.97	0.98		0.99	1.00
72	**<0.01**	0.50	0.81	0.81	0.91	0.99	0.89	0.97	1.00	0.97	0.99	0.98	0.96	0.95	0.98		0.99
84	**<0.01**	0.51	0.83	0.83	0.92	0.99	0.91	0.98	0.98	0.99	0.99	1.00	0.96	0.98	0.99	0.99	

Supplemental Table B. Excretion of radioactivity in fecal samples over time following injection of ^{3}H-CORT. Fisher's LSD pairwise comparison P values for the main effect of time following mixed-model ANOVA are presented (P values <0.05 are bolded); significant P values mean average proportion of radioactivity recovered during the two time points differed from one another. Results for mice injected at 0600h (n=4) are presented to the right of the grey boxes, and those for mice injected at 2000h (n=4) are displayed to the left of the grey boxes. Animals injected at 0600h showed two prominent spikes in excretion of radioactivity, one at 4-8 h post-injection and a second at 14-16h post-injection. Animals injected at 2000h excreted the majority of radioactivity between 2 and 6h post-injection.

	2	4	6	8	10	12	14	16	18	20	22	24	36	48	60	72	84
2		**<0.01**	**0.03**	0.07	0.37	0.21	**<0.01**	**<0.01**	**0.03**	0.24	0.73	0.87	0.98	0.45	0.93	0.91	0.91
4	0.60		0.44	0.26	**0.02**	**0.03**	**<0.01**	0.74	0.19	**0.03**	**<0.01**	**<0.01**	**<0.01**	**0.01**	**<0.01**	**<0.01**	**<0.01**
6	0.97	0.58		0.72	0.11	0.21	**<0.01**	0.57	0.67	0.18	**0.04**	**0.03**	**0.03**	**0.09**	**0.02**	**0.02**	**0.02**
8	**0.04**	0.11	**0.03**		0.24	0.40	**<0.01**	0.33	0.99	0.36	0.10	0.07	0.07	0.20	0.06	0.07	0.05
10	**<0.01**	**<0.01**	**<0.01**	**0.03**		0.69	**<0.01**	**0.01**	0.16	0.76	0.56	0.43	0.38	0.88	0.33	0.40	0.31
12	**<0.01**	**<0.01**	**<0.01**	0.06	0.95		**<0.01**	**0.03**	0.32	0.93	0.32	0.23	0.21	0.58	0.18	0.22	0.17
14	**<0.01**	**<0.01**	**<0.01**	**0.04**	0.84	0.90		**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
16	**<0.01**	**<0.01**	**<0.01**	**0.03**	0.83	0.91	0.99		0.23	**0.02**	**<0.01**	**<0.01**	**<0.01**	**0.01**	**<0.01**	**<0.01**	**<0.01**
18	**<0.01**	**<0.01**	**<0.01**	**0.02**	0.63	0.71	0.81	0.78		0.27	**0.05**	**0.03**	**0.03**	0.12	**0.02**	**0.03**	**0.02**
20	**<0.01**	**<0.01**	**<0.01**	**0.01**	0.71	0.81	0.92	0.89	0.86		0.37	0.27	0.25	0.65	0.21	0.25	0.19
22	**<0.01**	**<0.01**	**<0.01**	**0.01**	0.79	0.88	0.99	0.97	0.79	0.92		0.84	0.74	0.66	0.66	0.80	0.64
24	**<0.01**	**<0.01**	**<0.01**	0.08	0.63	0.64	0.55	0.51	0.38	0.39	0.45		0.89	0.52	0.80	0.96	0.78
36	**<0.01**	**0.01**	**<0.01**	0.20	0.35	0.41	0.33	0.29	0.21	0.20	0.23	0.66		0.46	0.92	0.93	0.89
48	**<0.01**	**<0.01**	**<0.01**	**0.01**	0.54	0.66	0.76	0.72	0.98	0.81	0.73	0.27	0.13		0.40	0.49	0.38
60	**<0.01**	**<0.01**	**<0.01**	**0.01**	0.53	0.65	0.75	0.70	0.97	0.79	0.71	0.26	0.12	0.99		0.84	0.98
72	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.44	0.56	0.66	0.61	0.88	0.68	0.61	0.21	0.09	0.87	0.89		0.81
84	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.45	0.57	0.67	0.62	0.89	0.70	0.62	0.21	0.09	0.89	0.90	0.99	

Supplemental Table C. Fisher's LSD pairwise comparison P values for the main effect of time following linear mixed-model analysis on pup body mass are presented (P values <0.05 are bolded). Results are for all pups (n=38) as fathers' treatment condition did not significantly affect pup body mass.

Post-Natal Day	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
4	**0.02**																
5	**<0.01**	0.11															
6	**<0.01**	**<0.01**	**0.01**														
7	**<0.01**	**<0.01**	**<0.01**	0.06													
8	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.01**												
9	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.03**											
10	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.02**										
11	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.09									
12	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.02**								
13	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.02**							
14	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.02**						
15	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.01**					
16	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.04**				
17	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.02**			
18	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.02**		
19	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.01**	
20	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.22