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Associations Between Paternal Responsiveness and Stress Responsiveness in the
Biparental California Mouse, *Peromyscus Californicus*

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

Doctor of Philosophy

in

Neuroscience

by

Miyetani Chauke

March 2012

Dissertation Committee:

Dr. Wendy Saltzman, Chairperson

Dr. Karen Bales

Dr. Edward Korzus

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The Dissertation of Miyetani Chauke is approved:

Committee Chairperson

University of California, Riverside

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To my parents.

ABSTRACT OF THE DISSERTATION

Associations Between Paternal Responsiveness and Stress Responsiveness in the
Biparental California Mouse, *Peromyscus Californicus*

by

Miyetani Chauke

Doctor of Philosophy, Graduate Program in Neuroscience
University of California, Riverside, March 2012
Dr. Wendy Saltzman, Chairperson

The mechanistic basis of paternal behavior in mammals is poorly understood. Assuming there are parallels between the factors mediating maternal and paternal behavior, it can be expected that the onset of paternal behavior is facilitated by reductions in stress responsiveness, as occurs in females of several mammalian species. This dissertation describes studies investigating the role of stress responsiveness in the expression of paternal behavior in biparental, monogamous California mice (*Peromyscus californicus*). I compared endocrine, neural, and behavioral responses to stress, as well as anxiety and neophobia, in males from different housing conditions, and between males that did or did not respond paternally to an unfamiliar pup. Results from the first study suggested that fatherhood attenuates behavioral responses, but does not influence corticosterone responses, to an acute predator-urine stressor. Cohabitation with a female mate, however, tended to reduce corticosterone responses to repeated predator-urine exposure. I next characterized neophobia, anxiety, and neuroendocrine responses to acute predator-urine exposure. Results indicated that being a father did not modulate neophobia, but did appear to be associated with re-

duced anxiety. Paternally responsive males exhibited more anxiety-related behavior than nonpaternally responsive males, but did not differ in levels of neophobia. Social isolation, but not fatherhood, modulated the neuroendocrine response to stress exposure, while no effect of paternal responsiveness was found. Finally, to determine whether fathers and non-fathers, or paternally responsive and nonpaternally responsive males, differentially perceive pups as stressful, I compared neural responses to 1-h exposure to a pup. No effect of fatherhood was found; however, paternally responsive virgin males showed lower Fos expression in the paraventricular nucleus of the hypothalamus and bed nucleus of the stria terminalis compared to nonpaternally responsive virgins. My findings suggest that fathers in this biparental species do not exhibit reductions in stress responsiveness similar to those in lactating females of several species. Nonetheless, paternally responsive males show reduced activation of stress-related brain areas in response to pup exposure, as well as altered patterns of neophobia and anxiety-related behavior, as compared to nonpaternally responsive males. These findings will further the understanding of the mechanistic basis of paternal behavior in mammals.

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Chapter 1

Introduction

Becoming a parent is an event accompanied by numerous hormonal, neurochemical, and behavioral changes that are associated with the onset of parental care in mammals. In some rodent species, inexperienced adults avoid alien pups (Fleming & Luebke, 1981). This avoidance is associated with fear and anxiety in response to the novel pup stimulus. Rodent mothers (and fathers in biparental species), however, are attracted to, rather than fearful of pups, and this reduced fear is believed to play a role in facilitating the onset of parental behavior (Numan & Insel, 2003; Numan, 2007). In addition, mothers in several species including rats, mice, humans, and sheep, exhibit a general reduction in stress-responsiveness, fear and anxiety in response to a variety of stressful, frightening, and anxiogenic stimuli (Hard & Hansen, 1985; Lightman, 1992; Lonstein, 2007). No prior studies have explored whether rodent fathers require a similar reduction in emotionality and stress responsiveness in order to behave paternally. The increased central and peripheral expression of the peptides prolactin (PRL) and oxytocin (OT), and decreased central expression of the peptide corticotropin-releasing hormone (CRH) during lactation in comparison to

virgin females, have been implicated in the reduction of emotionality in mother rats (Slattery & Neumann, 2008). Changes in peripheral PRL levels in fathers of several biparental mammalian species suggest that it might play a similar role in fathers (Gubernick & Nelson, 1989; Wynne-Edwards, 2000). It is also possible that fathers exhibit similarly reduced central CRH levels, but this possibility has not been tested previously.

The California mouse (*Peromyscus Californicus*) is a biparental, monogamous rodent that provides a useful model for studying paternal behavior. Males in this species engage in parental behavior to the same extent as mothers (i.e., licking, grooming, huddling the pups), and their paternal investment improves survival and development of offspring (Cantoni & Brown, 1997; Gubernick & Alberts, 1987; Ribble, 1991). The few studies that have been conducted on the mechanistic basis of paternal behavior in California mice have implicated the medial preoptic area (mPOA), nucleus accumbens, basolateral amygdala, lateral habenula, and bed nucleus of the stria terminalis (BnST; (de Jong et al., 2009, 2010; Lee & Brown, 2002, 2007)). There is little evidence of hormonal control of paternal behavior in California mice (Wynne-Edwards & Timonin, 2007). Note that PRL, progesterone (P), and dihydrotestosterone have all been correlationally associated with fatherhood in California mice (see (Gubernick & Nelson, 1989; Trainor et al., 2003)). Finally, no prior studies have examined the role stress responsiveness plays in the onset of paternal behavior in this species. The following three chapters of this dissertation will describe associations between being a new father, or behaving paternally, and behavioral and neuroendocrine stress responsiveness, as well as the neuroendocrine response to pup exposure.

These studies will substantially enhance our understanding of the biology of paternal care, and might have implications for understanding and even alleviating abusive

paternal behaviors in humans.

1.1 Stress

Stress can be defined in several ways, from a perturbation of homeostasis (Selye, 1936; Wingfield & Sapolsky, 2003) to the response to stimuli that elicit fear or anxiety (Muller et al., 2003; Timpl et al., 1998). Psychological and physiological stressors such as hypoglycemia, cold, forced swimming, restraint and novelty activate similar physiological responses at the hormonal level (Dronjak et al., 2004; Figlewicz et al., 2002; Muir & Pfister, 1987; Reeder & Kramer, 2005).

Following exposure to a stressor, the body launches a “stress response”, which involves activation of the hypothalamic-pituitary-adrenal (HPA) axis, as well as the sympatho-adrenomedullary system (SMS). The stress response is characterized by energy mobilization, enhanced cardiovascular tone, sharpened cognition and the inhibition of behaviors and physiological processes not immediately imperative for survival (Nelson, 2005; Sapolsky et al., 2000; Wingfield & Sapolsky, 2003). Pathological consequences of prolonged stress include suppressive effects on such processes as metabolism, growth, tissue repair and immune defenses (Sapolsky et al., 2000; Wingfield & Sapolsky, 2003).

1.1.1 The HPA axis

The paraventricular nucleus of the hypothalamus (PVN) contains magnocellular (larger cells) and parvocellular (smaller cells) regions. The magnocellular PVN (mPVN) and magnocellular region of the supraoptic nucleus (SON) send projections to the posterior pituitary, where they release OT and arginine vasopressin (AVP; (Armario, 2006)). Parvo-

cellular PVN (pPVN) neurons project to the median eminence, where they are in contact with capillaries of the portal blood system (Fulford & Harbuz, 2005). CRH is released by pPVN neurons into the capillaries of the portal blood system and travels down long pituitary portal vessels and into the anterior pituitary. CRH, a 41-amino-acid peptide (Vale et al., 1981), is released, along with pPVN AVP, in response to stressors as well as under basal conditions. CRH has been implicated in mediating the HPA response to stress and modulating fear and anxiety (Bale et al., 2000; Habib et al., 2000; Hammack et al., 2003; Heinrichs et al., 2002; Okuyama et al., 1999; Timpl et al., 1998), as has AVP (Appenrodt et al., 1998; Landgraf et al., 1995; Liebsch et al., 1996). CRH and AVP then bind to their receptors on corticotropes (adrenocorticotrophic hormone (ACTH)-synthesizing cells in the anterior pituitary; Abel, 2005). CRH alone can induce secretion of ACTH, but synergistic activity with AVP enhances this effect (Gillies et al., 1982; Rivier & Vale, 1983). ACTH is released and travels in the circulatory system to the adrenal cortex, where it stimulates the synthesis and release of glucocorticoids from the zona fasciculata (Fulford & Harbuz, 2005) by binding with the G-protein-coupled melanocortin 2 receptor.

Glucocorticoids regulate secretion of CRH from the PVN and ACTH from the anterior pituitary via negative feedback onto the hippocampus, hypothalamus, and pituitary (Herman et al., 2002; Sawchenko, 1987). Glucocorticoids act on two subtypes of receptors, mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) (Fulford & Harbuz, 2005). MRs have a higher affinity for glucocorticoids than do GRs, with MRs suggested to play a role in regulating the basal expression of CRH and parvocellular AVP (Dallman et al., 1989), and GRs to play a role in the termination of the HPA response to stress and the circadian peak (Fulford & Harbuz, 2005).

As the main integrating center for stressful stimuli, the PVN receives afferents from several brain centers responsible for processing stressors, including other regions of the hypothalamus, amygdala, hippocampus, forebrain, cingulate and prefrontal cortex, brainstem, dorsal raphe nucleus, and BnST (Forray & Gysling, 2004; Herman et al., 2005; Pacak & Palkovits, 2001).

1.1.2 The SMS response

The SMS response involves the activation of the sympathetic nervous system in response to acute stress via stimulation of the spinal cord and hindbrain by PVN efferents, resulting in the secretion of epinephrine (from the adrenal medulla) and norepinephrine (from peripheral nerves) (Reeder & Kramer, 2005). The SMS response is almost instantaneous and causes increases in cardiovascular tone, respiration rate, blood flow to the muscles, alertness and blood glucose levels (Nelson, 2005) in order to allow the organism to respond quickly to a stressful situation. Activation of the parasympathetic nervous system then down-regulates the sympathetic nervous system (Reeder & Kramer, 2005).

1.1.3 Interactions between reproductive condition and stress

Reproductive behavior, including sexual and parental behavior, and reproductive physiology can be inhibited by stress in many species (Ferin, 1999; Rivest & Rivier, 1995; Wingfield & Sapolsky, 2003). In humans, parental behavior is affected by psychosocial stressors such as poverty, domestic violence, and sexual assault, which lead to deficiencies in maternal behavior and increased occurrence of child abuse (Banyard et al., 2003; Brockington, 1996; Kotch et al., 1995; Tolan et al., 2006). Prepartum predator-odor stress in rat

dams reduced postpartum pup-directed maternal behaviors such as retrievals and sniffing (Patin et al., 2002), while rat dams stressed by crowding or restraint exhibited reduced maternal behavior (Moore & Power, 1986; Yamada et al., 2002).

There exists a reciprocal relationship between stress and reproduction, and sometimes reproductive condition or the act of engaging in parental behavior can affect an organism's response to stress. The effects of reproductive condition on stress responses and anxiety in mothers are well understood, as described below in Section 1.3 (Lonstein, 2007), although in mammalian fathers few studies have been conducted.

1.2 Neural and endocrine mechanisms of parental behavior

Maternal behavior in rodents is assessed by the level of retrieval, nest building, nursing and pup-grooming behavior that the female engages in (Numan & Insel, 2003). Hormonal changes in female rats during parturition facilitate the dams' maternal behavior immediately after the birth of the pups. PRL, secreted from the anterior pituitary, and the structurally similar placental lactogens, secreted by the placenta, are elevated throughout pregnancy, and are necessary for mammary gland development and lactogenesis (Southard & Talamantes, 1991). The combination of P withdrawal, high estrogen (E2) levels, and a sustained presence of lactogenic hormones is necessary for the rapid onset of maternal behavior in inexperienced postpartum females (Numan & Insel, 2003).

In the absence of hormonal priming, adult female rats, in contrast, actively avoid pups (Fleming & Luebke, 1981), but can be sensitized to engage in maternal behavior through prolonged or repeated exposure to pups. During sensitization, the virgin female

engages in approach and avoidance behaviors toward the pups over several days, until approximately day 7, when she overcomes her fear and avoidance of the pups and begins to behave maternally (Numan, 2007). Treatment of inexperienced, nulliparous (never given birth) female rats with a hormone regimen identical to that of parturient females, rapidly facilitates maternal behavior (Bridges & Freemark, 1995; Bridges et al., 1996). In addition to lactogenic hormones and gonadal steroids, OT is important in the regulation of maternal behavior (Numan & Insel, 2003). OT is a peptide hormone synthesized in the PVN and SON of the hypothalamus, and is released from the posterior pituitary into the bloodstream (Numan & Insel, 2003). In mammals its functions include milk ejection in response to suckling, and uterine contractions in response to vaginocervical stimulation during labor. Peripheral OT levels and central OT levels in the mPOA, BnST, SON and PVN increase greatly during lactation (Kendrick et al., 1992; Theodosis & Poulain, 2001). Intracerebroventricular (i.c.v) infusions of OT into virgin female rats rapidly facilitate the onset of maternal behavior (Pedersen & Prange, 1979).

The majority of data on the neural basis of maternal behavior were obtained from studies on rats (Numan & Insel, 2003). In virgin females, the medial amygdala (MeA) receives inputs from the primary and accessory olfactory systems, which relay information about the novel pup odor to the virgin female's MeA, which in turn inhibits maternal behavior and likely mediates the virgin female's fear of pups (Numan, 2007). Lesions of the MeA facilitate maternal behavior in non-hormonally-primed virgin females, further supporting the role of pup stimuli in activating fear-inducing mechanisms in the virgin female's brain (Fleming et al., 1980; Numan et al., 1993). The mPOA/BnST neurons play a role in both activating maternal behavior, as well as suppressing the avoidance of pups.

Projections from the anterior hypothalamic nucleus to the periaqueductal gray play a role in defensive behavior, fearfulness, and avoidance behavior (Numan & Sheehan, 1997), and there is a proposed network from the olfactory bulbs to the MeA, anterior hypothalamic nucleus and periaqueductal gray that mediates avoidance of pups (Numan, 2007; Sheehan et al., 2001). A neural circuit including the mPOA, BnST, nucleus accumbens, and ventral pallidum has been shown to facilitate the onset of maternal behavior (Olmos & Heimer, 1999; Winn et al., 1997).

1.3 Emotionality and stress responsiveness in mothers

An individual's response to novelty can be either proactive (approach) or reactive (avoidance; (Martin et al., 2007)). Adult virgin female rats without pup experience often require sensitization to pups before they behave maternally (Numan & Insel, 2003; Rosenblatt & Mayer, 1995). Non-sensitized virgin females engage in avoidance/approach behaviors in investigation of the pups, with avoidance prevailing (Numan & Insel, 2003). Thus, it can be said that pups are aversive to nulliparous females, which leads to their avoidance behavior until they eventually overcome their fear after repeated pup exposure (Fleming & Luebke, 1981). Peripartum females, however, are not as fearful of the pups as virgin females are, and quickly engage in maternal behavior (Fleming & Luebke, 1981). Data suggest that some of the factors underlying the peripartum female's reduced emotionality are associated with the change in concentrations of maternal hormones, such as elevated OT and PRL levels, that are also involved in facilitating the onset of maternal behavior (Figueira et al., 2008; Fleming et al., 1989).

1.3.1 Reduced neuroendocrine response to stress in lactating females

The lactating rat, sheep and human mother have been found to exhibit decreased HPA responses to stress, fear and anxiety (Lightman, 1992; Tilbrook et al., 2006; Tu et al., 2005), as compared to virgin and non-lactating females. Several stress paradigms have been performed on lactating rats, including immobilization stress, endotoxins, acoustic startle, predator urine, and open-field tests (Deschamps et al., 2003; Fleming & Luebke, 1981; Lightman, 1992; Toufexis et al., 1999b; Windle et al., 1997b), with these challenges representing both physiological and psychological stressors. A significantly blunted corticosterone and ACTH response to such stressors has been observed in comparison to virgin females (Lightman, 1992; Deschamps et al., 2003; Windle et al., 1997b). On the other hand, lactating female rats have higher baseline corticosterone and ACTH levels than virgin females (Fischer et al., 1995; Lightman, 1992; Windle et al., 1997c,b), primarily due to a flattening of the diurnal corticosterone rhythm, whereby a rise in nadir levels and a trough in peak evening levels occurs (Tilbrook et al., 2006). Baseline CRH mRNA levels are lower, and AVP mRNA levels higher, in the PVN of lactating compared to non-lactating rats (Fischer et al., 1995; Walker et al., 2001a,b; Windle et al., 1997c,b). The increased AVP expression possibly contributes to the elevated basal corticosterone and ACTH levels (Tilbrook et al., 2006).

The stage of lactation affects a female's HPA response to stress; early lactating female rats (one week or less postpartum) display a higher corticosterone (Walker et al., 1995) and ACTH (Deschamps et al., 2003) response to stress than late lactating females (approximately 2 weeks postpartum). Conflicting data describe the effect of the infants'

presence during exposure to a stressor as either eliminating (rats, (Deschamps et al., 2003)) or further accentuating (sheep, (Tilbrook et al., 2006)) neuroendocrine hyporesponsiveness.

The functional significance of the neuroendocrine hyporesponsiveness to stress could lie in the protection of the infant from glucocorticoids in the mother's milk (Numan & Insel, 2003). Treating rat dams with corticosterone during lactation has long-term effects on their offspring, such as reduced stress-induced corticosterone response, increased hippocampal corticosterone receptors, improved conditioned learning, and an attenuated fear-related behavioral response (Casolini et al., 1997; Catalani et al., 1993, 2000). It is also possible that reduced peripartum stress responsiveness could have evolved for the purpose of protecting parental behavior from disruption by stressors (Wingfield & Sapolsky, 2003), or to preserve energy essential for lactation (Numan & Insel, 2003). Studies focusing on the mechanisms underlying this hyporesponsiveness to stress have identified differences between lactating females and virgin females at the level of the pPVN. A decreased pPVN *cfos* response to limbic stimulation has been found in lactating rats (da Costa et al., 1996; Slattery & Neumann, 2008), as well as a reduction in CRH stimulation of pituitary corticotropes (Neumann et al., 1998) and reduced expression of PVN CRH mRNA in response to stress (da Costa et al., 2001; Lightman & Harbuz, 1993). In addition, reduced activity of afferent projections to the PVN (da Costa et al., 2001; Toufexis & Walker, 1996; Toufexis et al., 1998) and reduced pituitary sensitivity to ACTH secretagogues (i.e., CRH and AVP; (Johnstone et al., 2000; Toufexis et al., 1999a) have been observed during lactation.

Other possible mechanisms of hyporesponsiveness could involve the neuropeptides OT and PRL (Numan & Insel, 2003; Slattery & Neumann, 2008). Both OT and PRL have been implicated in reduced stress responsiveness, and are likely responsible for the

hyporesponsiveness period in lactating females (Grattan, 2001; Lightman et al., 2001; Pi & Grattan, 1999; Slattery & Neumann, 2008; Torner et al., 2002, 2004; Windle et al., 1997a,b).

1.3.2 Reduced emotionality in mothers

In addition to neuroendocrine hyporesponsiveness to stress, postpartum rats exhibit reduced anxiety-like behavior, possibly as an adaptation to increase their ability to care for their offspring (Fleming & Luebke, 1981). Parturient rats and mice exposed to acoustic startle, open-field tests, elevated-plus-maze (EPM) and the light-dark box conflict test have a lowered anxiety response compared to virgin females (Fleming & Luebke, 1981; Maestripieri & Damato, 1991; Toufexis et al., 1999b). In postpartum rats, anxiety is reduced specifically in the first few days or weeks (Lonstein, 2005; Picazo & Fernandezguasti, 1993). Infant contact appears to reduce anxiety in lactating rats, as separation from pups for 2-4 hours prior to testing causes an increase in anxiety (Lonstein, 2005; Neumann, 2003). On the other hand, lack of suckling stimulation by pups does not affect elevated plus maze behavior in thelectomized (surgically removed nipples) postpartum rats (Lonstein, 2005). It is possible that contact with infants and engaging in maternal behavior alone are sufficient to somewhat reduce anxiety in mothers, as ovariectomized alloparental virgin females were found to be less anxious than ovariectomized virgin females, but more anxious than lactating females (Pereira et al., 2005). Conflicting data exist, however, as Ferreira et al. (2002) found that alloparental rats do not exhibit reduced anxiety compared to non-maternal ovariectomized virgin rats, but do exhibit reduced fearfulness (Ferreira et al., 1989, 2002). Similar to their possible roles in mediating neuroendocrine hyporesponsiveness to stress, OT and PRL have been found to be anxiolytic (Torner et al., 2002). Interaction

with pups increases PRL mRNA and PRL release in the PVN and mPOA (Torner et al., 2004), and i.c.v infusions of ovine PRL are anxiolytic in virgin male and female rats (Torner et al., 2001). In ovariectomized virgin female rats, chronic i.c.v infusions of ovine PRL were anxiolytic, decreased basal *cfos* mRNA expression in the CeA, and decreased *cfos* and CRH mRNA levels in the pPVN (Donner et al., 2007). High central OT concentrations are anxiolytic in rats and mice (Windle et al., 1997a,b, 2006), OT knockout virgin females are more anxious than wild type, and this is reversed with i.c.v. OT infusion (Amico et al., 2004; Mantella et al., 2003). OT levels increase after infant contact in the cerebrospinal fluid, POA and BnST in sheep (Kendrick et al., 1992). The specific sites where OT acts to reduce anxiety in postpartum rats are unknown, but i.c.v. infusions of OT into the periaqueductal gray reduce anxiety in postpartum females but not in diestrous virgin rats (Figueira et al., 2008). High CRH levels are anxiogenic and reduced CRH levels are anxiolytic in rats and mice (Bakshi & Kalin, 2000; Holsboer, 1999; Schulkin et al., 1998). I.c.v infusion of CRH causes motor activation, increases in anxiety-like behavior, and altered cognitive performance (Koob & Heinrichs, 1999). Correspondingly, CRH1R antagonists, such as antalarmin and CP-154,526, were anxiolytic when administered to rats and mice (Griebel et al., 1998; Millan et al., 2001; Zorrilla et al., 2002). During lactation, some CRH activity is downregulated; however, there is not yet sufficient evidence implicating this reduced CRH activity in reduced anxiety during the postpartum period (Lonstein, 2007).

1.4 Paternal behavior

Paternal behavior is defined as males engaging in extensive behavioral investment in their young (Numan & Insel, 2003). Occurring in only 6% of mammals (Kleiman & Malcolm, 1981); (Ziegler, 2000), paternal behavior has been studied in a variety of species from insects to amphibians to birds to humans. In rodents, paternal behaviors often include grooming, anogenital licking, huddling, pup retrieval, and nest building (Brown, 1985); (Gubernick & Alberts, 1987). Paternal behavior is hypothesized to have evolved because the benefits to a male of engaging in parental behavior may outweigh the advantages of seeking multiple females to impregnate in some species (Maynard Smith, 1977). Research into the mechanisms of paternal behavior has been relatively scarce, and has mostly focused on the inhibition of infanticide rather than the onset of paternal behavior. The California mouse is a valuable rodent model for paternal behavior as it is a biparental species, with paternal males engaging regularly in all maternal behaviors with the exception of nursing (Gubernick & Alberts, 1987).

For many species, the onset of paternal behavior coincides with the inhibition of infanticide. In many mammalian species, males engage in infanticidal behavior in the presence of alien pups (Gubernick et al., 1994). These infanticidal tendencies must somehow be eliminated upon the birth of the male's own pups (Gubernick et al., 1994). Infanticidal behavior in male California mice is sometimes, but not always, inhibited by sexual experience or cohabitation with a pregnant mate (Gubernick et al., 1994). For the majority of males, exposure to their mate within the first 3 days postpartum is important for the maintenance of paternal behavior. Gubernick et al. (1994) hypothesized that neuroendocrine changes

associated with these experiences, such as elevated OT levels, which inhibit infanticide in female house mice (*Mus musculus*, (McCarthy, 1990)), could potentially underlie the onset of paternal behavior in California mice. Recent studies have found no associations between paternal responsiveness and OT immunoreactivity (OT-IR) in California mice (de Jong et al., 2009; Lambert et al., 2011); however, in one study, fathers were characterized by high levels of OT-IR in the PVN, while pup-exposed virgin males were characterized by high levels of AVP-IR in the PVN (Lambert et al., 2011).

1.4.1 Neuroendocrine basis of paternal behavior

The hormones testosterone, corticosterone, AVP, OT, P and PRL have been studied in relation to their possible role in initiating and maintaining paternal behavior.

Testosterone Correlational studies have shown a relationship between decreasing testosterone concentrations and paternal behavior in new fathers in several biparental species (Wynne-Edwards & Timonin, 2007). Experimental studies in the Mongolian gerbil (*Meriones unguiculatus*) found that testosterone implants decreased paternal behavior (Clark & Galef, 1999). In the California mouse, in contrast, testosterone, via intracellular conversion to E2, has been shown to promote the maintenance of paternal behavior in experienced fathers (Trainor & Marler, 2002). The study suggested, however, that testosterone does not play a role in the initiation of paternal behavior (Trainor & Marler, 2002), and previous studies found that testosterone levels do not change in males exposed to pups acutely (Gubernick & Nelson, 1989). Studies in the biparental djungarian hamster (*Phodopus sungorus*) have found that testosterone levels increase prior to the delivery of pups and decrease after

parturition, followed by an increase within 5 days (Reburn & Wynne-Edwards, 1999).

Other steroids Glucocorticoid (cortisol, corticosterone) levels have been shown to increase before the birth of the offspring and decrease afterwards in male djungarian hamsters, non-human primates, and men, but the functional significance, if any, is not known (Wynne-Edwards & Timonin, 2007; Ziegler et al., 1996b). The glucocorticoid (and progesterone) receptor antagonist RU-486 did not alter paternal care in prairie voles (Lonstein & Vries, 2000a), and neither did CORT treatment in California mouse fathers (Harris et al., 2011). Further studies are required to determine if corticosterone plays a causal role in paternal behavior (Wynne-Edwards & Timonin, 2007).

Associations between progesterone levels and parental status are species-specific. In California mice, fathers have lower P concentrations than nonfathers, while in biparental male dwarf hamsters (*Phodopus campbelli*) an increase in P is found around the time of birth (Schum & Wynne-Edwards, 2005; Trainor et al., 2003). P receptors in male mice have been implicated in mediating pup-directed aggression (Schneider et al., 2003).

Arginine vasopressin AVP appears to play a role in paternal behavior, as biparental male prairie voles (*Microtus ochrogaster*) were found to increase responsiveness to pups when infused with AVP into the lateral septum (Wang et al., 1993). In a combined sample of California mice and white-footed mice (*P. leucopus*), AVP-IR in the BnST has been correlated with composite scores of paternal behavior (Bester-Meredith & Marler, 2003).

Oxytocin Analyses of peripheral OT levels in California mice yielded no differences between paternal and non-paternal males, or between infanticidal and non-infanticidal males

(Gubernick et al., 1995). Studies examining the central patterns of OT-IR have yielded no associations with paternal responsiveness in California mice, as described above (de Jong et al., 2009; Lambert et al., 2011). Both AVP and OT receptor patterns have been implicated in paternal behavior in meadow voles (*Microtus pennsylvanicus*) (Parker et al., 2001).

Prolactin New California mouse fathers have increased peripheral PRL levels compared to expectant fathers and virgin males (Gubernick & Nelson, 1989). In other rodents and non-human primates, increased levels of PRL are also correlated with paternal behavior (Wynne-Edwards, 2000). However, in male prairie voles, the dopamine antagonist bromocriptine was administered in order to reduce PRL levels, and paternal behavior was not affected (Lonstein & Vries, 2000a). Data from studies of djungarian hamsters and common marmosets similarly do not support a role for involvement of PRL in paternal behavior (Numan & Insel, 2003). Future studies examining central PRL release and receptor binding are needed to assess the role PRL plays in paternal behavior.

Recently the idea of a causal role of the neuroendocrine system in paternal behavior has been hotly debated (Schradin, 2007; Wynne-Edwards & Timonin, 2007). The role of hormones in paternal behavior onset was questioned, as a significant portion of the data on the neuroendocrine regulation of paternal behavior were correlational, and as studies using antagonists against “paternal” hormones did not produce the expected decrease in paternal behavior. (Wynne-Edwards & Timonin, 2007). Wynne-Edwards & Timonin (2007) suggested that the role of the hormones released by paternal males may be involved in behaviors occurring concurrently with paternal behavior, rather than playing a causal role

in paternal behavior *per se*. A rebuttal by Schradin (2007) posited that the “switch” from infanticidal to paternal behavior is likely to be mediated by neural, rather than neuroendocrine, mechanisms, with hormones still being an important component in the modulation of paternal behavior. It was also postulated that the mechanism underlying paternal care may be similar to those underlying maternal care in experienced mothers, as both rely on experience and external stimuli for their activation (Schradin, 2007).

1.4.2 Neural mechanisms of paternal behavior

Few studies have investigated the neural circuitry underlying paternal behavior in biparental mammals. Fos staining in male prairie voles exposed to pups revealed elevated staining in the accessory olfactory bulbs, MeA and mPOA compared to males not exposed to pups (Kirkpatrick et al., 1994). Lesions in the mPOA, nucleus accumbens and basolateral amygdala were found to impair paternal behavior in California mice (Lee & Brown, 2007). The mPOA is sexually dimorphic in virgin California mice, with males exhibiting a greater number of neurons than females (Gubernick et al., 1993a). This difference is eliminated, however, when the animals become parents, with females exhibiting an increase in mPOA size (Gubernick et al., 1993a). It is likely that males have a similar neurocircuitry mediating parental behavior to females, as lesions in the mPOA in both male and female California mice disrupt parental behavior (Lee & Brown, 2002). Furthermore, Fos-IR data from recent studies implicate the mPOA, BnST, lateral habenula, and dorsal raphe nucleus in paternal behavior of California mice (de Jong et al., 2009, 2010; Lambert et al., 2011).

1.4.3 Emotionality and stress responsiveness in fathers

Almost no data have been reported on emotionality and stress responsiveness in fathers, in contrast to the vast literature on mothers. Recently, however, Bardi et al. (2011) showed that paternal experience in California mice is associated with a lower number of interrupted grooming sequences, described as an indicator of low stress, during exposure to a novel-object. In the same species, Lambert et al. (2011) showed that inverse associations exist between paternal responsiveness and activation of stress- and anxiety-related brain areas following 10-min of pup exposure. In this dissertation, I describe findings from studies exploring the role of stress responsiveness and emotionality in the onset of paternal behavior/fatherhood by addressing the following aims:

Aim 1 Determine whether new California mouse fathers exhibit reduced hypothalamic-pituitary-adrenal (HPA) axis responses to stress as compared to nonbreeding and virgin males.

Aim 2 Determine whether new fathers or paternally responsive males exhibit reduced anxiety, fearfulness, and neural responsiveness to threatening stimuli as compared to non-fathers and nonpaternally responsive males.

Aim 3 Determine the neuroendocrine response to pup exposure in new fathers and paternally behaving males compared to nonfathers and nonpaternally behaving males.

1.5 Significance of the proposed research

Understanding the relationships of parental behavior with stress, fear and anxiety has many implications in both animal and human research. The studies described are among the first to examine the associations between parental status and paternal responsiveness on a biparental male's behavioral and neuroendocrine response to anxiogenic and stressful stimuli. The results may have implications for understanding the role of stress in disrupting parental behavior in captive animals that fail to rear offspring to adulthood. The stress arising from aspects of the captive environment could result in inferior parental behavior, leading to premature deaths of the infants (Clubb & Mason, 2003).

Stress-related disruption of parental behavior in humans is manifested in a multitude of cases of child abuse and child neglect due to psychosocial stressors such as low socioeconomic status, natural disasters, sexual abuse, and domestic violence (Banyard et al., 2003; Brockington, 1996; Kotch et al., 1995; Tolan et al., 2006). Little research has been performed on human fathers to elucidate the effect of stress on child abuse, child neglect and deficient paternal behavior (Dufour et al., 2008). This research will provide a platform from which further studies in fathers can be performed, and will also provide some clues as to the possible hormonal or neurochemical imbalances that may exist in fathers that abuse or neglect their children.

Chapter 2

Effects of reproductive status on behavioral and endocrine responses to acute stress in a biparental rodent, the California mouse (*Peromyscus californicus*)

2.1 Abstract

In several mammalian species, lactating females show blunted neural, hormonal, and behavioral responses to stressors. It is not known whether new fathers also show stress hyporesponsiveness in species in which males provide infant care. To test this possibility,

we determined the effects of male and female reproductive status on stress responsiveness in the biparental, monogamous California mouse (*Peromyscus californicus*). Breeding ($N = 8$ females, 8 males), nonbreeding ($N = 10$ females, 10 males) and virgin mice ($N = 12$ females, 9 males) were exposed to a 5-min predator-urine stressor at two time points, corresponding to the early postpartum (5-7 days postpartum) and mid/late postpartum (19-21 days postpartum) phases, and blood samples were collected immediately afterwards. Baseline blood samples were obtained 2 days prior to each stress test. Baseline plasma corticosterone (CORT) concentrations did not differ among male or female groups. CORT responses to the stressor did not differ among female reproductive groups, and all three groups showed distinct behavioral responses to predator urine. Virgin males tended to increase their CORT response from the first to the second stress test, while breeding and nonbreeding males did not. Moreover, virgin and nonbreeding males showed significant behavioral changes in response to predator urine, whereas breeding males did not. These results suggest that adrenocortical responses to a repeated stressor in male California mice may be modulated by cohabitation with a female, whereas behavioral responses to stress may be blunted by parental status.

2.2 Introduction

Lactational hyporesponsiveness, or blunted responsiveness to stress in lactating females, has been described in a number of mammalian species, including sheep (*Ovis aries*), flying foxes (*Pteropus hypomelanus*), Columbian ground squirrels (*Spermophilus columbianus*), Norway rats (*Rattus norvegicus*), and humans (Deschamps et al., 2003; Hubbs

et al., 2000; Lightman, 1992; Reeder et al., 2004; Shanks et al., 1997; Tilbrook et al., 2006; Tu et al., 2005; Windle et al., 1997b). This phenomenon has been characterized most thoroughly in rats. Compared to virgin females, lactating female rats have been found to exhibit reduced adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) responses to a variety of stressors, including immobilization, endotoxins, acoustic startle, predator urine, and open-field tests (Deschamps et al., 2003; Lightman, 1992; Shanks et al., 1997; Toufexis et al., 1999b; Windle et al., 1997b). Lactating rats also exhibit reduced anxiety-like and stress-related behavior in open-field, elevated plus maze, noise stress, and acoustic startle response tests, compared to virgin females (Fleming & Luebke, 1981; Lonstein, 2005; Toufexis et al., 1999a; Windle et al., 1997b).

At the central level, lactating female rats show reduced stress-induced activation of afferent projections to the paraventricular nucleus of the hypothalamus (PVN), which activates the hypothalamic-pituitary-adrenal (HPA) axis through release of the ACTH secretagogues corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) (da Costa et al., 2001; Toufexis & Walker, 1996; Toufexis et al., 1998). In addition, baseline CRH mRNA levels are lower, and AVP mRNA levels higher, in the PVN of lactating compared to non-lactating female rats (Fischer et al., 1995; Walker et al., 2001a), while lactating rats express lower CRH mRNA levels in the PVN in response to hypertonic saline and restraint stress, compared to virgin females (da Costa et al., 2001; Lightman & Young, 1989). Lactating rats have also been found to exhibit reduced pituitary sensitivity to CRH compared to virgin females, thus partly explaining their lower CORT responses to stress (Toufexis et al., 1999b).

The functional significance of neuroendocrine hyporesponsiveness to stress during

lactation is unknown. One possibility is that it could protect infants from high glucocorticoid concentrations in the mother's milk (Lightman et al., 2001; Slattery & Neumann, 2008). CORT treatment of rat dams during lactation has long-term effects on HPA activity and anxiety-related behavior in their offspring (Brummelte et al., 2006; Casolini et al., 1997; Catalani et al., 1993, 2000). Another possibility is that reduced peripartum stress responsiveness functions to preserve energy essential for lactation (Numan & Insel, 2003), or to buffer parental behavior from disruption by stressors, as hormones of the HPA axis are thought to suppress parental behavior (Brunton et al., 2008; Carter et al., 2001; Lightman et al., 2001; Rivier & Rivest, 1991; Saltzman & Abbott, 2009; Wingfield & Sapolsky, 2003). In approximately 6% of mammalian species, fathers, in addition to mothers, engage in parental care (Kleiman & Malcolm, 1981). The neuroendocrine basis of paternal behavior in rodents is unclear, and the role of hormones in the regulation of paternal behavior is currently under debate (Schradin, 2007; Wynne-Edwards & Timonin, 2007). Moreover, little is known of the neuroendocrine and affective consequences for fathers of engaging in paternal behavior. If, as described above, lactational hypo-responsiveness evolved to buffer parental behavior from stress, a reasonable hypothesis is that fathers of dependent offspring, in addition to lactating females, exhibit reduced neuroendocrine and behavioral responses to stress in biparental species.

We tested this hypothesis in a biparental rodent, the California mouse (*Peromyscus californicus*). California mice are socially and genetically monogamous, and males engage in extensive parental behavior upon the birth of their pups (Gubernick & Alberts, 1987; Ribble, 1991). The presence of the father accelerates pup development and increases pup survival, especially under energetically challenging conditions (Cantoni & Brown, 1997; Dudley, 1974;

Gubernick et al., 1993b; Gubernick & Teferi, 2000; Wright & Brown, 2002). Importantly, both circulating prolactin concentrations and AVP-ir within the brain have been shown to correlate with paternal behaviors in California mouse fathers (Bester-Meredith & Marler, 2003; Gubernick & Nelson, 1989). Given that both of these peptides are involved in the modulation of stress responsiveness, it is plausible that they may play a dual role in facilitating paternal behavior and ameliorating stress-reactivity in new fathers (Appenrodt et al., 1998; Bales et al., 2004; Everts & Koolhaas, 1999; Landgraf, 2006; Parker & Lee, 2001; Torner et al., 2002).

We characterized behavioral and CORT responses to predator-odor stress in breeding male and female California mice as compared to (1) virgin males and virgin females housed in same-sex pairs, and (2) nonbreeding (vasectomized) males and their female pair-mates housed in heterosexual pairs. We chose to use predator urine as a stressor because it is ecologically relevant and because predator odors are known to be potent stressors in a variety of mammalian species (Figueiredo et al., 2003; Kavaliers et al., 2001; Ward et al., 1996; Zhang et al., 2003). Specifically, we used urine from bobcats and coyotes, two predators that are sympatric with California mice through at least part of the latter's range (including the region from which our colony was derived, the Santa Monica Mountains in southern California), and that prey heavily on rodents (Fedriani & Fuller, 2000). We tested the breeding animals in the presence of their pups to avoid separation-induced stress (Lonstein, 2005); however, conflicting data describe the effect of the infants' presence during exposure to a stressor as either eliminating (rats: (Deschamps et al., 2003)) or further accentuating (sheep: (Tilbrook et al., 2006)) neuroendocrine hyporesponsiveness in mothers.

Previous studies of stress hyporesponsiveness in other species focused on females

that were either pregnant or lactating, but not both (Lightman & Young, 1989; Shanks et al., 1999; Walker et al., 1992, 1995). In California mice and many other rodents, however, females commonly undergo a postpartum estrus and conceive shortly after giving birth, so that pregnancy and lactation coincide (Gubernick, 1988). Therefore, we characterized stress responsiveness in female California mice that were concurrently pregnant and lactating, in order to study the animals under more naturalistic reproductive conditions. In rats, the stage of lactation affects females' HPA responses to stress; early lactating females (≤ 1 week postpartum) display higher CORT and ACTH responses to stress than mid-lactating females (approximately 2 weeks postpartum; (Deschamps et al., 2003; Walker et al., 1995)). We therefore tested breeding pairs during both the females' early (5-7 days) and mid/late (19-21 days) postpartum periods, which coincided with early- and mid-pregnancy.

2.3 Materials and Methods

2.3.1 Animals

We used male and female California mice purchased as adults from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Mice were maintained as described previously (de Jong et al., 2009, 2010). Briefly, animals were housed in transparent, $29.2 \times 19.1 \times 12.7\text{cm}$ polycarbonate cages, with aspen shavings as bedding, cotton wool as nesting material, and food (Purina Rodent Chow 5001) and water available *ad libitum*. Lights were on from 0500h to 1900h (14:10), and room temperature and humidity were maintained at approximately $18 - 26^\circ\text{C}$ and 60-70%, respectively. Animals were inspected daily and weighed twice per week to monitor health and pregnancies, and cages

and water bottles were changed once per week. Mice had been weaned at 25-35 days of age, initially housed in same-sex groups of 2-3 animals, and then housed in same-sex pairs upon their arrival in our lab until the start of the study. Animals had been ear-punched at weaning, and were marked with non-toxic hair color (Bigen, Hoya Co. Ltd., Nagoya, Japan) at the start of the study for rapid identification. All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and were reviewed and approved by the University of California, Riverside (UCR) IACUC. UCR is fully accredited by AAALAC.

2.3.2 Design

Male and female mice were randomly assigned to three reproductive groups (same-sex siblings were assigned to different conditions): breeding pairs, nonbreeding pairs, and virgins. Breeding pairs (N=8) comprised a female (breeding females) and sham-vasectomized male (breeding males). Nonbreeding pairs (N=10) comprised a female (nonbreeding females) and vasectomized male (nonbreeding males). Virgin males (sham-vasectomized; N=9) and virgin females (N=12) were maintained in unrelated same-sex pairs from the time of arrival in our laboratory and throughout the experiment.

Each pair of mice underwent two stress tests (see below) separated by 14 days, with each test immediately followed by collection of a blood sample; a baseline blood sample was collected two days before each stress test. Breeding males and breeding females underwent their first baseline blood sample 2-5 days after the birth of their first litter of pups. Breeding females gave birth to their first litter 42.3 ± 6.3 days after pair formation (mean \pm SE), and all but two gave birth to their second litter 14 - 23 (16.2 ± 3.0) days after the second

stress test. The schedule of baseline blood samples and stress tests for nonbreeding males, nonbreeding females, virgin males and virgin females was established by matching each nonbreeding or virgin pair to specific breeding pairs. Cohorts of animals containing mice from each reproductive group were tested concurrently. Time from pair formation (first day of pair-housing) to the first baseline blood sample did not differ between breeding (48.8 ± 6.2 days) and nonbreeding (44.1 ± 2.5 days) pairs ($P=0.463$).

Mean age of males (381.4 ± 16.1 days) at the time of the first stress test did not differ significantly among the three reproductive groups ($P = 0.259$), and there were no significant differences in the mean length of time between surgery and the first stress test (61.4 ± 7.4 days) across male reproductive groups ($P=0.147$). Mean age of females, however, differed among breeding (385.1 ± 20.4 days), nonbreeding (312.9 ± 12.0 days), and virgin (341.4 ± 12.9 days) females ($F [2, 29] = 5.375, P = 0.011$). Breeding females were significantly older than nonbreeding females ($P = 0.009$), while virgin females did not differ significantly from the other two groups.

2.3.3 Surgeries

Vasectomies and sham vasectomies were performed using sterile conditions and standard surgical procedures. Mice were anesthetized with isoflurane, a ventral midline incision (approximately 1cm) was made, the vas deferens was sutured and cut, and the incision was closed with tissue glue. Ketoprofen (5mg/kg , s.c) was administered at the completion of the surgery to provide analgesia. Following one week of recovery, animals were reunited with their cagemates.

2.3.4 Stress Tests

Data collection was divided into two phases, with phase 1 corresponding to the early postpartum period in breeding pairs and phase 2 corresponding to the mid/late postpartum period, two weeks later. In each of the two phases, a baseline blood sample was obtained, a stress test was performed, and a post-stress blood sample was collected from each mouse. Animals in all reproductive groups were tested with their pairmate present, and breeding males and breeding females were tested with their pups also present (1-4 pups per litter). For each stress test, the pair (or family) of mice was placed in a clean cage with bedding, cotton, and a plastic cup (diameter: 4cm \times height: 2cm) containing a clean cotton ball, and taken to an unfamiliar room containing no other animals. Animals were allowed to acclimate to the environment for 5 min, after which two trained observers (inter-observer reliability scores $> 90\%$) each scored the behavior of one focal animal for 5 min on laptop computers using the JWatcher event-recorder program (Blumstein & Daniel, 2007). The cup containing a clean cotton ball was then replaced with an identical cup containing a cotton ball soaked with 1 ml predator urine (see below), and behavioral observations were continued for an additional 5 min. Duration of time that mice spent within 3cm of the plastic cup (distance measured with reference to a 3cm ruler), autogrooming, grooming its cagemate, and freezing, as well as number of jumps, was scored continuously for all focal animals before and during presentation of predator urine. In addition, every 30 sec, upon an audible signal from a timer, the observers recorded whether or not each test subject was in proximity to its cagemate, and whether it was immobile or locomoting. The length of the cage was demarcated into three equal zones, and the zone in which each subject was located

(zone containing the cup, zone furthest from the cup, or intermediate zone) was scored on every 30-sec scan. For breeding animals, duration of time spent licking, in proximity to (within 3cm), and nursing the pups was also scored.

Bobcat and coyote urine (Predatorpee.com, Lexington Outdoors, Robbinston, ME, USA) were used as predator odors in a stress-test protocol modified from that of Deschamps et al. (2003). The urine was collected through floor drains from bobcats and coyotes housed in game farms and refuges, and following collection, was filtered and stored at room temperature. Each pair of mice was exposed to one odor in its first stress test and the other odor in its second stress test. The order of odor presentations was randomly balanced across pairs within each reproductive group. We wiped down the testing area with 70% alcohol immediately following each test; nonetheless, we cannot rule out the possibility that animals may have been exposed to lingering predator urine scents from previous tests. All procedures were carried out between 0900h and 1100h, when baseline plasma CORT levels in our mice are low and incremental responses to stress are pronounced (unpublished data).

2.3.5 Blood Sampling

Mice were anesthetized with isoflurane, and blood samples of up to 140 μ l were collected from the retro-orbital sinus into heparinized microhematocrit tubes. All blood samples, except one, were obtained within 3 min of initial disturbance to the animals (baseline samples) or within 3 min following the end of a stress test (post-test samples) to prevent the capture procedures from influencing CORT levels (Good et al., 2003). The remaining blood sample was obtained in 3 min 40 sec following the end of a stress test; however, the CORT concentration of this sample fell within the range of other post-stress blood samples

in this study. Blood was centrifuged at 13,200 rpm for 12 min at $4^{\circ}C$, and plasma was aspirated and stored at $-80^{\circ}C$ until assayed.

2.3.6 Corticosterone Assays

Corticosterone concentrations in plasma ($100\mu l$ of a 1 : 400 dilution) were determined using a double-antibody ^{125}I -radioimmunoassay kit (MP Biomedicals, Costa Mesa, CA, USA). All samples were assayed in duplicate at the recommended volume. The kit standards generate a curve adequate to measure CORT concentrations between 25 and $1000ng/ml$. Preliminary data indicated that plasma CORT concentrations range from 40 to $2000ng/ml$ in the California mouse. To accommodate this range we diluted plasma 1 : 400 and extended the lower end of the standard curve to $12.5ng/ml$ by diluting the provided $25ng/ml$ standard by half.

To assess the biochemical validity of the kit with California mouse plasma, we performed three validation procedures, parallelism, accuracy, and precision, following the methods of Harper & Austad (2000) and Good et al. (2003). In brief, parallelism was determined by comparing the log-logit slope of serially diluted California mouse plasma to the log-logit slope of the standard curve (see Figure 2.1). Slope equality was determined using linear regression in GraphPad Prism (version 5, GraphPad Software, Inc., La Jolla, CA, USA). The log-logit-transformed slope of the standard curve was statistically indistinguishable ($P = 0.363$) from the log-logit-transformed slope of serially diluted California mouse plasma. Accuracy samples were generated by adding $10\mu l$ of California mouse plasma with a known CORT concentration to each point on the standard curve. Accuracy was determined by comparing the observed CORT concentration in each sample to the expected

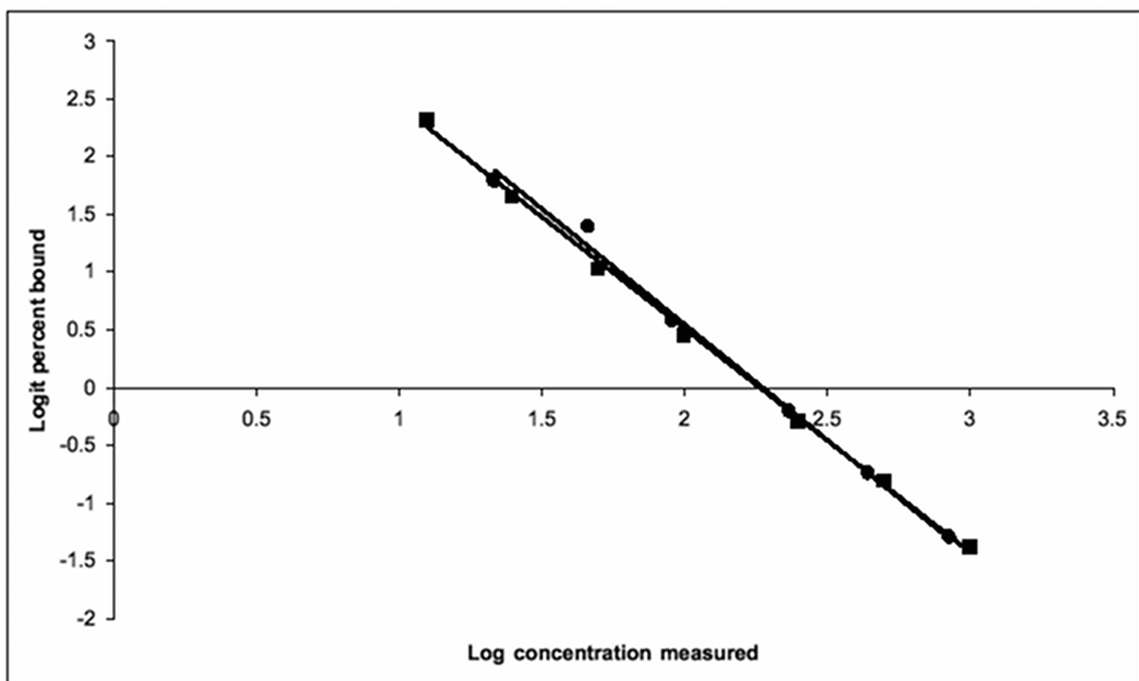


Figure 2.1: Parallelism of corticosterone levels from serial dilutions of California mouse plasma (circles) and standards from a commercially available double-antibody ^{125}I -radioimmunoassay kit (squares). Data were transformed using the log-logit method. California mouse plasma: $y = -1.9966x + 4.5394$. Standard curve: $y = -1.9243x + 4.3507$. Difference between slopes: $P = 0.363$.

concentration. Average assay accuracy was 99.1%, and the difference between the observed and expected CORT concentrations per tube was statistically indistinguishable from zero ($P = 0.620$). Assay precision was determined by calculating intra-assay variation as the coefficient of variation (CV) between replicate samples in an assay. Inter-assay variation was determined by calculating the CV for the average CORT levels in a laboratory-maintained California mouse plasma pool. The intra- and inter-assay CVs were 4.2% and 5.1%, respectively. All samples from each animal were run in the same assay, and each assay included samples from at least one animal from each sex and reproductive condition.

2.3.7 Data Analysis

CORT data were log-transformed to achieve normality and analyzed by 3-way repeated-measures ANOVA for each sex separately, with reproductive group (breeding, nonbreeding, virgin) as a between-groups factor, and phase (early postpartum [phase 1]), mid/late postpartum [phase 2]) and baseline vs. post-test (basal_test) as within-groups factors. We report both main effects and interactions from each ANOVA in the results section. CORT data were tested for outliers (Sokal & Rohlf, 1995), and test subjects with outlying or missing hormonal data were removed from the analysis. Final numbers of animals used in CORT analyses were 8 breeding males, 9 nonbreeding males, 5 virgin males, 7 breeding females, 9 nonbreeding females, and 11 virgin females.

All behavioral scores, except jumping, are expressed as percentages of total observation duration or percentages of total number of 30-sec instantaneous scans; jumps are expressed as total number of occurrences within each 5-minute testing period before or during predator-urine exposure. Behavioral data were compared among groups non-

parametrically, using the Kruskal-Wallis test; significant overall effects were followed by nonparametric pairwise post-hoc comparisons (Siegel & Castellan, 1988). Within reproductive groups, behavior was compared between phases and before vs. during predator-urine exposure using Wilcoxon Signed Ranks tests. Statistical analyses were performed using SPSS (IBM Corporation, Somers, NY, USA) and Systat (Systat Software, Inc., Chicago, IL, USA), and were evaluated at the 0.05 level (2-tailed).

2.4 Results

2.4.1 Males

Corticosterone Initial comparisons of baseline plasma CORT concentrations revealed that baseline levels did not differ among breeding, nonbreeding, and virgin males (main effect of group: $F[2, 19] = 1.463$, $P = 0.256$) or between phase 1 and phase 2 (main effect of phase: $F[1, 19] = 3.956$; $P = 0.061$; group X phase interaction: $F[2, 19] = 1.984$, $P = 0.165$; Figure 2.2A). Predator-urine tests significantly elevated plasma CORT above basal levels in male California mice (main effect of basal_test: $F[1, 19] = 405.649$, $P < 0.001$; compare Figures 2.2A and 2.2B; note different y-axes). The magnitude of the CORT response to stress relative to basal levels differed between phase 1 and phase 2 (basal_test X phase interaction: $F[1, 19] = 10.635$, $P = 0.004$), and this effect differed among the three groups of males (group X basal_test X phase interaction: $F[2, 17] = 3.901$, $P = 0.031$). Virgin males tended to increase the magnitude of their CORT response to stress from phase 1 to phase 2 ($F[1, 4] = 5.712$, $P = 0.075$), while breeding and nonbreeding males did not (breeding males: $F[1, 7] = 0.118$, $P = 0.745$; nonbreeding males: $F[1, 8] = 1.485$, $P = 0.258$; Figure

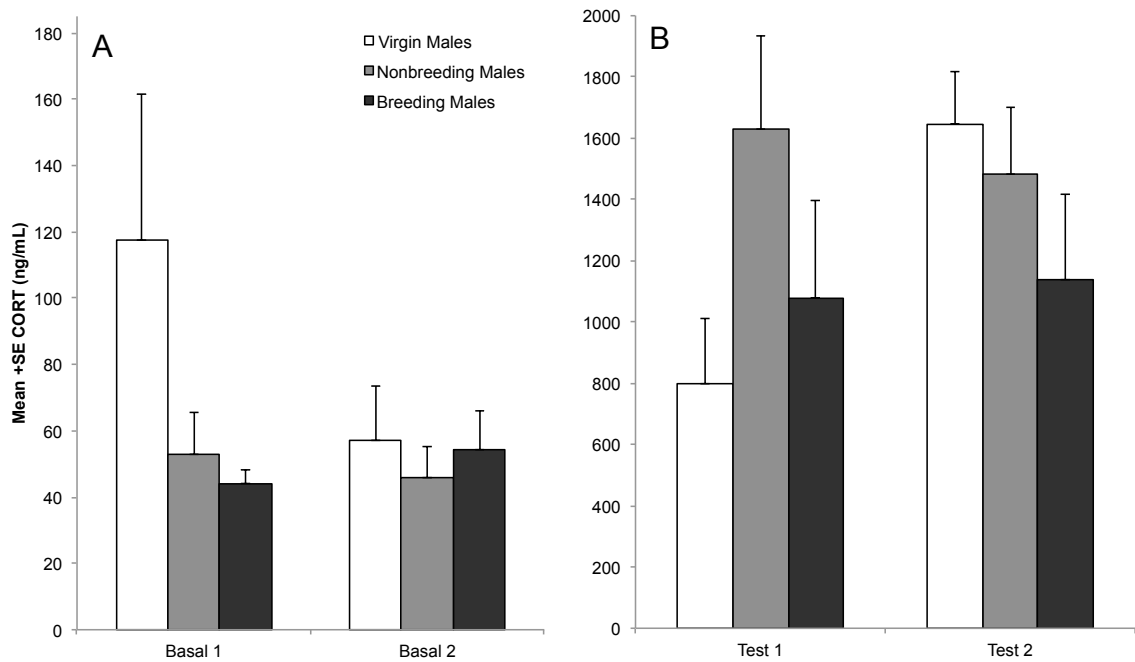


Figure 2.2: Plasma corticosterone concentrations of breeding, nonbreeding and virgin male California mice in phase 1 and phase 2. A: Baseline CORT concentrations; B: CORT concentrations immediately following exposure to predator urine. CORT levels were significantly higher after predator-urine exposure than under baseline conditions ($P < 0.001$). A group X phase X basal_test interaction ($P = 0.031$) revealed that the change in CORT responses to stress from test 1 to test 2 differed among groups. Virgin males tended to increase their CORT response to stress from phase 1 to phase 2 ($P = 0.075$), whereas breeding and nonbreeding males did not. Note difference between graphs in y-axis scale.

2.2B). Planned comparisons revealed that virgin males had a significantly greater increase in the magnitude of the stress response from phase 1 to phase 2, as compared to nonbreeding males (group X basal_test X phase interaction: $F[1, 12] = 6.412$, $P = 0.026$), and tended to have a greater increase in the magnitude of the stress response as compared to breeding males (group X basal_test X phase interaction: $F[1, 11] = 4.620$, $P = 0.055$).

Behavior Locomotion, autogrooming, approaches to within 3 cm of the cup (containing a clean or predator-urine-soaked cotton ball), proximity to cagemate, grooming cagemate, and freezing behavior did not differ among groups of males in either phase 1 or phase 2, either before or during predator-urine exposure (Table 2.1). To determine whether repetition of the stressor or exposure to predator urine affected behavior, we compared behavior in the 5 min before vs. the 5 min during predator-urine exposure, as well as in phase 1 vs. phase 2, using Wilcoxon tests for each group of males. Phase did not significantly influence behavior either during or before urine exposure in any of the groups of males (see Table 1). Predator urine did, however, affect behavior in the nonbreeding and virgin male groups. During predator-urine exposure, as compared to the 5 min prior, virgin and nonbreeding males spent significantly less time in proximity to the cup (nonbreeding males, phase 1: $Z = -2.380$, $P = 0.017$, $N = 10$; nonbreeding males, phase 2: $Z = -2.547$, $P = 0.011$, $N = 9$; virgin males, phase 2: $Z = -2.201$, $P = 0.028$, $N = 8$), and less time autogrooming (virgin males, phase 1: $Z = 2.366$, $P = 0.018$, $N = 9$; nonbreeding males, phase 2: $Z = -2.521$, $P = 0.012$, $N = 9$). In contrast, no differences were found between behaviors before and during stressor exposure in breeding males. Breeding males did not differ in the duration of time spent licking their pup(s) or in proximity to their pup(s) before vs. during

Table 2.1: Behavior (median, range) before and during exposure to predator urine in breeding (N=8), nonbreeding (N=9-10), and virgin (N=8-9) male California mice.

Behavior	Breeding males		Nonbreeding males		Virgin males		Kruskal-Wallis, χ^2		P	
	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2
Within 3 cm of cup ^a										
Pre-stress	2.2 (0.6, 18.5)	7.0 (0.4, 17.1)	6.0 (0.0, 47.4)	5.2 (0.78, 14.0)	2.6 (0.0, 50.6)	1.5 (0.0, 14.6)	0.065	3.707	0.968	0.157
During stress	4.0 (0.0, 15.2)	1.6 (0.0, 12.5)	0.7 ^d (0.0, 19.7)	0.4 ^d (0.0, 3.4)	1.5 (0.0, 11.3)	0.2 ^d (0.0, 4.4)	2.396	1.783	0.302	0.410
Autogroom ^a										
Pre-stress	0.9 (0.0, 7.0)	1.5 (0.0, 22.1)	1.9 (0.0, 7.2)	3.6 (0.0, 21.0)	2.8 (0.0, 18.9)	1.7 (0.0, 17.8)	2.864	2.332	0.239	0.312
During stress	0.4 (0.0, 7.5)	0.1 (0.0, 3.9)	0.0 (0.0, 2.9)	0.0 ^d (0.0, 0.0)	0.0 ^d (0.0, 4.3)	0.0 (0.0, 4.0)	3.142	5.248	0.208	0.073
Groom partner ^a										
Pre-stress	0.0 (0.0, 0.9)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 1.9)	0.0 (0.0, 0.0)	2.368	0.000	0.306	1.000
During stress	0.0 (0.0, 2.8)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	2.375	0.000	0.305	1.000
Freeze ^a										
Pre-stress	0.0 (0.0, 1.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.3)	0.0 (0.0, 1.7)	0.0 (0.0, 0.0)	0.0 (0.0, 0.6)	2.817	1.895	0.244	0.388
During stress	0.2 (0.0, 32.6)	0.0 (0.0, 2.8)	0.0 (0.0, 21.5)	0.3 (0.0, 13.6)	0.0 (0.0, 2.9)	0.6 (0.0, 26.1)	1.503	1.342	0.472	0.511
Jump ^b										
Pre-stress	29.0 (0.0, 93.0)	19.0 (2.0, 86.0)	29.5 (0.0, 52.0)	25.0 (3.0, 86.0)	12.0 (0.0, 83.0)	22.5 (0.0, 134.0)	3.605	0.051	0.165	0.975
During stress	40.5 (0.0, 99.0)	15.5 (0.0, 63.0)	35.0 (0.0, 139.0)	20.0 (0.0, 258.0)	15.0 (0.0, 88.0)	5.5 (0.0, 100.0)	0.590	0.151	0.745	0.927
Locomotion ^c										
Pre-stress	40.0 (0.0, 60.0)	40.0 (0.0, 70.0)	30.0 (0.0, 60.0)	30.0 (0.0, 70.0)	10.0 (0.0, 100.0)	5.0 (0.0, 40.0)	1.840	5.303	0.398	0.071
During stress	38.9 (0.0, 100.0)	11.1 (0.0, 66.7)	50.0 (0.0, 66.7)	22.2 (0.0, 66.7)	11.1 (0.0, 88.9)	0 (0.0, 66.7)	2.279	1.865	0.320	0.394
Proximity to partner ^c										
Pre-stress	80.0 (10.0, 90.0)	75.0 (40.0, 100.0)	60.0 (0.0, 90.0)	70.0 (20.0, 100.0)	60.0 (40.0, 100.0)	85.0 (30.0, 90.0)	0.368	0.501	0.832	0.779
During stress	72.3 (0.0, 100.0)	66.7 (0.0, 88.9)	72.3 (0.0, 100.0)	77.8 (0.0, 100.0)	66.7 (33.3, 88.9)	61.1 (11.1, 100.0)	0.116	0.721	0.944	0.697
In cage zone furthest from predator urine ^c										
Pre-stress	40.0 (10.0, 90.0)	50.0 (20.0, 70.0)	40.0 (10.0, 100.0)	60.0 (0.0, 100.0)	60.0 (0.0, 100.0)	70.0 (20.0, 100.0)	0.324	1.434	0.850	0.488
During stress	38.9 (0.0, 100.0)	66.7 (11.1, 100.0)	33.3 (11.1, 100.0)	44.4 ^d (11.1, 100.0)	66.7 (33.3, 100.0)	94.5 (11.1, 100.0)	3.543	1.313	0.170	0.519

^a Percent of time during the 5-min period before or during predator-urine exposure.

^b Total number of occurrences.

^c Percent of 30-sec instantaneous scans during the 5-min period before or during predator-urine exposure.

^d Significantly different from pre-stress scores for the same group of males (P<0.05)

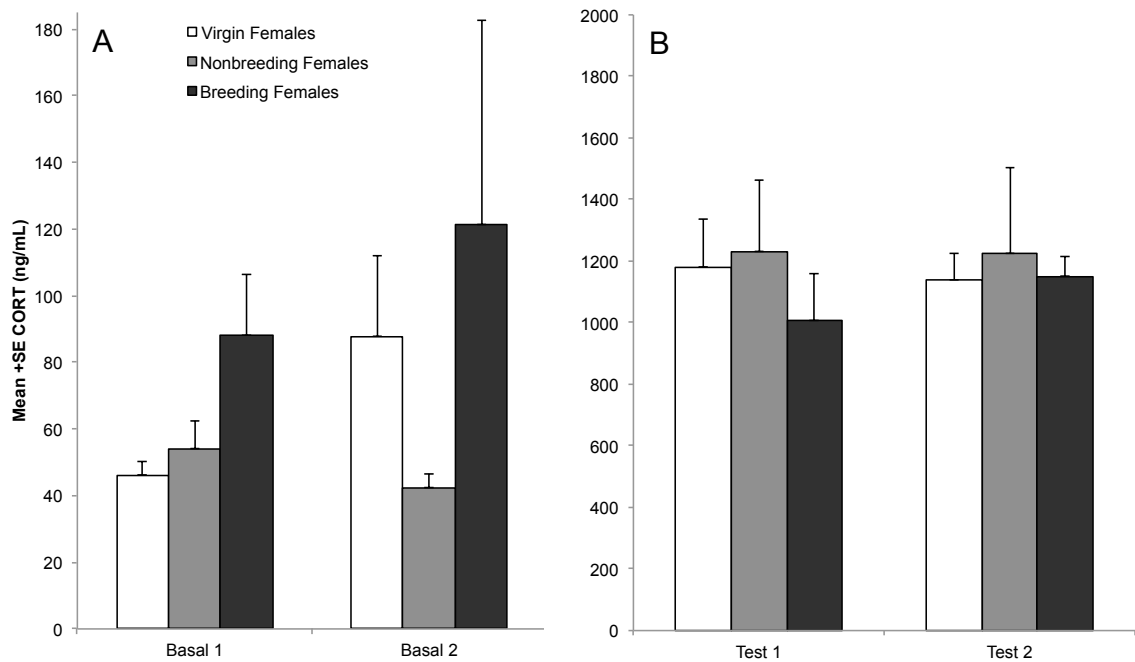


Figure 2.3: Plasma corticosterone concentrations of breeding, nonbreeding and virgin female California mice in phase 1 and phase 2. A: Baseline CORT concentrations; B: CORT concentrations immediately following exposure to predator urine. CORT levels were significantly higher after predator-urine stress exposure than under baseline conditions ($P < 0.001$) but did not differ between phases or among groups. Note difference between graphs in y-axis scale.

predator-urine exposure (Table 2.3).

2.4.2 Females

Corticosterone Initial comparisons of baseline plasma CORT concentrations revealed that CORT levels did not differ among breeding, nonbreeding, and virgin females (main effect of group: $F[2, 23] = 0.446$, $P = 0.645$) or between phase 1 and phase 2 (main effect of phase: $F[1, 23] = 0.746$, $P = 0.397$; group X phase interaction: $F[2, 23] = 1.417$, $P = 0.263$; Figure 2.3A). Exposure to predator urine significantly raised females' plasma CORT

concentrations above basal levels (main effect of basal_test: $F[1, 23] = 9.277$, $P < 0.001$) (compare Figures 2.3A and 2.3B; note different y-axes). However, neither overall CORT levels nor the difference between basal and post-stress CORT levels differed among breeding, nonbreeding, and virgin females (main effect of group: $F[2, 23] = 0.871$, $P = 0.432$; group X basal_test interaction: $F[2, 23] = 0.181$, $P = 0.836$) or between phases (main effect of phase: $F[1, 23] = 0.086$, $P = 0.771$; phase X basal_test interaction: $F[1, 23] = 0.071$, $P = 0.291$) (Figure 2.3B). Because female groups differed significantly in age (see Materials and Methods), age was used as a covariate in the analyses; however, no significant effects of age were found.

Locomotion, approaches to within 3 cm of the cup, proximity to cagemate, grooming cagemate, and freezing behavior did not differ among groups of females in either phase 1 or phase 2, either before or during predator-urine exposure (Table 2.2). During phase 1, however, female reproductive groups differed in the amount of time spent autogrooming during stressor exposure ($\chi^2 = 6.022$, $P = 0.049$). Pairwise post-hoc comparisons revealed that breeding females engaged in more autogrooming than virgin females ($Z = -2.007$, $P = 0.045$), while nonbreeding females did not differ from either of these groups.

Phase did not significantly influence behavior in breeding and nonbreeding females. In contrast, virgin females engaged in more locomotor activity during stressor exposure in phase 1 than in phase 2 ($Z = -2.588$, $P = 0.010$, $N = 12$), and spent more time in the cage zone furthest from the predator urine during stressor exposure in phase 2 than in phase 1 ($Z = -2.106$, $P = 0.035$, $N = 12$). Acute exposure to predator urine altered behavior in all three groups of females. During predator-urine exposure, as compared to the preceding 5 min, virgin females significantly reduced their locomotor activity (phase

Table 2.2: Behavior (median, range) before and during exposure to predator urine in breeding ($N = 8$), nonbreeding ($N = 9 - 10$), and virgin ($N = 12$) female California mice.

Behavior	Breeding females		Nonbreeding females		Virgin females		Kruskal-Wallis, χ^2		P	
	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2
Within 3 cm of cup ^a										
Pre-stress	7.6 (0.0, 40.8)	5.7 (0.0, 18.8)	4.9 (0.0, 14.1)	2.3 (0.0, 8.4)	3.3 (0.0, 32.8)	5.1 (0.3, 22.1)	0.669	4.756	0.716	0.093
During stress	2.5 (0.0, 59.4)	0.1 (0.0, 5.0)	0.5 (0.0, 7.8)	0.8 (0.0, 4.9)	2.7 (0.0, 17.3)	0.9 ^d (0.0, 20.1)	1.679	0.988	0.432	0.610
Autogroom ^a										
Pre-stress	2.0 (0.0, 8.8)	1.4 (0.0, 6.0)	3.6 (0.0, 8.8)	2.8 (0.0, 17.9)	1.2 (0.0, 4.9)	2.1 (0.0, 6.6)	0.510	0.484	0.775	0.785
During stress	1.1 (0.0, 6.2)	0.0 (0.0, 46.5)	0.0 ^d (0.0, 5.2)	0.0 (0.0, 20.1)	0.0 (0.0, 4.4)	0.2 (0.0, 5.2)	6.022	0.188	0.049	0.910
Groom partner ^a										
Pre-stress	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 1.3)	0.0 (0.0, 10.7)	0.0 (0.0, 4.9)	0.0 (0.0, 0.0)	0.762	2.222	0.683	0.329
During stress	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 5.2)	0.0 (0.0, 16.9)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	2.000	2.222	0.368	0.329
Freeze ^a										
Pre-stress	0.0 (0.0, 1.0)	0.0 (0.0, 0.3)	0.0 (0.0, 0.8)	0.0 (0.0, 16.9)	0.0 (0.0, 5.9)	0.0 (0.0, 0.2)	0.224	1.126	0.894	0.569
During stress	1.0 (0.0, 18.2)	0.4 (0.0, 27.9)	0.2 (0.0, 37.6)	1.0 ^d (0.0, 25.6)	0.0 (0.0, 0.6)	0.1 ^d (0.0, 5.5)	2.021	0.544	0.364	0.762
Jump ^b										
Pre-stress	0.0 (0.0, 50.0)	2.0 (0.0, 91.0)	4.5 (0.0, 81.0)	7.0 (0.0, 119.0)	13.5 (0.0, 84.0)	5.0 (0.0, 120.0)	3.894	1.137	0.143	0.566
During stress	0.0 (0.0, 137.0)	0.0 (0.0, 31.0)	19.5 (0.0, 110.0)	25.0 (0.0, 83.0)	2.0 (0.0, 92.0)	3.0 (0.0, 241.0)	2.872	4.341	0.238	0.114
Locomotion ^c										
Pre-stress	20.0 (0.0, 60.0)	35.0 (0.0, 70.0)	30.0 (0.0, 70.0)	30.0 (0.0, 50.0)	40.0 (0.0, 100.0)	20.0 (0.0, 70.0)	2.217	0.705	0.330	0.703
During stress	0.0 (0.0, 77.8)	5.6 (0.0, 55.6)	22.2 (0.0, 77.8)	22.2 (0.0, 55.6)	33.3 ^d (0.0, 88.9)	5.6 ^e (0.0, 77.8)	0.920	0.634	0.631	0.728
Proximity to partner ^c										
Pre-stress	80.0 (10.0, 90.0)	75.0 (40.0, 100.0)	60.0 (0.0, 90.0)	70.0 (20.0, 100.0)	90.0 (40.0, 100.0)	75.0 (30.0, 90.0)	2.235	0.471	0.327	0.790
During stress	72.3 (0.0, 100.0)	66.7 (0.0, 88.9)	72.3 (0.0, 100.0)	77.8 (0.0, 100.0)	77.8 (11.1, 100.0)	66.7 (0.0, 88.9)	0.143	1.023	0.931	0.600
In cage zone furthest from predator urine ^c										
Pre-stress	45.0 (0.0, 100.0)	30.0 (0.0, 80.0)	55.0 (20.0, 100.0)	60.0 (10.0, 100.0)	50.0 (0.0, 100.0)	50.0 (10.0, 90.0)	0.088	2.534	0.957	0.282
During stress	66.7 (22.2, 100.0)	72.3 ^d (33.3, 100.0)	44.5 (0.0, 100.0)	66.7 (0.0, 100.0)	55.6 (11.1, 100.0)	83.4 ^e (0.0, 100.0)	0.390	0.158	0.823	0.924

^a Percent of time during the 5-min period before or during predator-urine exposure.

^b Total number of occurrences.

^c Percent of 30-sec instantaneous scans during the 5-min period before or during predator-urine exposure.

^d Significantly different from pre-stress scores for the same group of females ($P < 0.05$).

^e Significantly different from phase 1 scores for the same group of females ($P < 0.05$).

Table 2.3: Parental behavior (median, range) before and during exposure to predator urine in breeding males ($N = 8$) and breeding females ($N = 8$).

Behavior	Breeding males		Breeding females	
	Phase 1	Phase 2	Phase 1	Phase 2
Lick pup(s) ^a				
Pre-stress	0.0 (0.0, 6.7)	0.0 (0.0, 2.4)	6.3 (0.0, 35.1)	3.1 (0.0, 59.3)
During stress	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	1.8 (0.0, 36.5)	0.2 (0.0, 44.19)
Nursing ^a				
Pre-stress	-	-	99.7 (98.6, 99.8)	99.7 (98.0, 99.7)
During stress	-	-	99.7 (99.1, 100.0)	99.7 (99.6, 100.0)
Proximity to pup(s) ^b				
Pre-stress	25.0 (0.0, 100.0)	30.0 (0.0, 70.0)	100.0 (90.0, 100.0)	95.0 (40.0, 100.0)
During stress	27.8 (0.0, 100.0)	33.3 (0.0, 100.0)	100.0 (100.0, 100.0)	100.0 (88.9, 100.0)

^a Percent of time during the 5-min period before or during predator-urine exposure.

^b Percent of 30-sec instantaneous scans during the 5-min period before or during predator-urine exposure.

1: $Z = -2.296$, $P = 0.022$, $N = 12$) and proximity to the cup (phase 2: $Z = -2.667$, $P = 0.008$, $N = 12$), nonbreeding females decreased the amount of time spent autogrooming (phase 1: $Z = -1.992$, $P = 0.046$, $N = 10$), and both virgin and nonbreeding females increased the amount of time spent freezing compared to their behavior before stressor exposure (phase 2, virgin females: $Z = -2.197$, $P = 0.028$, $N = 12$; phase 2, nonbreeding females: $Z = -1.992$, $P = 0.046$, $N = 9$). Breeding females spent more time in the cage zone furthest from the cup during predator-urine exposure, compared to the preceding 5 min (phase 2: $Z = -2.240$, $P = 0.025$, $N = 8$; see Table 2.2), but maternal behaviors (licking the pup(s), proximity to the pup(s), and nursing) were not affected by predator-urine exposure (Table 2.3).

2.5 Discussion

The present study is among the first to investigate the effects of reproductive state on responses to acute stress in males of a biparental mammalian species. We predicted that we would find blunted CORT and behavioral responses to a predator-urine stressor in new fathers and mothers, based on the hypothesis that reduced stress responsiveness found in lactating mammals may have evolved to protect parental behavior from disruption by stressors. We found partial support for this hypothesis: virgin males tended to increase their CORT response from the first to the second stress test, while breeding and nonbreeding males did not. In addition, virgin and nonbreeding males showed significant behavioral changes in response to predator urine, whereas breeding males did not. In contrast, no differences were found in CORT responses to stress in females, and all female groups showed behavioral changes in response to predator urine. Our findings point to a role of reproductive status in the modulation of behavioral and endocrine stress responsiveness in males, but do not provide evidence for an effect of reproductive state on stress responsiveness in female California mice.

2.5.1 Baseline corticosterone levels

Initially, we compared baseline CORT levels among reproductive groups within each sex. Breeding females tended to have higher mean baseline CORT concentrations than virgin and nonbreeding females during both the early lactational period and the late lactational period, but these differences did not reach statistical significance. Elevated baseline levels of CORT have been found in lactating female rats (Fischer et al., 1995; Stern

et al., 1973; Walker et al., 1995) and have been attributed to the high energetic costs of lactation (Walker et al., 1992). The breeding females in the current study were simultaneously lactating and pregnant, however, and in other rodents, early-pregnant females have low baseline CORT levels (Ogle & Kitay, 1977; Stefanski et al., 2005; Waddell & Atkinson, 1994). Thus, our use of breeding females that were simultaneously pregnant and lactating could have been responsible for the lack of significant differences in baseline CORT among female groups in the current study.

Baseline plasma CORT concentrations did not differ among breeding, nonbreeding and virgin males. This finding suggests that fatherhood, pair bonds, and same-sex relationships do not influence baseline CORT differentially in male California mice. This is similar to findings in prairie voles (*Microtus ochrogaster*), in which baseline CORT levels did not differ between isolated males and males housed with a female pairmate (Vries et al., 1997), but contrasts with findings from Glasper & Vries (2005) in which male California mice paired with an ovariectomized female had significantly lower baseline CORT than isolated males. In that study, social isolation, rather than the absence of a female pairmate, might have resulted in elevated baseline CORT levels (McCormick et al., 2006; Ruscio et al., 2007). In biparental cotton-top tamarins (*Saguinus oedipus*), baseline urinary cortisol levels were not found to differ between new fathers and males paired with a female (Ziegler et al., 1996a), although in biparental black tufted-ear marmosets (*Callithrix kuhlii*), males' baseline urinary cortisol levels increased after the birth of their first litter (Nunes et al., 2001). Altogether, these findings suggest that being a father does not influence baseline CORT levels consistently across species, and that paternal behavior may be relatively disengaged from hormones of the HPA axis, as has been proposed previously (Wynne-Edwards

& Timonin, 2007).

Baseline CORT concentrations in California mice in this study were considerably lower than those in other studies of the same species (Glasper & Vries, 2005; Oyegbile & Marler, 2006; Trainor et al., 2010). Differences in methodology might explain this disparity. In the Glasper & Vries (2005) study, baseline plasma samples were obtained from animals that had previously been subject to wounding procedures; however, the times of day at which wounding and blood collection were performed, as well as the latency from disturbance to blood collection, were not specified. Without this information, it is difficult to compare baseline CORT measures in the two studies. Predictably high baseline CORT levels were found by (Oyegbile & Marler, 2006; Trainor et al., 2010) shortly after lights out, corresponding to the rise in CORT around the time of waking in nocturnal rodent species ((Dallman et al., 1993; Malisch et al., 2008); unpublished data). Furthermore, (Trainor et al., 2010) obtained baseline plasma samples $> 7min$ following initial disturbance to the animals. In contrast, baseline blood samples in the present study were collected several hours after lights-on, near the nadir of the daily CORT rhythm ((Dallman et al., 1993; Malisch et al., 2008); unpublished data), and were collected within 3 min of initial disturbance to the animals.

2.5.2 Predator-urine stressor

Predator odor is an ecologically relevant stressor that represents a threat to the individual mouse and its pups, and that elicits a conserved and stereotyped neuroendocrine response across rodent species (Deschamps et al., 2003; Kavaliers et al., 2001; Masini et al., 2005; Perrot-Sinal et al., 1999). Among female California mice in the current study, CORT

levels were markedly increased following predator-urine stress tests in all reproductive groups. No differences were found in the magnitude of the CORT response to stress among breeding, nonbreeding, and virgin females. These results contrast with numerous findings in rats, in which lactating dams were shown to have a reduced CORT response to noise, endotoxin, elevated plus maze, and light/dark box tests compared to virgin females (Lightman, 1992; Shanks et al., 1997; Toufexis et al., 1999a; Windle et al., 1997b). Again, our findings might reflect the fact that the breeding females in the present study were concurrently pregnant and lactating, unlike the lactating females used in previous studies of other rodent species. In rats, stress responsiveness differs between lactating and pregnant females: pronounced CORT responses to stress, similar to those seen in virgins, have been found in early-pregnant rats (Nakamura et al., 1997; Neumann et al., 1998), while blunted CORT responses to stress, similar to those of lactating females, have been found in late-pregnant rats (Brunton et al., 2008).

Another difference between the present study and most previous studies of lactational hyporesponsiveness in rodents is that breeding females in our experiment were exposed to the predator-urine stressor while in the presence of their pups (and mate), which might have sensitized the lactating females' CORT response to stress, possibly circumventing any adaptations for stress hyporesponsiveness. Deschamps et al. (2003) found that early lactating rats tested in the presence of their pups had a significantly higher CORT response to predator urine than early lactating females separated from their pups. In sheep, however, the lambs' presence and suckling greatly attenuated their mothers' cortisol responses to isolation and restraint stress, compared to ewes tested in the absence of their lambs or with their lambs present but unable to suckle (Tilbrook et al., 2006). Finally, the differ-

ences among studies may also be due to the nature of the stressor. For example, while the CORT response to repeated predator odor stressors is often sustained or sensitized in rodents (Blanchard et al., 1998; Figueiredo et al., 2003; Plata-Salaman et al., 2000), the response to other stressors such as chronic restraint or isolation typically habituates (Cole et al., 2000; Fernandes et al., 2002; Girotti et al., 2006; McCormick et al., 2006). As so few studies on lactational hyporesponsiveness to stress have utilized predator odor as a stressor, however, comparisons have to be made with studies applying different types of stress paradigms.

Studies of several other species have failed to detect reduced glucocorticoid responses to stressors in lactating as compared to non-lactating females (mice: (Douglas et al., 2003); rhesus macaques, *Macaca mulatta*: (Maestripietri et al., 2008); humans: (Altemus et al., 2001; Kaye et al., 2004)). (Note that Douglas et al. (2003) found that lactating mice had a significantly reduced ACTH response, but not CORT response, to forced swimming compared to virgin females). This, in addition to a number of studies in which blunted anxiety and fear were not found in lactating rats (Sibolboro-Mezzacappa et al., 2003; Silva et al., 1997; Young & Cook, 2004; Zuluaga et al., 2005), suggests that the occurrence of lactational stress hyporesponsiveness is highly variable and dependent on experimental design, as well as species.

In the present study, the inclusion of nonbreeding pairs of California mice (i.e., vasectomized males and their female mates) allowed us to evaluate possible effects of a pair bond, in addition to parenthood, on stress reactivity. Being a parent and engaging in a pair bond did not differentially modulate stress responsiveness in females, as no differences were found in the stress reactivity of breeding females compared to nonbreeding and virgin

females. Social isolation has been found to greatly sensitize adrenocortical responses to stress in female prairie voles, Siberian hamsters (*Phodopus sungorus*), and black tufted-ear marmosets, in comparison to females housed with a male or female pairmate (Detillion et al., 2004; Grippo et al., 2007; Smith et al., 1998). Few studies, however, have attempted to characterize differential effects of male-female pair bonds, same-sex cagemates, and parental status on stress responsiveness. Importantly, the nonbreeding females in our study may have become pseudopregnant following copulation (Kenney et al., 1977). Elevated prolactin levels commonly found in pseudopregnant females could have dampened the nonbreeding females' neuroendocrine response to stress, similar to what was expected in breeding females. As neither breeding nor nonbreeding females differed from virgin females in their behavioral or hormonal responses to stress, however, this scenario seems unlikely.

We found that the change in males' CORT responses to predator urine from phase 1 to phase 2 differed among reproductive groups. Virgin males tended to become sensitized to the stressor, with an increased CORT response in phase 2 compared to phase 1, while males pair-housed with a female (either breeding or nonbreeding) responded similarly in the two phases. These data suggest an effect of mating or male-female pair bonding in modulating the response to repeated predator-urine stress in males. As described above, social support in the form of a male-female pair bond or same-sex social bond typically reduces the CORT response to acute stress in male prairie voles in comparison to socially isolated males (Carter, 1998; Vries et al., 1997). Oxytocin released within the brain during mating and/or pair formation in prairie voles (Cho et al., 1999; Waldherr & Neumann, 2007) has been implicated in the social buffering and modulation of stress responsiveness (Vries et al., 1997; Petersson et al., 1999; Windle et al., 1997a), and this might be the mechanism

through which breeding and nonbreeding male California mice avoided sensitization to the predator-urine stressor. Furthermore, central AVP associated with the facilitation and maintenance of pair bonds (Cho et al., 1999; Liu et al., 2001; Winslow et al., 1993) may have modulated the stress response in breeding and nonbreeding males, although it is unclear whether it would play a stimulatory or inhibitory role (Landgraf, 2006). Finally, exposure to estrous, pseudopregnant, or pregnant females could have played a part in dampening the CORT responses to repeated predator-urine stress in breeding and nonbreeding males. Such an effect has previously been found in male mice (*Mus musculus*), whereby brief exposure to odors from an estrous female facilitated the dampening of CORT responses to predator odor (Kavaliers et al., 2001).

Reproductive status also modulated behavioral responses to the predator-urine stressor in male, but not female, California mice. Predator urine affected behavior in nonbreeding and virgin males, causing acute reductions in time spent in proximity to the cup and in time spent autogrooming. In contrast, breeding males showed no significant behavioral changes in response to predator urine. These results, along with recent findings showing that paternal experience correlates with altered behavioral responses to novelty, but not fear, in pup-exposed California mice (Bardi et al., 2011), suggest that fatherhood, engaging in paternal behavior, or exposure to a pregnant female blunts the effects of stress on behavior in males.

Neuroendocrine hyporesponsiveness to stress in lactating female mammals is thought to have evolved as a mechanism to protect parental behavior from disruption in the face of stressors (Altemus et al., 1995; Brunton et al., 2008; Carter et al., 2001; Lightman et al., 2001; Wingfield & Sapolsky, 2003). If this hypothesis is correct, we would expect fathers in

biparental species to exhibit reduced stress responsiveness compared to non-fathers. The results of this study suggest that fatherhood, or engaging in paternal behavior, does buffer the behavioral response to stress, but that this effect is not clearly associated with, or mediated by, diminished glucocorticoid responses to stress, at least in this biparental species. The reduction in anxiety-related behavior found in lactating females of other species is likely mediated by a combination of maternal hormones and contact with their pups (Figueira et al., 2008; Lonstein, 2005). Therefore, it is likely that stimuli from and interaction with the pups may have contributed to breeding males' reduced stress-related behavior in the current study. These findings suggest that differential mechanisms underlie the lactation-induced suppression of behavioral and neuroendocrine responses to stressors in parents, with parental behavior or stimuli from pups possibly mediating an altered behavioral response, but not an altered adrenocortical response to stress.

2.5.3 Conclusion

In summary, the results of this study suggest that parenthood does not modulate corticosterone responses to an acute stressor in male and female California mice. Cohabitation with a female pairmate, however, does appear to reduce the adrenocortical response to repeated stress in males, while fatherhood appears to mitigate the behavioral response to stress. Ongoing studies further characterizing HPA function, stress-induced neural activation, and anxiety-related behavior in California mouse fathers and non-fathers are being conducted to more fully ascertain the extent to which fatherhood may influence stress responsiveness and emotionality in this monogamous, biparental mammal.

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Chapter 3

Paternal responsiveness is associated with, but not mediated by reduced neophobia in male California mice (*Peromyscus californicus*)

3.1 Abstract

Hormones associated with pregnancy and parturition have been implicated in facilitating the onset of maternal behavior via reductions in neophobia, anxiety, and stress responsiveness. To determine whether the onset of paternal behavior has similar associ-

ations in biparental male California mice (*Peromyscus californicus*), we compared paternal responsiveness, neophobia (novel-object test), and anxiety-like behavior (elevated plus maze, EPM) in isolated virgins (housed alone), paired virgins (housed with another male), expectant fathers (housed with pregnant pairmate), and new fathers (housed with pairmate and pups). Corticotropin-releasing hormone (CRH) and Fos immunoreactivity (IR) were quantified in brain tissues following exposure to a predator-odor stressor or under baseline conditions. New fathers engaged in more nest-building during paternal-behavior tests than all other males, and showed lower anxiety-like behavior than expectant fathers and isolated virgins in EPM tests. In all housing conditions, stress elevated Fos-IR in the hypothalamic paraventricular nucleus (PVN). Social isolation reduced overall (baseline and stress-induced) Fos- and colocalized Fos/CRH-IR, and increased overall CRH-IR, in the PVN. In the central nucleus of the amygdala, social isolation increased stress-induced CRH-IR and decreased stress-induced activation of CRH neurons. Across all housing conditions, paternally behaving males displayed more anxiety-related behavior than nonpaternal males but showed no differences in CRH- or Fos-IR. Finally, the latency to engage in paternal behavior was positively correlated with the latency to approach a novel object. These results suggest that being a new father does not reduce anxiety, neophobia, or neural stress responsiveness. Low levels of neophobia, however, were associated with, but not necessary for paternal responsiveness.

3.2 Introduction

Maternal behavior in lactating rats is associated with reduced fearfulness, anxiety, and stress responsiveness (Fleming & Luebke, 1981; Hard & Hansen, 1985; Lonstein, 2005; Toufexis et al., 1999a; Windle et al., 1997b). In contrast to lactating dams, virgin female rats avoid and are fearful of unfamiliar pups due to the aversive properties of the infants' odors and vocalizations (Fleming & Luebke, 1981; Fleming & Rosenblatt, 1974; Numan & Insel, 2003). As would be expected, inhibiting the neural circuitry mediating fear and avoidance, as well as the neural pathways processing olfactory stimuli, facilitates the onset of maternal behavior in virgin female rats (Fleming & Rosenblatt, 1974; Fleming et al., 1979, 1980; Numan, 2007). Under natural conditions, inhibition of the fear/avoidance circuitry is most likely mediated by the high estrogen:progesterone ratio and the elevated central concentrations of prolactin and oxytocin found during the peripartum and postpartum periods (Bridges et al., 1996, 2001; Numan, 2007; Numan & Insel, 2003).

Maternal hormones also serve to downregulate stress reactivity in postpartum females. In several species, the hormonal and neuronal changes occurring at parturition and lactation are accompanied by profoundly reduced behavioral and neural responses to stress. Compared to virgin females, for example, lactating female rats show a blunted stress-induced activation of the hypothalamo-pituitary-adrenal (HPA) axis, manifest in reduced synthesis and release of corticotropin-releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), and glucocorticoids (da Costa et al., 2001; Deschamps et al., 2003; Lightman, 1992; Shanks et al., 1997, 1999; Toufexis et al., 1999b). These changes are accompanied by attenuated levels of anxiety and fear in comparison to virgin females (Fleming & Luebke,

1981; Hard & Hansen, 1985; Lonstein, 2005; Toufexis et al., 1999a; Windle et al., 1997b). The function of lactational stress hyporesponsiveness is unknown. However, as anxiety and neophobia are associated with pup-avoidance behaviors in virgin females (Fleming et al., 1980; Fleming & Luebke, 1981; Numan & Insel, 2003; Numan et al., 1993), it is likely that lactational hyporesponsiveness facilitates the onset of maternal behavior.

Spontaneous paternal behavior by males occurs in approximately 6% of mammalian species, including humans (Kleiman & Malcolm, 1981; Numan & Insel, 2003). Little is known about the neuronal or endocrine mechanisms underlying the onset and maintenance of paternal behavior, or its associations with stress responsiveness. The current literature on the mechanistic basis of paternal behavior has implicated the bed nucleus of the stria terminalis (BnST), medial preoptic area (mPOA), lateral habenula, medial amygdala and nucleus accumbens (Bester-Meredith & Marler, 2003; de Jong et al., 2009, 2010; Kirkpatrick et al., 1994; Lee & Brown, 2007). This corresponds to findings in female rats, which showed that the BnST, mPOA, and nucleus accumbens were important for the expression of maternal behavior (Numan, 2007) in response to pup sights, sounds and odors. It is unknown whether these similarities between the neural mechanisms controlling maternal and paternal behavior extend to the relationship between reduced stress responsiveness and the onset of parental behavior. Initial studies of the monogamous, biparental California mouse (*Peromyscus californicus*) suggest that paternal experience is associated with changes in anxiety-related behavior (Bardi et al., 2011) and behavioral responses to acute stress (Chauke et al., 2011), but does not markedly affect the corticosterone response to acute stress (Chauke et al., 2011); however, no studies have attempted to elucidate the role that anxiety and stress responsiveness may play in the onset and maintenance of paternal

behavior, and it is unknown whether engaging in paternal behavior is either a consequence or a cause of reduced behavioral and neural stress responses.

We therefore tested two hypotheses, which are not mutually exclusive:

1. Parental status influences stress responsiveness and emotionality. Specifically, fatherhood reduces neophobia, anxiety-like behavior, and neural responses to stress.
2. Individual differences in stress responsiveness and/or emotionality are associated with individual differences in paternal responsiveness. Specifically, males that show spontaneous paternal behavior toward a foster pup exhibit less neophobia, less anxiety-related behavior, and smaller neural responses to stress, as compared to males that do not behave paternally.

To test these hypotheses, we characterized behavioral responses to a foster pup, neophobia, anxiety-like behavior, and neural stress responsiveness in males that were housed (1) with a pregnant/lactating pairmate and pups, (2) with a female pairmate that was pregnant for the first time (3) with a same-sex pairmate, or (4) individually. Males housed with a first-time pregnant pairmate were used to control for effects of housing with newborn pups, males housed with a same-sex pairmate were used to control for reproductive experience and chemosensory cues from a pregnant female, and individually housed males were included to control for potential effects of social housing in general on behavior and neural stress responsiveness. We performed paternal-behavior, novel-object, and elevated-plus-maze tests to characterize paternal responsiveness, neophobia, and anxiety, respectively, and predator-urine exposure was used as an ethologically relevant stressor to characterize behavioral and neural (CRH, Fos) responses to stress.

3.3 Methods

3.3.1 Animals

We used 70 male and 36 female California mice, descendants of males and females that were purchased as adults from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Mice were housed in $44 \times 24 \times 20\text{cm}$ polycarbonate cages containing aspen shavings for bedding and cotton wool for nesting material, with food (Purina Rodent Chow 5001) and water available *ad libitum*. Lighting was on a 14:10 cycle, with lights on from 0500h to 1900h, room temperature at $18 - 26^\circ$, and humidity at 60-70%. At 27-33 days of age, prior to the birth of the next litter of siblings, animals were ear-punched for identification, removed from their parents' cage, and housed in groups of four same-sex, age-matched cagemates until the start of the study. Animals were inspected daily, and cages and water bottles were changed once per week. Beginning at the time of pair formation or isolation (see Section 3.3.2), mice were weighed twice per week to monitor health and pregnancies and to habituate the animals to handling. At least 2 days elapsed between cage-changing and any experimental procedures. All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and were reviewed and approved by the University of California, Riverside (UCR) Institutional Animal Care and Use Committee. UCR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

3.3.2 Experimental design

At 105.4 ± 2.1 (*mean* \pm *SE*) days of age, male California mice were randomly assigned to four housing conditions. New fathers ($N = 18$), were housed with a female pairmate and underwent data collection following the birth of their first litter; expectant fathers ($N = 18$), were housed with a female pairmate and underwent data collection when the female was pregnant with their first litter; paired virgins ($N = 16$), were housed with an unrelated male; and isolated virgins ($N = 18$), were housed alone for the duration of the study. Each experimental animal underwent a series of behavioral tests (see Sections 3.3.3, 3.3.4 and 3.3.5 below) at intervals of 24-48h. First, each male was exposed to a foster pup in a paternal-behavior test, followed 29-31h later by exposure to a novel object, and subsequently, 24-26h later by an elevated-plus-maze (EPM) test. Approximately 19h following EPM tests, animals were perfused transcardially and brains were collected (see Section 3.3.6 below for details). Within each housing condition, approximately half of the males were euthanized under stress conditions, and the other half were left undisturbed prior to perfusion.

Mice underwent the experimental procedures in cohorts consisting of age-matched males in the new father, expectant father, paired virgin, and isolated virgin conditions. Female pairmates of males in the new father group gave birth 41.6 ± 2.2 (*mean* \pm *SE*) days following pair formation. New fathers underwent their first behavioral test, the paternal-behavior test, 2-3 days following the birth of their first litter of pups. The start of behavioral testing for all other groups was based on the number of days from pair formation to the onset of testing in the new father group (44.3 ± 2.1 days). Animals were weighed twice per week

beginning at 3.3 ± 2.0 days following pair formation (new fathers, expectant fathers, and paired virgins), or isolation (isolated virgins). Neither mean age at the time of the paternal-behavior test (146.5 ± 2.2 days) nor duration of the period of biweekly weighing (38.5 ± 1.9 days) differed among the four housing conditions (age at paternal test: $F[3, 69] = 2.039$, $P = 0.117$; duration of weighing period: $F[3, 69] = 0.731$, $P = 0.537$).

Female pairmates of new fathers and expectant fathers were euthanized after the males were removed from their cages for perfusion; their uterine horns were dissected out, and wet weights were obtained. Females were classified as pregnant following visual identification of embryos within uterine embryo sacs. Pregnant females had uterine horns weighing a total of $2.4 \pm 0.6g$, compared to a previously identified nonpregnant weight of $0.001 \pm 0.0g$ ($t = 2.141$, $P = 0.044$) typically found in virgin females (unpublished data). The specific stage of pregnancy, however, was not determined. Males in the expectant father condition whose mates were not confirmed as pregnant were omitted from data analysis.

3.3.3 Paternal-behavior tests

Paternal-behavior tests were carried out as described previously (de Jong et al., 2009, 2010). Briefly, the tests were performed between 1000h and 1200h, during the light phase, in which California mice typically engage in a high degree of paternal behavior (Wright & Brown, 2002). Each male was removed from its home cage and placed in a clean cage containing bedding and cotton nesting material. The mouse, in its cage, was then carried to a separate room and allowed to habituate for 10 min. An unrelated pup, age 1-4 days, was placed in the corner of the cage furthest from the male. Mice were videotaped for the duration of the 10-min test, after which the male and pup were immediately returned to

their respective home cages. In five tests, experimental males attacked stimulus pups ($N = 3$ isolated virgin males, 2 paired virgin males). These tests were terminated immediately following initiation of the attacks, and the pups were euthanized with pentobarbital (ca. 200 – 300mg/kg ip Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI, USA).

For paternal-behavior tests as well as all other behavioral tests, behavioral measures were scored from videotapes by an observer blind to housing condition, using the event-recorder program JWatcher (Blumstein & Daniel, 2007). For paternal-behavior tests, latencies to approach the foster pup, huddle the foster pup, and engage in paternal behavior (i.e., lick, huddle, or mouth-carry the foster pup), and duration of time spent mouth-carrying, sniffing, licking, huddling the pup, in kyphosis and nest-building were scored as previously described (de Jong et al., 2009, 2010).

3.3.4 Novel-object tests

Novel-object tests took place in the colony room between 1630h and 1830h, nearing the start of the dark phase, when the mice begin to increase their physical activity (unpublished data). Each male's cagemate(s) were removed from the home cage, and 5 min later a stainless steel, wire-mesh tea-ball (diameter: 8cm) was placed in the corner of the cage furthest from the mouse. Behavioral responses to the novel object were recorded on videotape for the duration of the 5-min test. Immediately following testing, the male was reunited with its cagemate(s) in the home cage. Behavioral parameters scored included latency to approach to within 2cm of the novel object, as well as duration of time spent sniffing and touching the novel object, and duration of rearing behavior.

3.3.5 Elevated-plus-maze (EPM) tests

EPM tests were conducted between 1630h and 1830h in an unfamiliar room. The EPM apparatus was constructed from dark, opaque polycarbonate material and consisted of two open arms ($51 \times 9\text{cm}$) and two closed arms ($51 \times 9 \times 20\text{cm}$), raised 1m above the floor. A lamp containing a 60W bulb was placed directly above the center of the apparatus to provide even illumination to all arms. Each experimental male was removed from its home cage in the colony room, carried in a small container to an unfamiliar room, and then placed in the center of the maze, facing an open arm as per the protocol of Walf & Frye (2007). Mice were videotaped in the EPM for 5min , after which they were reunited with their cagemates. Behaviors scored in JWatcher included number and duration of open-arm, closed-arm and EPM center entries; duration of time spent immobile; and number of fecal boli expelled and head dips performed in the open arms.

3.3.6 Predator-urine exposure

At 1000-1100h, animals assigned to the stress condition, along with their cagemate(s), were carried in their home cage to an unfamiliar room and allowed to habituate for 2h. A plastic cup containing a cotton ball soaked with 1mL coyote urine (PredatorPee.com, Lexington Outdoors, Robbinston, ME, USA) was placed in a corner of the cage for 5min and then removed; this procedure elicits rapid and marked elevations in plasma corticosterone concentrations in California mice (Chauke et al., 2011). Animals in the undisturbed condition were left undisturbed in their home cage in the colony room. One hour following the end of predator-odor exposure for stressed mice, or between 1200h and 1300h for undisturbed mice, males were deeply anesthetized with pentobarbital (ca. $200 - 300\text{mg/kg}$,

ip), followed by transcardial perfusion with 0.1M phosphate-buffered saline and later, 4% paraformaldehyde. Brains were processed for immunohistochemistry as described below (Section 3.3.7).

3.3.7 Fos and CRH Immunohistochemistry

Immunohistochemistry was performed as described previously (de Jong et al., 2009, 2010). Briefly, brains were sliced into 30um coronal sections on a cryostat and collected in five series. Brain slices in one series were colocalized for Fos and CRH. All slices from the series were incubated overnight with rabbit-anti-Fos antibody (sc-253, 1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by donkey-anti-rabbit second antibody (1:1500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), then stained with 3,3'-diaminobenzidine (DAB) and ammonium nickel sulfate in order to mark Fos-positive cells as blue-black in color. To achieve Fos and CRH colocalization, the brain slices were later incubated overnight with goat-anti-CRH (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by the donkey-anti-goat second antibody (1:1500; Jackson ImmunoResearch, West Grove, PA, USA) and stained with DAB, but without ammonium nickel sulfate. This resulted in a brown cytoplasmic staining that was distinguishable from the blue-black nuclear Fos staining. Individual brain sections were then mounted on gelatin/chrome-alum-coated glass slides, dehydrated, cleared in ethanol and xylene, embedded in entellan (EMS, Hatfield, PA, USA), and coverslipped.

3.3.8 Quantification of immunoreactivity

Due to the absence of a *P. californicus* brain atlas, the *Mouse Brain in Stereotaxic Coordinates* (Franklin & Paxinos, 2008) for the neuroanatomically similar C57BL/J6 mouse was used to locate relevant brain areas (de Jong et al., 2009). Bregma levels in the text refer to levels specified in the atlas, and not actual Bregma levels in *P. californicus*.

For the quantification of Fos-, CRH- and colocalized Fos/CRH-immunoreactivity (Fos-IR, CRH-IR, and Fos/CRH-IR, respectively), brain areas were selected based on their known functions and associations with stress responsiveness and anxiety (Dielenberg et al., 2001; Kovacs, 1998): the bed nucleus of the stria terminalis (BnST, mouse Bregma level +0.14), paraventricular nucleus of the hypothalamus (PVN, mouse Bregma level -0.82), and central nucleus of the amygdala (CeA, mouse Bregma level -1.46).

Standardized digital photographs of each brain area, as well as a millimeter scale, were taken at a magnification of 20 \times with a digital camera (Canon EOS-40D) mounted on a microscope (Leica Leitz DMRB). Using image analysis software (GNU Image Manipulation Program, Version 2.6, GIMP), a grid of lines equivalent to $0.2 \times 0.2\text{mm}$ was placed in each photograph so that it contained either all or the majority of immunoreactive neurons in the selected area, or, in case of larger brain areas, a portion containing a representative spread of immunoreactive neurons within specific brain regions. Numbers of Fos-positive neurons as well as numbers of CRH-positive and Fos/CRH-colocalized neurons within the square were counted manually by an observer blind to identity, housing condition, and stress condition of the mice. Data for Fos- and CRH-positive cells are expressed as *counts*/ $200\mu\text{m}^2$, while data for Fos/CRH-colocalized neurons are expressed as the percentage of Fos-positive CRH

neurons out of the total number of CRH *neurons*/200 μm^2 .

The BnST and CeA showed higher densities of CRH-IR than the PVN, such that the vast majority of neurons counted in these two regions possessed either CRH-IR or Fos/CRH colocalization. Analysis of colocalization in the PVN was therefore based on the quantification of Fos-immunoreactive cells within CRH cell bodies, and for the CeA and BnST, colocalized cells were defined as those in which Fos-positive cells appeared to be immersed in CRH-rich regions.

3.3.9 Statistical analyses

The litters of two of the new fathers died before the end of data collection; in these cases, we used only those data that were collected prior to the death of the pups. In the expectant father group, three of the female pairmates gave birth prior to the completion of behavioral tests and brain collection. For the males in these pairs, we used only those data that were collected before their pairmates gave birth. Again, data from these males were comparable to those from other expectant fathers.

Behavioral and immunohistochemical data were analyzed using the Statistical Package for the Social Sciences (SPSS) v. 16.0 (IBM Corporation, Armonk, NY, USA). Variables were tested for normality using Levene's test for homogeneity of variance and the Skewness-Kurtosis test. Latencies to engage in paternal behavior (i.e., lick, huddle, or mouth-carry the foster pup), approach the foster pup, and approach the novel object were normally distributed among the four housing conditions and therefore were analyzed using ANOVAs. Significant effects were followed up using Tukey's HSD tests. The remaining behavioral data were not normally distributed and therefore were analyzed using nonpara-

metric tests. Behavioral scores from paternal-behavior, novel-object, and EPM tests were compared among males in the four housing conditions using Kruskal-Wallis tests, and significant effects were followed up by nonparametric post hoc pairwise comparisons (Siegel & Castellan, 1988).

To characterize paternal responsiveness in the paternal-behavior test, for each male we calculated a composite score of total time spent engaging in licking, huddling, kyphosis, nest-building, and mouth-carrying (i.e., full paternal behavior). Each male spent either $< 1.0\%$ or $> 20.0\%$ of the 10min test engaging in full paternal behavior (see Results Section 3.4.1). Therefore, we categorized each male as being “paternal” or “nonpaternal”, based on whether or not it engaged in full paternal behavior for more than 20% of the paternal-behavior test. Mann-Whitney tests were used to compare behavior of paternal and nonpaternal males (pooled across all four housing conditions) in novel-object and EPM tests.

Fos-IR data from the BnST and CeA were not normally distributed. Fos-IR data from the CeA were normalized by square-root transformation and were therefore analyzed parametrically, whereas Fos-IR data from the BnST remained non-normally distributed following transformation and were therefore analyzed nonparametrically. Numbers of Fos- and CRH-positive cells in the PVN and CeA, numbers of CRH-positive cells in the BnST, and the percentage of CRH neurons colocalized with Fos (Fos/CRH-positive cells) in the PVN, BnST and CeA were compared among all four housing conditions using 2-way ANOVAs with either (1) stress (stressed, undisturbed) and housing condition (new fathers, expectant fathers, paired virgins, isolated virgins) or (2) stress and paternal responsiveness (paternal, nonpaternal) as factors. Significant main effects of housing condition were followed up by

Tukey's post hoc tests.

Associations between paternal responsiveness, neophobia, and anxiety were quantified from correlations of paternal-behavior, novel-object, and EPM test results using Spearman's rho. The relationship between the neural response to stress and paternal responsiveness was examined by correlating stress-induced Fos-IR in the PVN and colocalized Fos/CRH-IR in the CeA with several measures of paternal behavior (see Section 3.7 below). Correlations were performed using bivariate Pearson's correlations for data representing latencies to engage in paternal behavior and approach the novel object (normally distributed), and using Spearman's rho for all other data sets (non-normally distributed). To reduce the probability of Type 1 error from performing multiple comparisons, the Bonferroni correction was applied to adjust critical P values. For all other analyses, effects were accepted as statistically significant when P (two-tailed) was less than 0.05 ($Z \pm 1.996$).

3.4 Results

3.4.1 Paternal-behavior tests

Results of paternal-behavior tests are summarized in Table 3.1. Nest-building behavior differed significantly among new fathers, expectant fathers, paired virgins, and isolated virgins (Kruskal-Wallis test, $\chi^2 = 9.746$, $P = 0.021$). New fathers had higher total durations of nest-building behavior compared to expectant fathers, paired virgins, and isolated virgins; however, these differences were not statistically significant in post hoc pairwise tests. An overall significant difference among housing conditions was also found in the latency to huddle the foster pup ($\chi^2 = 8.993$, $P = 0.029$). New fathers showed

	Isolated virgins N=18	Paired virgins N=16	Expectant fathers N=17-18	New fathers N=17-18	Kruskal-Wallis P	Paternal N=52-54	Nonpaternal N=16	Mann-Whitney P
Paternal-behavior test (s)								
engage in any paternal behavior - latency ^a	206.7 ± 52.7	226.6 ± 67.3	151.2 ± 50.8	111.1 ± 47.0	0.430 ^b	27.5	600.0	<0.001
approach pup - latency ^a	63.5 ± 17.7	121.4 ± 50.0	90.9 ± 44.4	105.0 ± 47.4	0.793 ^b	6.0 - 401.0	28.0 - 600.0	0.001
huddle pup - latency	149.7	271.3	83.3	54.6	0.029	16.8	103.1	<0.001
sniff pup - duration	51.7 - 600.0	21.6 - 600.0	23.0 - 600.0	23.7 - 600.0	0.089	1.6 - 394.2	2.1 - 600.0	<0.001
	23.9	15.8	27.9	7.4		63.1	600.0	
	0.0 - 78.0	0.0 - 71.0	0.0 - 116.0	0.0 - 58.0		22.0 - 573.0	600.0 - 600.0	
						23.9	0.0	0.001
						4.0 - 116.0	0.0 - 71.0	
Full paternal behavior (s)								
lick pup - duration	298.6	225.5	267.7	307.6	0.907	346.4	0.0	<0.001
huddle pup - duration	45.2	16.2	55.3	88.4	0.115	21.9 - 575.4	0.0 - 5.0	<0.001
kyphosis - duration	0.0	0.0	0.0	0.0	0.169	78.3	0.0	<0.001
mouth-carry - duration	0.0	0.0	0.0	0.0	0.503	11.9 - 443.9	0.0 - 0.0	0.066
build nest - duration	0.0	0.0	0.0	0.0	0.021	0.0	0.0	0.537
	0.0 - 0.7	0.0 - 70.8	0.0 - 14.3	0.0 - 7.6		0.0 - 121.0	0.0 - 0.0	
	0.0 - 0.0	0.0 - 0.0	0.0 - 29.2	0.0 - 173.3		0.0 - 70.8	0.0 - 1.0	0.132
	0.0 - 0.0	0.0 - 0.0	0.0 - 29.2	0.0 - 173.3		0.0	0.0	
	0.0 - 0.0	0.0 - 0.0	0.0 - 29.2	0.0 - 173.3		0.0 - 173.3	0.0 - 0.0	
Composite score (full paternal behavior)	346.9	306.5	419.5	442.6	0.519	490.5	0.0	<0.001
	0.0 - 584.1	0.0 - 591.6	0.0 - 587.8	0.0 - 581.2		130.8 - 591.6	0.0 - 5.0	
Novel-object test (s)								
approach object - latency ^a	97.4 ± 28.2	80.4 ± 28.1	48.2 ± 23.1	57.1 ± 16.8	0.472 ^b	23.7	31.0	0.426
sniff object - duration	12.3	38.0	50.9	43.6	0.252	2.1 - 300.0	2.6 - 300.0	0.085
touch object - duration	0.1	19.0	38.9	56.2	0.239	39.1	8.8	0.243
rear - duration	0.0	0.2	0.5	0.5	0.029	0.0 - 191.2	0.0 - 122.5	
	0.0 - 171.2	0.0 - 186.5	0.0 - 232.1	0.0 - 222.4		33.7	6.9	
	0.0 - 0.9	0.0 - 2.6	0.0 - 5.2	0.0 - 8.0		0.0 - 232.1	0.0 - 154.1	
						0.2	0.1	0.921
						0.0 - 8.0	0.0 - 6.6	

^aData represented as mean ± SE for comparisons among housing conditions

^bANOVA results (P)

Table 3.1: Comparisons among housing conditions and paternal vs. nonpaternal animals for paternal-behavior and novel-object tests in male California mice. Data are represented as median and range, unless otherwise indicated.

shorter latencies to huddle the pup than isolated virgins (post hoc pairwise test, $Z = 2.944$, $P = 0.003$), and showed nonsignificant tendencies toward shorter latencies to huddle the pup as compared to expectant fathers ($Z = 1.749$, $P = 0.080$) and paired virgins ($Z = -1.827$, $P = 0.068$). Latency to approach the foster pup and latency to engage in paternal behavior (any one of: licking, huddling, mouth-carrying) did not differ among the four housing conditions. Furthermore, time spent huddling, licking, mouth-carrying, and sniffing the pup, and performing kyphosis did not differ among housing conditions (see Table 3.1).

A composite score of full paternal behavior, compiled from durations of licking, huddling, nest-building, kyphosis and mouth-carrying, did not differ among new fathers, expectant fathers, paired virgins, and isolated virgins (see Table 3.1). We did, however, find a nonsignificant decrease in the proportions of males that were categorized as paternally responsive (i.e., spent $\geq 20\%$ of the 10min test engaging in paternal behavior; see Methods) from new fathers (16/18, 88.9%) to expectant fathers (15/18, 83.3%) to isolated virgins (13/18, 72.2%) and finally to paired virgins (10/16, 62.5%).

By definition, paternal males ($N = 54$) had significantly higher composite scores for full paternal behavior than did nonpaternal ($N = 16$) males. Not surprisingly, paternal and nonpaternal males also showed robust differences in the latency to approach the pup, huddle the pup, and engage in paternal behavior; and the duration of time spent sniffing, licking, and huddling the pup (see Table 3.1). No differences were found in time spent mouth-carrying, nest-building, or in kyphosis between paternal and nonpaternal males.

3.4.2 Novel-object tests

New fathers, expectant fathers, paired virgins, and isolated virgins did not differ in the latency to approach the novel object or time spent touching or sniffing the novel object (see Table 3.1). Housing condition did, however, influence the duration of time spent rearing during the novel-object test ($\chi^2 = 9.055$, $P = 0.029$), generally considered to be a measure of exploratory behavior (Espejo, 1997). Post hoc pairwise comparisons revealed that new fathers showed a nonsignificant tendency to spend more time rearing than isolated virgins ($Z = -1.783$, $P = 0.075$), but did not differ from expectant fathers ($Z = -1.189$, $P = 0.234$) or paired virgins ($Z = 0.466$, $P = 0.641$). Rearing behavior did not differ among the remaining housing conditions.

Paternal and nonpaternal animals showed no significant differences in their behavioral responses to the novel object. Interestingly, however, paternal males showed a nonsignificant tendency to sniff the novel object for longer durations than nonpaternal males (Mann-Whitney test, $Z = -1.724$, $P = 0.085$; see Table 3.1).

3.4.3 Elevated-plus-maze tests

Male California mice tended to fall and/or jump off the open arms of the EPM, resulting in 14 animals (4 isolated virgins, 2 paired virgins, 4 expectant fathers, 4 new fathers) being omitted from statistical analyses. A further five animals (1 isolated virgin, 2 expectant fathers, 2 new fathers) remained completely immobile for extended durations in the center or open arms of the EPM ($> 40\%$ of testing time), and were thus eliminated from analyses of EPM data. It should be noted that two new fathers and one expectant father were not subjected to EPM tests due to other factors described above (see Section 3.3.9),

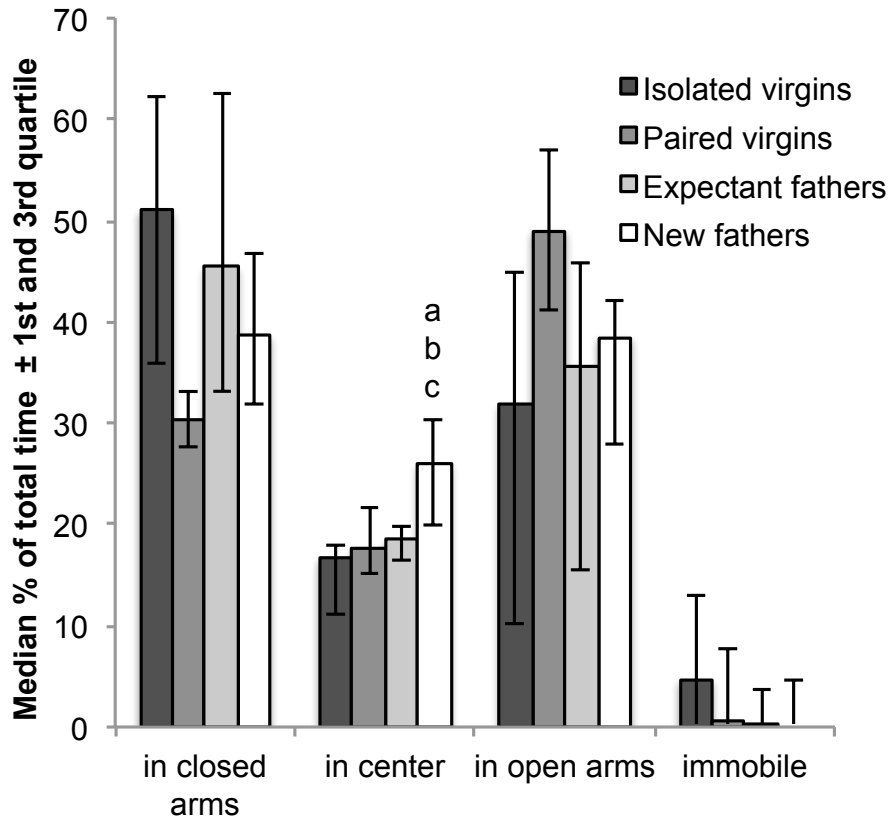


Figure 3.1: Durations (percent of total time) of behaviors (median \pm first and third quartile) displayed in 5min elevated-plus-maze tests in isolated virgin males, paired virgin males, expectant fathers, and new fathers. a = significantly different from isolated virgins, $P < 0.05$; b = significantly different from paired virgins, $P < 0.05$; c = significantly different from expectant fathers, $P < 0.05$.

and data from one paired virgin male were omitted due to technical issues with the video recording. The final analyses used data from 13/18 isolated virgins, 13/16 paired virgins, 11/18 expectant fathers, and 10/18 new fathers.

New fathers, expectant fathers, paired virgins and isolated virgins differed in the frequency of open-arm entries ($\chi^2 = 9.894$, $P = 0.019$) and head dips ($\chi^2 = 11.514$, $P = 0.009$), and in the time spent in the center of the EPM ($\chi^2 = 11.243$, $P = 0.010$). Post hoc pairwise comparisons showed that new fathers spent significantly more time in the center of the maze than expectant fathers ($Z = -2.4644$, $P = 0.014$), paired virgins ($Z = 2.482$,

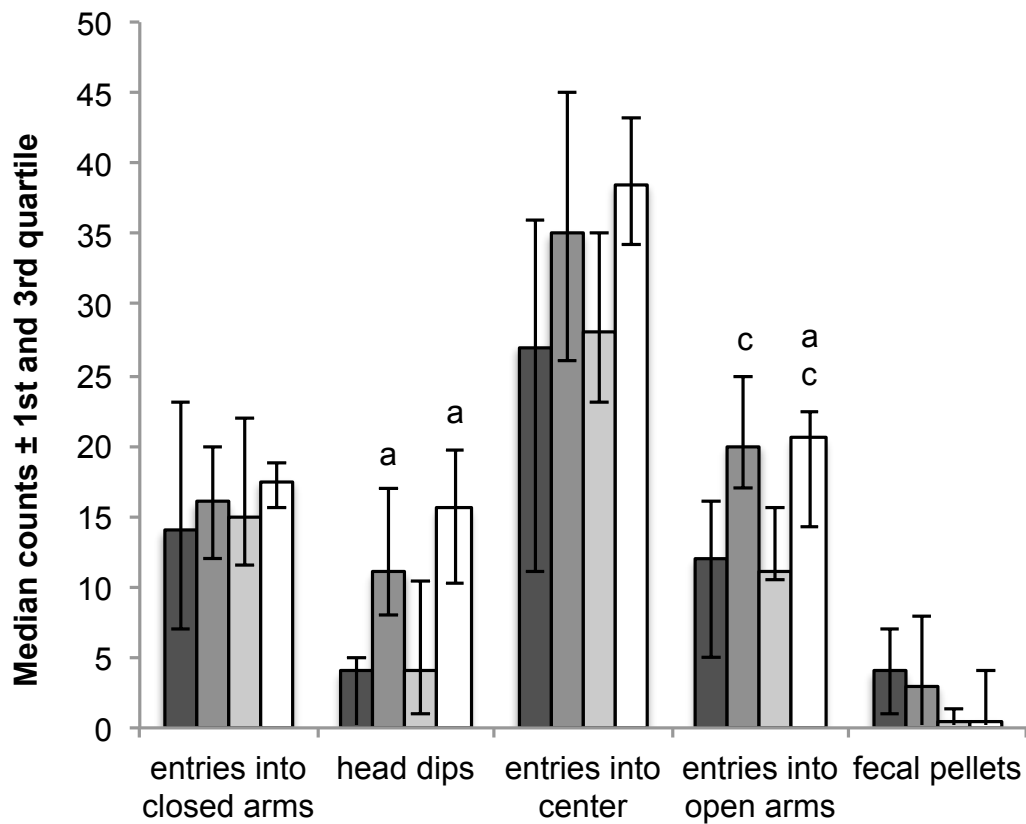


Figure 3.2: Numbers (median \pm first and third quartile) of behaviors displayed and of fecal pellets released during 5min elevated-plus-maze tests in isolated virgin males, paired virgin males, expectant fathers, and new fathers. a = significantly different from isolated virgins, $P < 0.05$; b = significantly different from paired virgins, $P < 0.05$; c = significantly different from expectant fathers, $P < 0.05$.

$P = 0.013$), and isolated virgins ($Z = -2.727$, $P = 0.006$). Both new fathers and paired virgins performed a greater number of head dips, considered to be an index of exploratory behavior (Brown, 1999), as compared to isolated virgins ($Z = -2.759$, $P = 0.006$; $Z = -2.127$, $P = 0.003$, respectively) but not expectant fathers ($Z = -1.656$, $P = 0.098$; $Z = -1.536$, $P = 0.125$, respectively). Finally, new fathers and paired virgins entered the open arms of the EPM at greater frequencies than expectant fathers ($Z = -2.254$, $P = 0.024$; $Z = -2.320$, $P = 0.020$, respectively) and isolated virgins (new fathers only: $Z = -2.110$, $P = 0.035$; see Figures 3.1 and 3.2).

Paternal and nonpaternal animals differed in the number and duration of open-arm entries. Interestingly, nonpaternal males ($N = 12$) spent more time in ($Z = -2.001$, $P = 0.045$), and had a greater number of entries into ($Z = -2.273$, $P = 0.023$), the open arms of the EPM than paternal males ($N = 35$). Paternal and nonpaternal males did not differ in any other behavior in the EPM.

3.4.4 Predator-urine stress-induced Fos and CRH expression in stress-related brain regions

Representative photomicrographs of Fos and CRH staining in the PVN and CeA are shown in Figure 3.3. Results are summarized in Figures 3.4, 3.5 and 3.6, and in Table 3.2.

Paraventricular nucleus of the hypothalamus (PVN) As expected, numbers of Fos-IR neurons in the PVN were higher in stressed mice than in undisturbed mice (main effect of stress: $F[1, 48] = 18.354$, $P < 0.001$, see Figure 3.4). For the stressed and undisturbed

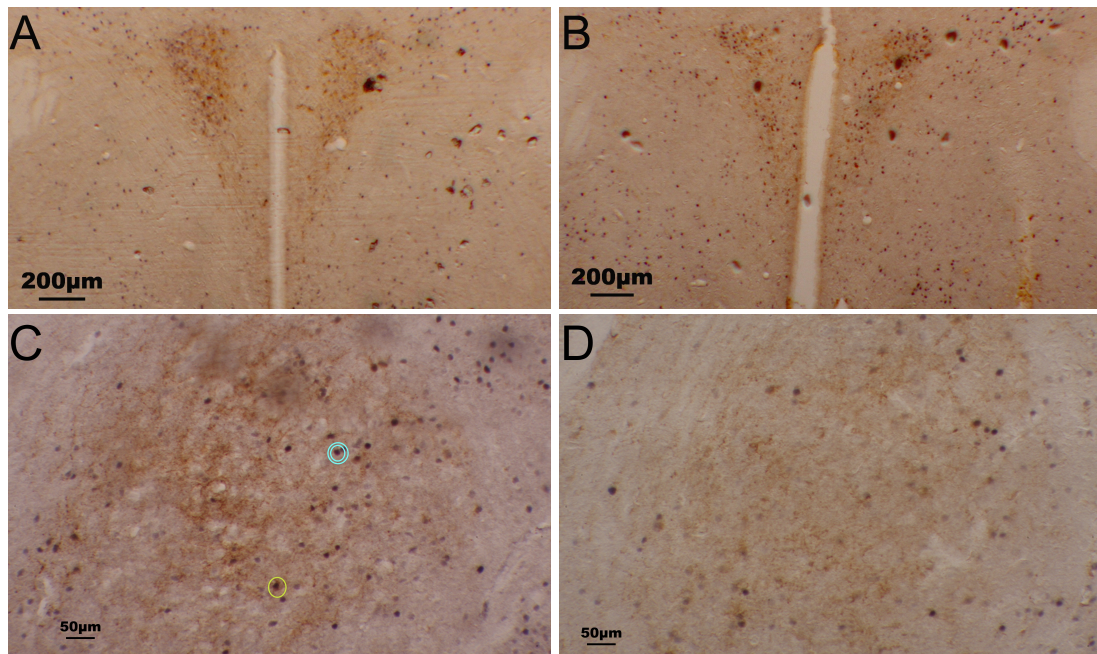


Figure 3.3: Representative photomicrographs of immunohistochemical staining of Fos and corticotropin-releasing hormone (CRH) in the paraventricular nucleus of the hypothalamus (A, B) and central nucleus of the amygdala (C, D) of undisturbed (A, C) and stressed (B, D) male California mice. Fos is stained blue-black (nuclear staining); CRH is stained reddish-brown (cytoplasmic staining). A, B: equivalent of mouse Bregma $-0.82mm$; C, D: equivalent of mouse Bregma $-1.46mm$. Figs. A and B were taken at $2.5\times$ magnification; Figs. C and D were taken at $10\times$ magnification. Fos/CRH colocalized cells are circled once, and Fos-only-immunoreactive cells are circled twice in C.

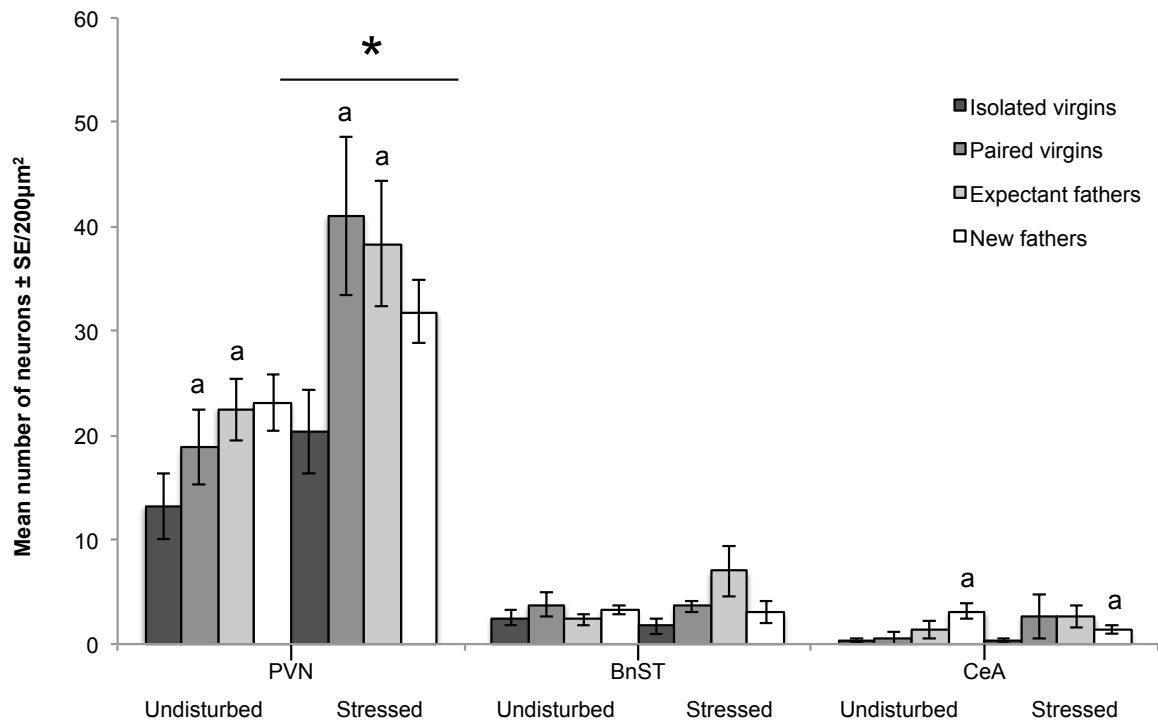


Figure 3.4: Number of Fos-IR cells (*mean* \pm *SE*) in a $0.2 \times 0.2\text{mm}$ square in the paraventricular nucleus of the hypothalamus (PVN), bed nucleus of the stria terminalis (BnST), and central nucleus of the amygdala (CeA) of male California mice in isolated virgin, paired virgin, expectant father, and new father housing conditions, following either no disturbance or exposure to a predator-urine stressor. * = significantly different from corresponding group under “undisturbed” condition, $P < 0.05$; a = significantly different from isolated males under undisturbed and stressed conditions combined, $P < 0.05$.

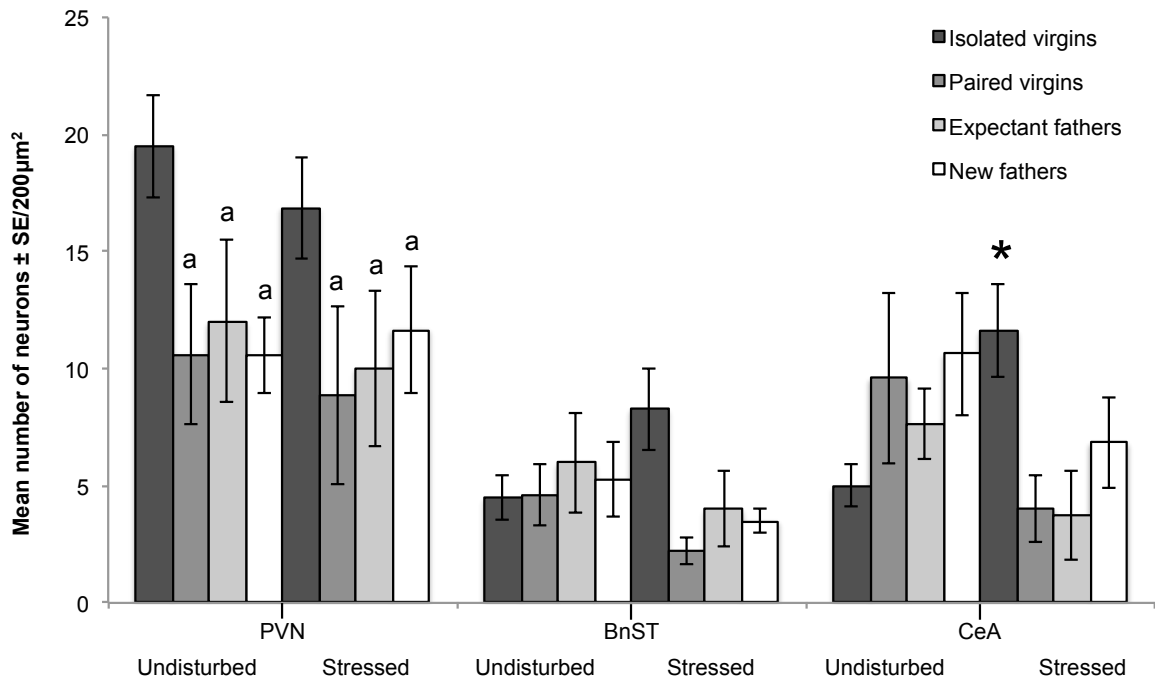


Figure 3.5: Number of CRH-IR cells ($mean \pm SE$) in a $0.2 \times 0.2mm$ square in the paraventricular nucleus of the hypothalamus (PVN), bed nucleus of the stria terminalis (BnST), and central nucleus of the amygdala (CeA) of male California mice in isolated virgin, paired virgin, expectant father, and new father housing conditions, following either no disturbance or exposure to a predator-urine stressor. * = significantly different from corresponding group under “undisturbed” condition, $P < 0.05$; a = significantly different from isolated males under undisturbed and stressed conditions combined, $P < 0.05$.

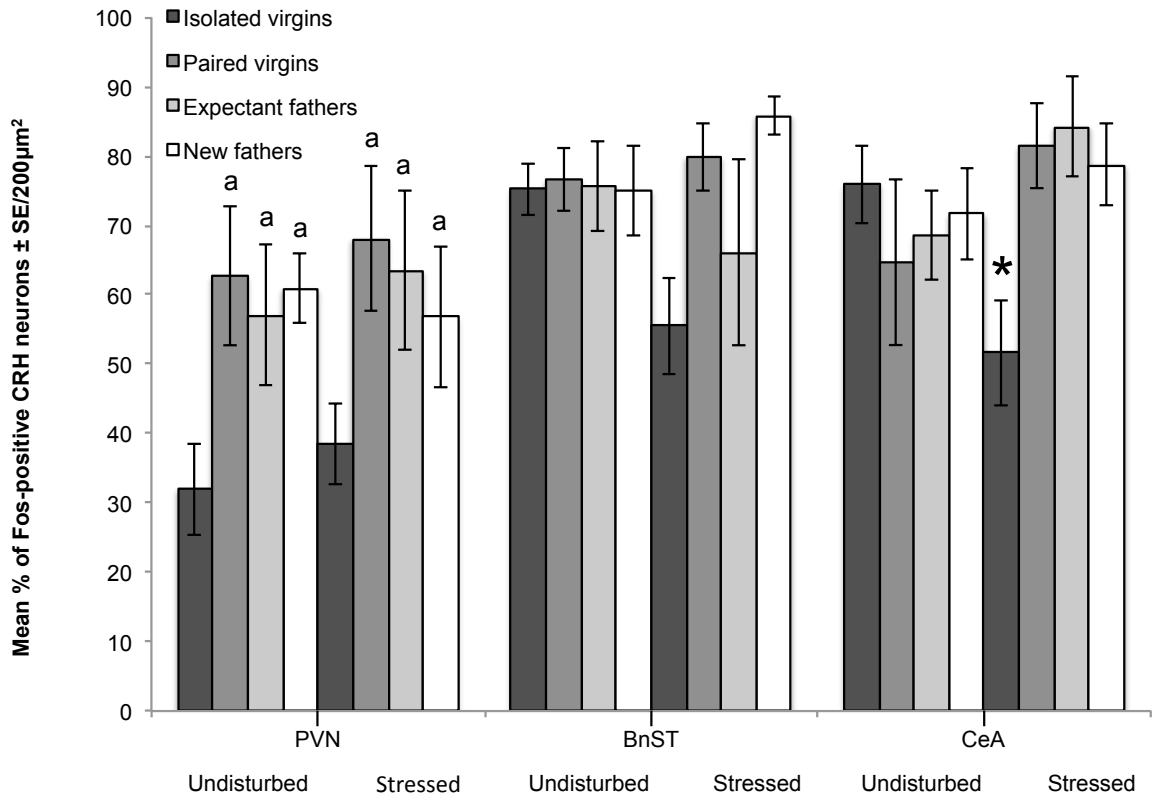


Figure 3.6: Percentage of colocalized Fos/CRH cells ($mean \pm SE$) in a $0.2 \times 0.2 mm$ square in the paraventricular nucleus of the hypothalamus (PVN), bed nucleus of the stria terminalis (BnST), and central nucleus of the amygdala (CeA) of male California mice in isolated virgin, paired virgin, expectant father, and new father housing conditions, following either no disturbance or exposure to a predator-urine stressor. * = significantly different from corresponding group under “undisturbed” condition, $P < 0.05$; a = significantly different from isolated males under undisturbed and stressed conditions combined, $P < 0.05$.

Brain regions and Measure	Paternal		Nonpaternal		ANOVA:	
	Undisturbed (N=23-25)	Stressed (N=18-20)	Undisturbed (N=5)	Stressed (N=6-8)	Paternal vs. Nonpaternal	Paternal x Stress ^d
PVN - Fos-IR ^a	18.6 ± 1.9	28.8 ± 3.2	20.8 ± 4.0	41.5 ± 6.3	P = 0.065	P = 0.189
PVN - CRH-IR ^a	13.7 ± 1.5	13.8 ± 2.0	14.6 ± 4.4	7.8 ± 2.0	P = 0.310	P = 0.169
PVN - Fos/CRH-IR ^b	49.7 ± 4.8	51.7 ± 5.9	56.2 ± 10.6	67.2 ± 9.2	P = 0.171	P = 0.573
BnST - Fos-IR ^a	2.8 ± 0.4	4.2 ± 1.1	3.2 ± 0.6	2.8 ± 1.0	P = 0.946 ^c	n/a
BnST - CRH-IR ^a	5.3 ± 0.8	4.6 ± 1.0	4.0 ± 1.3	5.0 ± 1.1	P = 0.740	P = 0.545
BnST - Fos/CRH-IR ^b	74.5 ± 2.8	71.2 ± 6.2	79.9 ± 6.0	68.7 ± 5.0	P = 0.833	P = 0.552
CeA - Fos-IR ^a	1.5 ± 0.4	1.6 ± 0.4	1.0 ± 0.6	1.9 ± 1.6	P = 0.865	P = 0.862
CeA - CRH-IR ^a	8.0 ± 1.2	6.3 ± 1.3	7.6 ± 2.2	8.7 ± 1.9	P = 0.596	P = 0.475
CeA - Fos/CRH-IR ^b	73.4 ± 3.3	75.5 ± 4.8	64.6 ± 10.2	64.6 ± 8.8	P = 0.132	P = 0.874

^aTotal number of immunoreactive neurons in a 0.2 x 0.2 mm square

^bPercent of Fos-positive CRH-containing neurons in a 0.2 x 0.2 mm square

^cStatistical results from Mann-Whitney test

^dPaternal x Stress interaction in 2-way ANOVA

Table 3.2: Numbers (*mean ± SE*) of Fos-IR and CRH-IR neurons, and percentage of Fos/CRH-IR neurons, in the hypothalamic paraventricular nucleus (PVN), bed nucleus of the stria terminalis (BnST), and central nucleus of the amygdala (CeA) of paternally responsive and nonpaternally responsive male California mice either following 5min exposure to a predator-urine stressor or under undisturbed conditions.

conditions combined, numbers of Fos-IR neurons in the PVN differed significantly among males in the four housing conditions (main effect of housing condition: $F[3, 48] = 4.634$, $P = 0.006$): paired virgins and expectant fathers had higher numbers of Fos-IR neurons in the PVN compared to isolated virgins (Tukey's post hoc test, $P = 0.014$, $P = 0.028$, respectively). We did not, however, find a significant interaction between housing condition and stress ($F[3, 48] = 1.211$, $P = 0.316$). Paternal males showed a nonsignificant tendency to exhibit lower Fos-IR in the PVN than nonpaternal males ($F[1, 52] = 3.559$, $P = 0.065$); again, however, no interaction with stress was found (see Figure 3.4 and Table 3.2).

Numbers of CRH-IR neurons in the PVN showed a significant main effect of hous-

ing condition ($F[3, 48] = 4.211$, $P = 0.010$). Post hoc tests revealed that new fathers, expectant fathers, and paired virgins had lower overall CRH-IR for the stressed and undisturbed conditions combined than isolated virgins ($P = 0.023$, $P = 0.036$, $P = 0.009$, respectively). CRH-IR in the PVN did not differ between stressed and undisturbed animals or between paternal and nonpaternal animals, and no significant interactions were found between stress and housing condition or stress and paternal responsiveness (see Figure 3.5 and Table 3.2).

A main effect of housing condition was found in the percentage of CRH neurons colocalized with Fos in the PVN ($F[3, 48] = 5.431$, $P = 0.003$). New fathers, expectant fathers, and paired virgins showed greater activation of CRH neurons than isolated virgins ($P = 0.012$, $P = 0.014$, $P = 0.002$, respectively) for the stressed and undisturbed conditions combined. Again, Fos/CRH-IR in the PVN did not differ between stressed and undisturbed animals or between paternal and nonpaternal animals (see Figure 3.6 and Table 3.2).

Central nucleus of the amygdala (CeA) For the stressed and undisturbed conditions combined, numbers of Fos-IR neurons in the CeA differed significantly among housing conditions ($F[3, 48] = 3.178$, $P = 0.032$): isolated virgins had fewer Fos-IR cells than new fathers ($P = 0.021$), similar to our findings in the PVN. CeA Fos-IR did not differ between stressed and undisturbed mice or between paternal and nonpaternal mice (see Figure 3.4 and Table 3.2).

No main effects of stress or housing condition were observed on numbers of CRH- and colocalized Fos/CRH-IR neurons in the CeA; however, significant interactions between stress and housing condition were found (CRH-IR: $F[3, 48] = 4.323$, $P = 0.009$; Fos/CRH-

IR: $F[3, 48] = 5.637$, $P = 0.002$). Independent-samples t-tests revealed that among isolated virgins, stressed mice exhibited significantly more CRH-IR cells ($t = -3.281$, $P = 0.005$) and a significantly lower percentage of colocalized Fos/CRH-IR cells ($t = 3.286$, $P = 0.005$) in the CeA than undisturbed mice. In contrast, new fathers, expectant fathers, and paired virgins showed the opposite pattern, although these trends were not statistically significant (see Figures 3.4, 3.5, and 3.6). Again, no differences were found between paternal and nonpaternal animals (see Table 3.2).

Bed nucleus of the stria terminalis (BnST) Analyses of numbers of Fos-IR and CRH-IR neurons, and percentages of colocalized Fos/CRH-IR neurons in the BnST, revealed no significant effects of stress, housing condition, or paternal responsiveness (see Figures 3.4, 3.5, 3.6, and Table 3.2).

3.4.5 Correlational analyses

Results of correlational analyses are summarized in Table 3.3. To evaluate associations between paternal behavior and emotionality, we performed correlational analyses using the behavioral parameters that we considered the best measures of neophobia (novel-object test: time spent sniffing and latency to approach the novel object), anxiety (EPM test: number of entries into and total time spent in the open arms) and paternal responsiveness (paternal-behavior test: latency to engage in paternal behavior, and duration of time spent licking the foster pup and in full paternal behavior). Interpreting the data with the traditional critical P value of 0.05, we found a significant positive relationship between the latency to engage in paternal behavior and the latency to approach a novel object for

A.

Behavior	<i>Time spent</i>		<i>Latency</i> Paternal behavior
	Licking pup	Full paternal behavior	
Latency to approach novel object (N=68)	-0.129	-0.202 ^b	0.281 ^{§a}
Time spent sniffing novel object (N=68)	0.195	0.223 ^b	-0.194
Number of entries into open arm of EPM (N=47)	-0.124	-0.146	-0.011
Time spent in open arm of EPM (N=47)	-0.195	-0.263 ^b	0.077

B.

Measure/ Brain region	<i>Time spent</i>		<i>Latency</i> Paternal behavior
	Licking pup	Full paternal behavior	
Fos-IR in PVN (N=26)	-0.231	-0.253	0.218
Fos/CRH-IR in CeA (N=27)	0.148	0.162	-0.191

^a0.025>P>0.01

^b0.1>P>0.05

[§]Pearson correlation

Table 3.3: A. Spearman correlation coefficients for comparisons of behavior in paternal tests, novel-object tests, and elevated-plus-maze (EPM) tests for all male California mice. B. Spearman correlations in all male California mice ($N = 26$) for comparisons of number of Fos-IR neurons in the paraventricular nucleus of the hypothalamus (PVN) and percentage of colocalized Fos/CRH neurons in the central nucleus of the amygdala (CeA) per $200\mu m^2$, following exposure to a predator-urine stressor, with behavior in the paternal behavior test.

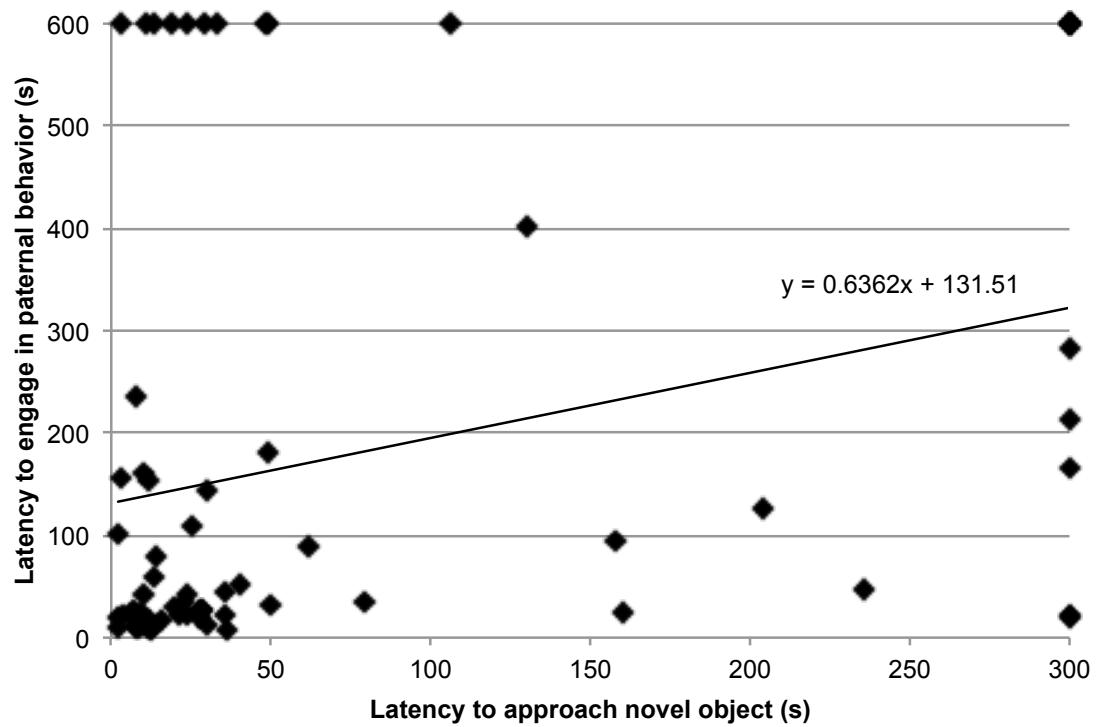


Figure 3.7: Scatter plot showing the association between the latency to engage in paternal behavior during paternal-behavior tests and the latency to approach the novel object during novel-object tests for all animals in the study.

all animals analyzed together ($R^2 = 0.281$, $P = 0.020$, $N = 68$; Figure 3.7). Following Bonferroni correction (adjusted critical $P=0.004$), however, this correlation was not significant. The remaining data were not normally distributed, and therefore Spearman's rho was used for all other correlations. No other associations between paternal behavior and neophobia or anxiety were found for all four housing conditions combined, with either a P value of 0.05, or a Bonferroni-corrected P value of 0.004.

To evaluate possible associations between paternal behavior and stress responsiveness, we compared numbers of Fos-IR PVN neurons and the percentage of colocalized Fos/CRH-IR CeA neurons in response to stress with paternal behavior (latency to engage in paternal behavior, and duration of time spent licking the foster pup and in full paternal behavior). No significant correlations were found between paternal responsiveness and stress-induced Fos-IR in the PVN or the percentage of Fos/CRH-colocalized neurons in the CeA among stressed animals from all four housing conditions combined, with either a P value of 0.05, or a Bonferroni-corrected P value of 0.008.

3.5 Discussion

Reductions in neophobia, anxiety, and stress responsiveness have been implicated in facilitating the onset of maternal behavior in female rodents. In this study, we attempted to determine whether levels of neophobia, anxiety, and stress responsiveness are similarly associated with the degree of paternal responsiveness or housing condition in biparental, monogamous, male California mice. We hypothesized that neophobia, anxiety-like behavior, and neural stress responsiveness would be (1) lower in new fathers than in nonfathers,

and (2) lower in paternally responsive males as compared to nonpaternally responsive males. Overall, we found little evidence in support of either hypothesis, although we did find evidence that individual differences in latency to engage in paternal behavior may be associated with individual differences in generalized neophobia.

Effects of housing condition In the current study, no differences were found in several common measures of paternal behavior among males in the four housing conditions. Latency to engage in paternal behavior and time spent huddling and licking the foster pup were similar in isolated virgin males, paired virgin males, expectant fathers, and new fathers. In contrast, Gubernick & Nelson (1989) found that new fathers had shorter latencies to approach foster pups than both expectant fathers and paired virgins, a finding that was replicated in a recent study in our lab (de Jong et al., 2009). In the present study, however, we did find that the proportion of paternally behaving males decreased non-significantly from new fathers to expectant fathers to isolated virgins to paired virgins. Moreover, new fathers spent significantly more time building a nest during the paternal-behavior test than the other groups, and had significantly shorter latencies to huddle the foster pup than isolated virgins. This latter finding suggests that social isolation caused some reductions in paternal responsiveness. Overall, these results are consistent with previous findings that new fathers behave more paternally than nonfathers, although not as robustly as previously described (de Jong et al., 2009; Gubernick & Laskin, 1994; Gubernick et al., 1994).

Interestingly, Bardi et al. (2011) and Lambert et al. (2011) found no differences in paternal responsiveness between new fathers and virgin males, including virgin males that either had or had not previously interacted with pups. The high level of paternal behavior

observed in virgins in the current study, along with that observed by Bardi et al. (2011) and Lambert et al. (2011) however, contrasts strikingly with findings by Gubernick and colleagues (Gubernick & Laskin, 1994; Gubernick et al., 1994), in which more than half of adult, pair-housed virgin males and expectant fathers ignored or attacked foster pups. The California mice used by Gubernick and colleagues were likely genetically closer to the wild-type population than our animals, as their studies were performed on ninth-generation descendants of wild-caught California mice (Gubernick et al., 1995), in contrast to animals in our colony (and that of (Bardi et al., 2011) and (Lambert et al., 2011)) that are descendants of animals captured between 1979 and 1987. Another possible contributing factor is the time of day at which paternal-behavior tests were performed, as California mice engage in greater durations of paternal behavior during the light phase of the cycle than during the dark phase (Wright & Brown, 2002). Neither Gubernick and colleagues (Gubernick & Laskin, 1994; Gubernick et al., 1994), nor Bardi et al. (2011) and Lambert et al. (2011) specified the time at which paternal-behavior tests were performed; it is therefore difficult to conclude if this could provide an explanation for the differences in findings.

Neophobia, as assessed on the basis of behavioral responses to a novel, wire-mesh tea-ball, did not differ significantly among the four housing conditions. Correspondingly, a prior study in our lab found that behavioral and neural (Fos) responses to the same novel wire-mesh ball did not differ across housing conditions (new fathers, expectant fathers, and paired virgins) in male California mice unless a foster pup was present inside the ball (de Jong et al., 2009). Recently, Bardi et al. (2011) found no differences in neophobic responses to a wooden half-log stimulus in California mice among fathers, pup-exposed paired virgin males, and pup-naïve paired virgin males. Taken together, these data suggest

that neophobia and fearfulness in males of this species are not strongly influenced by social housing conditions or reproductive status. In contrast, avoidance of foster pups and longer latencies to engage in maternal behavior in virgin female rats, as compared to lactating females, have been attributed to virgins' increased fearfulness and neophobia (Fleming & Luebke, 1981; Numan & Insel, 2003). A possible explanation for this difference in findings could lie in the absence of distinct elevations in centrally acting hormones/neuropeptides, such as oxytocin and prolactin, in new fathers comparable to those occurring in lactating dams (Bridges et al., 2001; Grattan, 2001; Insel, 1986; Landgraf et al., 1991; Neumann & Landgraf, 1989; Pi & Grattan, 1999; Slattery & Neumann, 2008; Torner et al., 2002; van Leengoed et al., 1987). While oxytocin and prolactin likely work in concert to mediate the reductions in fear, anxiety and stress responsiveness observed in lactating females (Neumann et al., 2000; Slattery & Neumann, 2008; Torner et al., 2002), it is yet to be determined whether elevated levels of plasma prolactin found in California mouse fathers (Gubernick & Nelson, 1989) potentially perform a similar central function. No differences in peripheral oxytocin levels were found in new fathers compared to nonfathers (Gubernick et al., 1995), and effects of fatherhood on central expression of oxytocin or prolactin have not been described (but see (de Jong et al., 2009).

In EPM tests, paired virgin males and new fathers had greater frequencies of entries into the open arms and performed more head-dips than isolated virgin males and expectant fathers, indicating reduced anxiety and increased exploration, respectively, in the paired virgins and new fathers (Lister, 1990; Pellow et al., 1985; Walf & Frye, 2007). These data are difficult to interpret: we had expected new fathers and expectant fathers to exhibit similar behavioral profiles, as males in both of these groups had prior sexual experience,

engagement in a pair bond, and exposure to a pregnant female, whereas paired virgins and isolated virgins had experienced none of these. Rats in the late stages of pregnancy exhibit higher levels of anxiety-like behavior in the elevated plus maze than lactating and virgin females (Neumann, 2001; Neumann et al., 1998; Zuluaga et al., 2005); thus, it is possible that anxiety levels increase during the prepartum period in new fathers as well as new mothers in biparental species. The finding that isolated virgin males showed more anxiety-like behavior in the EPM than paired virgins is consistent with previous findings that social isolation increases anxiety and enhances behavioral stress responses in rodents (Pan et al., 2009; Rogers & Cole, 1993; Starkey et al., 2007), and suggests that male-male relationships can have beneficial effects on emotionality in this monogamous species. In sum, the results of the EPM tests suggest that long-term social relationships in general may reduce anxiety in male California mice, but that anxiety-like behavior may increase during the prepartum period. It should be noted that acute separation from their pups might have increased anxiety in new fathers, who otherwise may have shown lower anxiety than nonfathers. This is unlikely as the same procedures have been carried out in rat dams, which still exhibit reduced anxiety compared to virgin females (Lonstein, 2005). It should also be noted, however, that the EPM has not previously been validated as an anxiety test in California mice, and as this species is considered to be semi-arboreal (Clark, 1936), it is possible that confinement in an EPM is not a stressful experience.

The results from the current study did not support our hypothesis that fatherhood blunts the neural response to stress. New fathers did not show significant differences in the Fos, CRH, or colocalized Fos/CRH response to predator odor in the PVN, BnST or CeA in comparison to expectant fathers or paired virgins. Virgin males that were individually

housed, however, showed elevated CRH in the CeA and reduced activation of CRH neurons in the CeA in response to stress compared to the other housing conditions. Furthermore, overall numbers of Fos-positive cells were reduced, numbers of CRH-positive cells increased, and activation of CRH neurons decreased in the PVN of stressed and undisturbed isolated virgins compared to the other housing conditions. Typically, stressors such as immobilization and predator odor elicit increased activation of CRH neurons in the PVN, as well as increased Fos and CRH mRNA in the PVN, BnST, amygdala, and hippocampus (Day et al., 2004; Figueiredo et al., 2003; Rivalland et al., 2007; Rotllant et al., 2007). It is likely that prolonged social isolation was a form of chronic stress that caused a dysregulation of the HPA axis and its central regulatory mechanisms in our isolated virgin males (Albeck et al., 1997; Choi et al., 2006; Dallman, 1993; Grippo et al., 2007; Ladd et al., 2005; Lightman, 2008; Pournajafi-Nazarloo et al., 2011; Ruscio et al., 2007; Tilders et al., 1993). Together, these findings suggest that social isolation functions as a chronic stressor in the California mouse, a highly social species, and that isolation stress increases central expression of CRH while downregulating neuronal activation. Exposure to stress in the presence of a familiar conspecific, meanwhile, likely preserved normal functioning of the HPA system in new fathers, expectant fathers, and paired virgins.

Individual differences in paternal responsiveness We had anticipated that paternally behaving males, regardless of housing condition, would show less neophobic behavior (e.g., reduced latencies to approach the novel object), less anxiety-like behavior (e.g., increased entries into and increased time spent in the open arms of the EPM), and reduced stress responsiveness (e.g., less Fos-IR and Fos/CRH colocalization in the PVN and CeA

following exposure to predator urine), as compared to nonpaternally behaving males. Contrary to these predictions, we found that individual differences in paternal responsiveness were not associated with differences in neophobic behaviors or in the neural response to stress. These results suggest that reductions in fearfulness and neophobia are not necessary for the display of paternal responsiveness in California mice. This does not rule out the possibility that some associations do exist between paternal responsiveness and neophobia, however, as we found that, for all animals, the latency to approach the novel object during novel-object tests correlated positively with the latency to engage in paternal behavior in paternal-behavior tests, although this correlation was not significant when we employed a Bonferroni correction. Correspondingly, maternally experienced female rats characterized by high novelty-seeking behavior were also found to have shorter latencies to engage in maternal behavior and longer durations of maternal behavior towards foster pups (although not towards their own pups) than maternally experienced females characterized by less novelty-seeking behavior (Clinton et al., 2007).

Reduced anxiety-related behavior, however, was found in nonpaternal males compared to paternal males. Compared to paternal males, nonpaternal males made more entries into and spent more time in the open arm of the EPM, typically thought to signify low anxiety (Lister, 1990; Walf & Frye, 2007). In contrast, lactating female rats (exposed to their own pups) were found to exhibit reduced anxiety-related behavior in the EPM when compared to virgin females (exposed to no pups (Byrnes & Bridges, 2006; Lonstein, 2005; Wartella et al., 2003), although animals in these studies were not compared on the basis of individual differences in maternal responsiveness. Interestingly, both pup-sensitized virgins and lactating female rats were found to spend more time in, and perform more entries into

the open arms of the EPM than ovariectomized females (Pereira et al., 2005). Bardi et al. (2011) found reduced numbers of interrupted grooming sequences, described as indicating low stress-related behavior, in paternally experienced male California mice and “adequate parents” (males that behaved paternally) as compared to both paternally inexperienced animals and “inadequate” parents (males that attacked pups). These findings from Pereira et al. (2005) and Bardi et al. (2011) suggest that animals that behave parentally, as well as animals that have parental experience, show dampened behavioral stress responsiveness. This appears to contradict our findings, which suggested that nonpaternal males exhibited less anxiety-related behavior in EPM tests. One possible explanation is that EPM data from numerous mice were omitted, as they fell or jumped off the EPM during testing, possibly skewing behavioral results in favor of housing conditions or behavioral phenotypes in which animals were less likely to fall off the maze. It is also possible that, as mentioned above, entries into and time spent in the open arms of the EPM are not appropriate measures of anxiety in this species. Future studies are needed to further clarify the relationship between paternal responsiveness and anxiety in male California mice.

We did not find any significant relationships between paternal responsiveness and stress-induced neuronal activation in the PVN or CeA. A recent study in male California mice showed that composite scores of paternal behavior correlated negatively with Fos immunoreactivity in the PVN in response to 10 min of pup-exposure (Lambert et al., 2011). This suggests that paternal responsiveness may be associated with reduced neural stress responses to an unfamiliar pup stimulus. These data point to a relationship between decreased paternal responsiveness and increased activation of anxiety-related brain areas following exposure to a pup. These findings, in conjunction with the results of the present

study, suggest that behavioral responses to a foster pup correlate with neural responses to the same stimulus and are inversely associated with anxiety induced by the pup, but do not correlate with neural activation or anxiety levels in response to other, non-pup-related stimuli.

3.6 Conclusions

We conclude that paternal responsiveness in the biparental California mouse, unlike maternal responsiveness in female rats, is not associated with systematic alterations or modifications of the stress and anxiety systems. Furthermore, anxiety, as assessed on the basis of elevated-plus-maze tests, was not lower in paternally responsive males as compared to nonpaternally responsive males. Nonetheless, rapidity to approach a novel object was positively correlated with rapidity to engage in paternal behavior, suggesting that attraction to novelty, typically indicative of reduced fearfulness, may play a role in the initiation of paternal behavior. Finally, the findings in this study point to a lack of homology between the mechanisms underlying the onset of maternal and paternal behavior in rodents.

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Chapter 4

The neuroendocrine response to pup-exposure in biparental, male California mice (*Peromyscus californicus*)

4.1 Abstract

In some species, such as rats, reproductively inexperienced females avoid unfamiliar pups and find them stressful or anxiogenic; this avoidance is processed by olfactory- and anxiety-related brain regions. It is unknown whether individual variation in paternal responsiveness in males from biparental species is mediated by similar processes. In this study we compared the expression of Fos, corticotropin-releasing hormone (CRH), and argi-

nine vasopressin (AVP) in the parvocellular and magnocellular paraventricular nuclei of the hypothalamus (pPVN and mPVN, respectively), the central nucleus of the amygdala (CeA), and the bed nucleus of the stria terminalis (BnST) in male California mice (*Peromyscus californicus*), a biparental, monogamous species. New fathers (housed with a female pairmate and their first litter), nonbreeding males (housed with a tubally ligated female pairmate), and virgin males (housed with an unrelated male) were either exposed to a foster pup (aged 0-4 days) for 1 h (PUP condition) or left undisturbed (CTRL condition) before perfusion and collection of brains. Neither reproductive condition nor exposure to a pup differentially affected Fos-, CRH-, or AVP-immunoreactivity (IR) in most brain areas, although new fathers overall (PUP and CTRL) had higher Fos-IR in the pPVN compared to nonfathers. Within the virgin male group, however, males that engaged in paternal behavior had lower Fos-IR in the mPVN and BnST than those that did not. Paternal behavior in virgin males was inversely correlated with Fos-IR in the mPVN and BnST, while duration of time spent sniffing the pup correlated positively with Fos-IR in the BnST. These data suggest that while fatherhood does not modulate the acute neuroendocrine response to a foster pup, paternal responsiveness is associated with reduced activation of stress- and anxiety-related brain areas.

4.2 Introduction

Paternal care is rare in mammals, occurring in only about 6% of mammalian species (Kleiman & Malcolm, 1981). The mechanistic basis of paternal behavior is poorly understood, while neural and endocrine factors mediating the onset and maintenance of

maternal behavior have been described in detail (Numan & Insel, 2003). Numerous studies have pointed to the existence of a neuroendocrine circuit that mediates the activation of maternal behavior via hormonal cues in female rats (Numan, 2007; Numan & Insel, 2003). At the time of parturition, high estrogen levels, combined with progesterone withdrawal, function to inhibit fearfulness and avoidance of novel pup odors, while facilitating the expression of maternal behavior (Bridges et al., 1996, 2001; Landgraf et al., 1991; Numan, 2007; Numan & Insel, 2003; Torner et al., 2002). This occurs in the presence of elevated central and peripheral levels of oxytocin and prolactin, neuropeptides associated with both maternal behavior and reduction of neuroendocrine stress responsiveness (Bridges et al., 1996, 2001; Neumann & Landgraf, 1989; Neumann et al., 2000; Windle et al., 1997a,b).

To date, little evidence for hormonal involvement in the onset and maintenance of paternal behavior has been found (Wynne-Edwards & Timonin, 2007), although the neurohormone arginine-vasopressin (AVP) has been implicated in the control of paternal behavior in California mice (*Peromyscus californicus*), prairie voles (*Microtus ochrogaster*) and meadow voles (*M. pennsylvanicus* (Bales et al., 2004; Bester-Meredith & Marler, 2003; Parker & Lee, 2001; Wang et al., 1994)). In contrast, several brain areas involved in maternal responsiveness have also been implicated in the facilitation of paternal behavior; these include the medial preoptic area, bed nucleus of the stria terminalis (BnST), basolateral amygdala, and nucleus accumbens (de Jong et al., 2009; Lee & Brown, 2007; Lee et al., 2000; Numan & Insel, 2003; Stack & Numan, 2000). Overall, there appear to be some parallels in the neurobiological control of maternal and paternal behavior, although the findings have not been consistent.

As mentioned above, the onset of maternal behavior in female rats involves the

suppression of fear and anxiety in the face of novel sounds and odors from pups (Fleming & Luebke, 1981). In contrast, recent findings in our laboratory suggest that modifications in behavioral and neuroendocrine stress responsiveness are not necessary for the onset of paternal behavior in the biparental, monogamous California mouse (Chauke et al., Chapter 3, In Revision), a species in which new fathers immediately engage in full paternal care following the birth of their pups (Gubernick & Alberts, 1987; Ribble, 1991). Some associations, however, were found between paternal responsiveness and novelty-seeking behavior in our study (Chauke et al., Chapter 3, In Revision). This suggests that paternally and non-paternally responsive animals (or new fathers as compared to nonfathers) may differentially perceive pup stimuli aversive.

The physiological response to stress is mediated largely by the hypothalamic-pituitary-adrenal axis. In response to psychological and physiological stressors, corticotropin-releasing hormone (CRH), often in conjunction with AVP, is released from parvocellular neurons of the paraventricular nucleus of the hypothalamus (pPVN) into the portal blood stream via the median eminence. CRH then stimulates the synthesis and secretion of adrenocorticotrophic hormone from the anterior pituitary, which in turn stimulates the release of glucocorticoids from the adrenal glands.

We previously compared behavioral and neuroendocrine (CRH) responses to a predator-odor stressor among new fathers, expectant fathers, paired virgin males and isolated virgin males, and also between paternally responsive and nonpaternally responsive male California mice. Our results showed that neither fatherhood nor paternal responsiveness was associated with reduced behavioral and neuroendocrine stress responses (although a moderate correlation between paternal responsiveness and reduced neophobia was found;

(Chauke et al., Chapter 3, In Revision). In contrast, Lambert et al. (2011) recently reported that paternal behavior during a 10-min test with an unfamiliar pup was negatively associated with activation of stress- and anxiety-related brain areas in male California mice. Together, these findings suggest that paternal responsiveness is not associated with altered responses to non-pup-related stressors, but that paternally responsive males may have reduced stress and anxiety responses to pups, as compared to nonpaternally responsive males.

In the current study, we set out to test the hypothesis that new California mouse fathers or paternally responsive males find pup stimuli less stressful than do nonfathers or nonpaternally responsive males, respectively. The neuroendocrine (Fos, CRH, and AVP) response to 1 h of exposure to an unfamiliar pup was compared among virgin males housed with an unrelated male, nonbreeding males housed with a female pairmate, and new fathers, as well as between paternally responsive and nonpaternally responsive males. Nonbreeding males were included in the study to control for effects of housing with newborn pups, and virgin males were used to control for effects of cohabitation and mating with a female.

4.3 Methods

4.3.1 Animals

Male (N=72) and female (N=43) California mice, descendants of males and females purchased as adults from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA), were used. All animals were housed in $44 \times 24 \times 20\text{cm}$ polycarbonate cages containing aspen shavings for bedding and cotton wool for nesting material, with food (Purina Rodent Chow 5001) and water available *ad libitum*. Following a

14:10 light/dark cycle, lights were on from 0500h to 1900h, with room temperature set at $18 - 26^{\circ}\text{C}$ and humidity at 60-70%. Animals were removed permanently from their parents' cage and ear-punched for identification at 27-33 days of age, prior to the birth of the next litter of siblings. They were then housed in groups of four same-sex, age-matched cage-mates until they were assigned to one of three reproductive conditions for the experiment (see below). Animals in the study were inspected daily, and after being assigned to reproductive conditions, were weighed twice per week to monitor health and pregnancies and to habituate animals to handling. Cages and water bottles were changed once per week, and at least 2 days elapsed between cage-changing and any experimental procedures. All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, and were reviewed and approved by the University of California, Riverside (UCR) Institutional Animal Care and Use Committee. UCR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

4.3.2 Experimental design

Male California mice were randomly assigned to three reproductive conditions – new fathers ($N = 20$), housed with a female pairmate and, eventually, their first litter of pups; nonbreeding males ($N = 23$), housed with a tubally ligated female pairmate; and virgin males ($N = 29$), housed with an unrelated male pairmate. Because virgin males show the greatest variability in paternal responsiveness (unpublished data), we used a total of 20 virgin males in the PUP condition (compared to 11 new fathers and 12 nonbreeding males) to increase the likelihood that we would have enough paternally responsive and nonpaternally responsive males to allow comparisons between the two categories. Female

cagemates of nonbreeding males underwent bilateral tubal ligation one week prior to pair formation (see Surgery section for details). Behavioral and neural responses to either a pup or no stimulus were assessed in new fathers following the birth of their first litter, along with age-matched virgin and nonbreeding males in cohorts containing at least one animal from each reproductive condition (see below).

4.3.3 Behavioral tests and collection of brains

When their first litter of pups was 2-3 days of age, new fathers, along with virgin and nonbreeding males in their cohort, were removed from their home cage at 1100h and isolated in the colony room for 24h in clean cages containing bedding, food and water. Between 1100h and 1200h the following day, each experimental male in the PUP condition was exposed to an unrelated pup age 0-4 days, placed in the corner of the cage furthest from the male, for 1h. If an animal attacked a stimulus pup, the pup was immediately euthanized with pentobarbital (ca. 200 – 300mg/kg i.p; Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI, USA). Animals in the CTRL condition (new fathers, $N = 9$; nonbreeding males, $N = 11$; virgin males, $N = 9$) were left undisturbed, except for brief manual manipulation of the bedding material at the beginning of behavioral testing to control for cage disturbance during the pup-exposure procedure. All mice were videotaped for the duration of the 1h test. Pup- and activity-related behaviors were scored as described below.

Approximately 1 h following introduction of the pup into the male's cage exposure (PUP condition) or manipulation of bedding (CTRL condition), males were deeply anesthetized with pentobarbital (ca. 200-300 mg/kg, i.p.), followed by transcardial perfusion with 0.1M phosphate-buffered saline and later, 4% paraformaldehyde. Brains were

Category	Measure	Description
Proximity to pup	Latency to approach pup	Latency to approach within 3 cm of pup
	In pup zone	In the same third of cage as pup
	Out of pup zone	Not in same third of cage as pup
Pup-directed paternal behavior	Huddle pup	Crouching over pup
	Kyphosis	Nursing posture over pup
	Lick pup	Licking pup
	Mouth-carry	Carrying pup in mouth
Other	Sniff pup	Sniffing pup
Activity levels	Active	Generally active: running, jumping, or walking
	Rest	Sleeping or sitting; immobile

Table 4.1: Ethogram of behaviors scored in the paternal-behavior test.

processed for immunohistochemistry as described below. To confirm that tubally ligated female mates of nonbreeding males were not pregnant, these females were euthanized and dissected to check for the presence of fetuses or embryo sacs.

4.3.4 Behavioral analyses

Behavioral measures were scored from videotapes by an observer blind to reproductive condition, using the event-recorder program JWatcher (Blumstein & Daniel, 2007). Behaviors from the first 20 min of the test were scored as durations, to ensure that each male's initial interactions with the foster pup were characterized thoroughly. For the remaining 40 min of the paternal-behavior test, behaviors were scored on 5-min instantaneous scans in order to determine whether the initial response to the foster pup was sustained throughout the test. For the first 20 min of each paternal-behavior test, the latency to approach the foster pup; total time spent mouth-carrying, sniffing, licking, and huddling the pup; and total time in kyphosis (nursing posture) were scored as described in Table 4.1.

For the last 40 min of paternal-behavior tests, the following behaviors were scored in instantaneous scans every 5 min: proximity to the pup (≤ 1 body-length), whether or not the male was engaging in paternal behavior (licking, huddling, or mouth-carrying the pup), and whether the mouse was active or inactive (for descriptions see Table 4.1). For CTRL animals, total duration of time spent active (i.e., running, jumping, walking) or inactive during the first 20 min of the test was scored.

A total of five animals ($N = 2$ virgin males, 2 nonbreeding males, 1 new father) attacked the foster pup during paternal-behavior tests. These animals were scored as having engaged in 0 sec of paternal behavior. Other behavioral data (e.g., latency to approach the foster pup and time spent in the pup zone, resting, and sniffing the pup) from these animals were not included in behavioral analyses.

4.3.5 Surgeries

Tubal ligations were performed under sterile conditions using standard surgical procedures. Females to be paired with nonbreeding males were anesthetized with isoflurane, a ventral midline incision (approximately 1cm) was made, each oviduct was sutured and cut, and the incision was closed with tissue glue. Ketoprofen (5mg/kg, s.c.) was administered at the completion of the surgery to provide analgesia. Following one week of recovery in isolation, females were paired with intact virgin males to form nonbreeding pairs.

4.3.6 Immunohistochemistry

Immunohistochemistry was performed as described previously (de Jong et al., 2009, 2010; Chauke et al., Chapter 3, In Revision). Brains were sliced into 30um coronal sections

on a cryostat and collected in five series. Brain slices in one series were double-stained for Fos and CRH, and slices from a second series were double-stained for Fos and AVP. For Fos/CRH colocalization, slices were incubated overnight with rabbit-anti-Fos antibody (sc-253, 1:5000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by donkey-anti-rabbit second antibody (1:1500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and then stained with 3,3'-diaminobenzidine (DAB) and ammonium nickel sulfate in order to mark Fos-positive cells as blue-black in color. To achieve Fos and CRH colocalization, the brain slices were later incubated overnight with goat-anti-CRH (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by the donkey-anti-goat second antibody (1:1500; Jackson ImmunoResearch, West Grove, PA, USA) and stained with DAB, but without ammonium nickel sulfate. For Fos/AVP colocalization, a separate series of brains was incubated overnight with rabbit-anti-AVP (1:200,000; Peninsula Laboratories, Torrance, CA, USA), followed by donkey-anti-rabbit second antibody (1:1500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) after the initial Fos-staining step. The result was a brown cytoplasmic neuropeptide staining that was distinguishable from the blue-black nuclear Fos staining. Individual brain sections were then mounted on gelatin / chrom-alum-coated glass slides, dehydrated, cleared in ethanol and xylene, embedded in entellan (EMS, Hatfield, PA, USA), and coverslipped.

4.3.7 Quantification of immunoreactivity

We used the *Mouse Brain in Stereotaxic Coordinates* (Franklin & Paxinos, 2008) for the neuroanatomically similar C57BL/J6 mouse to locate relevant brain areas (de Jong et al., 2009), as currently no brain atlas has been prepared for *P. californicus*. Bregma

levels in the text are in reference to levels specified in the atlas, and not actual Bregma levels in *P. californicus*.

For the quantification of Fos and CRH immunoreactivity, brain areas were selected based on their known functions and associations with stress responsiveness and anxiety: parvocellular paraventricular nucleus of the hypothalamus (pPVN, mouse Bregma level -0.46) and central nucleus of the amygdala (CeA, mouse Bregma level -1.46). For quantification of Fos and AVP immunoreactivity, brain areas associated with paternal behavior and/or stress responsiveness were selected: bed nucleus of the stria terminalis (BnST, mouse Bregma level -0.22) and magnocellular paraventricular nucleus of the hypothalamus (mPVN, mouse Bregma level -0.82), respectively.

An observer blind to reproductive condition and test condition selected the region with the highest density of Fos/neuropeptide colocalization within each brain area. Standardized digital photographs of each brain area, as well as a millimeter scale, were taken at a magnification of $20\times$ with a digital camera (Canon EOS-40D) mounted on a microscope (Leica Leitz DMRB). Image analysis software (GNU Image Manipulation Program, Version 2.6, Free Software Foundation, Boston, MA, USA) was used to form a grid of lines equivalent to $0.2 \times 0.2\text{mm}$ in each photograph so that it contained either all or the majority of immunoreactive neurons in the selected area, or, in case of larger brain areas, a portion containing a representative spread of immunoreactive (IR) neurons. Numbers of Fos-positive, CRH-positive, or AVP-positive neurons, as well as numbers of colocalized Fos/CRH and Fos/AVP neurons within the square, were counted manually. Data for Fos-, CRH-, and AVP-positive cells are expressed as *counts*/ $200\mu\text{m}^2$, while data for Fos/CRH and Fos/AVP colocalized neurons are expressed as the percentage of Fos-positive CRH or AVP neurons

out of the total number of CRH or AVP *neurons/200 μm^2* . Analysis of Fos/neuropeptide colocalization was based on the quantification of Fos dots within CRH/AVP cell bodies as described previously (de Jong et al., 2009, 2010; Chauke et al., Chapter 3, In Revision).

4.3.8 Statistical analyses

Behavioral and immunohistochemical data were analyzed using the Statistical Package for the Social Sciences (SPSS) v. 16.0 (IBM Corporation, Armonk, NY, USA). Variables were tested for normality using Levene’s test for homogeneity of variance and the Skewness-Kurtosis test. Normally distributed data were analyzed using parametric statistical tests, while non-normally distributed data were analyzed using nonparametric statistical tests.

Each animal in the PUP condition was categorized as being “paternal” or “nonpaternal” based on its level of paternal responsiveness during the first 20 min of the paternal-behavior test. Paternal males were defined as those that spent $\geq 30\%$ of the 20-min-period engaging in pup-directed paternal behavior (huddling, licking, mouth-carrying), while nonpaternal males spent $< 30\%$ of the period licking the pup. We used this cut-off point as none of the animals spent between 9% and 30% of the 20-min-period engaging in pup-directed paternal behavior. Animals that attacked pups ($N = 5$) were considered nonpaternal. Behavioral scores from the first 20 min of paternal-behavior tests were compared among males in the three reproductive conditions (new father, nonbreeding male, virgin male) using Kruskal-Wallis tests, and significant effects were followed up by nonparametric post hoc pairwise comparisons (Siegel & Castellan, 1988). Kruskal-Wallis tests were also used to compare behaviors scored in 5-min scans during the remaining 40 min of paternal-behavior

tests, and to compare activity levels among CTRL animals from the three reproductive conditions. Behavioral data from paternal and nonpaternal males were compared using Mann-Whitney tests.

Immunohistochemical data that were normally distributed among the three reproductive conditions were analyzed using two-way ANOVAs with reproductive condition (new father, nonbreeding male, virgin male) and test condition (PUP, CTRL) as factors. Significant main effects of reproductive condition were followed up by Tukey's post hoc tests. Immunohistochemical data from paternal and nonpaternal virgin males were compared using t-tests. Associations between paternal responsiveness and the neuroendocrine response to pup-exposure were examined in virgin males by correlating pPVN and BnST Fos-IR with paternal behavior. Correlations were performed using bivariate Spearman's rho for all data sets.

For all analyses, effects were accepted as statistically significant when P (two-tailed) was less than 0.05 ($Z \pm 1.996$), unless otherwise specified.

4.4 Results

4.4.1 Paternal-behavior tests

Results of paternal-behavior tests are summarized in Table 4.2. New fathers, nonbreeding males, and virgin males did not differ in paternal behavior, i.e., latency to approach the foster pup, or total time spent licking, huddling, or mouth-carrying the pup during the first 20 min of pup-exposure (Table 4.2). Moreover, no differences among reproductive conditions were found in other behavioral measures in the first 20 min. None

	Virgin males N=18-20	Nonbreeding males N=10-12	New fathers N=10-11	Kruskal- Wallis P	Paternal N=32	Nonpaternal N=6-11	Mann- Whitney P
<u>Paternal behaviors</u>							
Approach pup - latency	46.1 16.8-486.6	16.3 3.4-186.2	48.2 4.0-1200.0	0.113	45.1 3.4-486.6	32.5 25.5-1200.0	0.800
Lick pup - duration	584.3 0.0-1099.0	794.8 0.0-1059.9	728.7 0.0-1066.4	0.567	794.8 466.2-1099.0	0.0 0.0-296.9	<0.001
Huddle pup - duration	842.1 0.0-1156.0	825.2 0.0-1166.7	955.3 0.0-1177.9	0.604	991.5 510.0-1177.9	0.0 0.0-0.0	<0.001
Mouth-carry - duration	0.0 0.0-10.2	0.0 0.0-4.6	0.0 0.0-7.5	0.419	0.0 0.0-10.2	0.0 0.0-0.0	0.288
<u>Other behaviors</u>							
Sniff pup - duration	23.0 0.0-130.8	11.1 0.0-60.5	10.2 0.0-573.7	0.524	11.6 0.0-573.7	39.1 0.0-130.8	0.653
In pup zone - duration	1086.1 110.0-1183.7	1160.6 992.6-1194.3	1127.6 0.0-1195.9	0.076	1149.6 715.5-1195.9	421.9 0.0-993.3	<0.001
Rest - duration	36.6 0.0-443.0	12.9 0.0-762.9	71.8 4.1-1200.0	0.151	36.6 0.0-443.0	80.4 0.0-1200.0	0.097

Table 4.2: Comparisons among reproductive conditions, and between paternal and nonpaternal male California mice in all three reproductive conditions together, for the first 20 min of the paternal-behavior test. All measures are in seconds and are presented as median and range.

of the animals were observed entering into a nursing posture (kyphosis) during this time. During the last 40 min of the test, levels of activity differed among reproductive conditions ($\chi^2 = 7.621$, $P = 0.022$). Post hoc pairwise comparisons revealed that nonbreeding males were more active than virgin males ($Z=2.560$, $P=0.011$); neither group differed from new fathers. No differences in paternal behavior ($\chi^2 = 3.130$, $P = 0.209$) or proximity to the pup ($\chi^2 = 0.339$, $P = 0.844$) were found among the three reproductive conditions during this period. For CTRL animals (exposed to no stimulus), no differences in activity were found among reproductive conditions ($\chi^2 = 0.258$, $P = 0.879$).

As described in Section 4.3, males in the PUP condition were categorized as paternal ($N = 9/11$ new fathers, $9/12$ nonbreeding males, and $14/20$ virgin males) or nonpaternal ($N = 2/11$ new fathers, $3/12$ nonbreeding males, and $6/20$ virgin males) based on a compos-

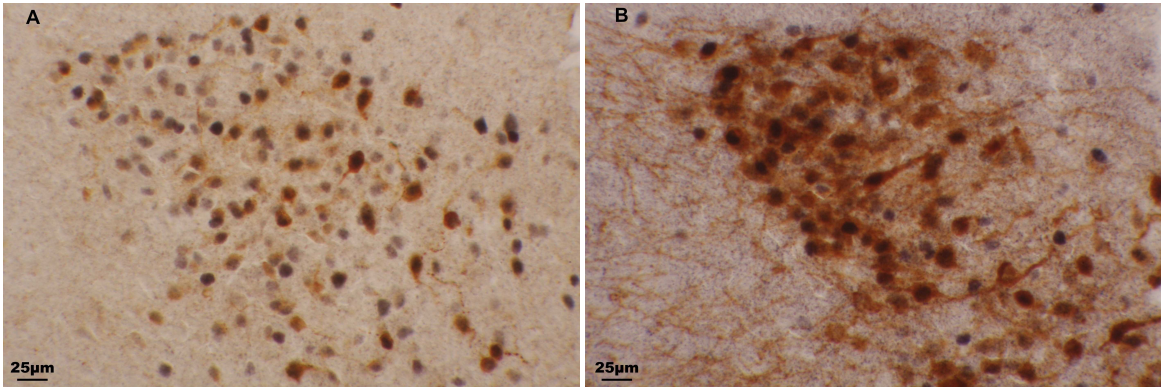


Figure 4.1: Representative photomicrographs of double-labeling of Fos and corticotropin-releasing hormone (CRH; A) and Fos and vasopressin (B) in the hypothalamus of male California mice. Fos is stained blue-black (nuclear staining); CRH and vasopressin are stained reddish brown (cytoplasmic staining). A: equivalent of mouse Bregma $-0.46mm$; B: equivalent of mouse Bregma $-0.82mm$.

ite score of time spent huddling, licking, and mouth-carrying the foster pup during the first 20min of the paternal-behavior test. Not surprisingly, paternal males spent significantly more time in the pup zone (i.e., the third of the cage containing the pup; $Z = -3.643$, $P < 0.001$), huddling ($Z = -4.490$, $P < 0.001$), and licking ($Z = -4.915$, $P < 0.001$) the foster pup than nonpaternal males. Paternal and nonpaternal males did not, however, differ in the latency to approach ($Z = -0.280$, $P = 0.800$) or time spent sniffing the pup ($Z = -0.481$, $P = 0.653$; see Table 4.2 for details).

4.4.2 Neuroendocrine response to pup-exposure

Representative photomicrographs depicting Fos, CRH, and AVP staining in the hypothalamus are shown in 4.1. Exposure to a foster pup did not elevate Fos-, CRH-, or AVP-IR, or the percentage of colocalized Fos/CRH or Fos/AVP neurons, in any brain region, either for all three reproductive conditions combined or within individual reproductive conditions (Table 4.3)

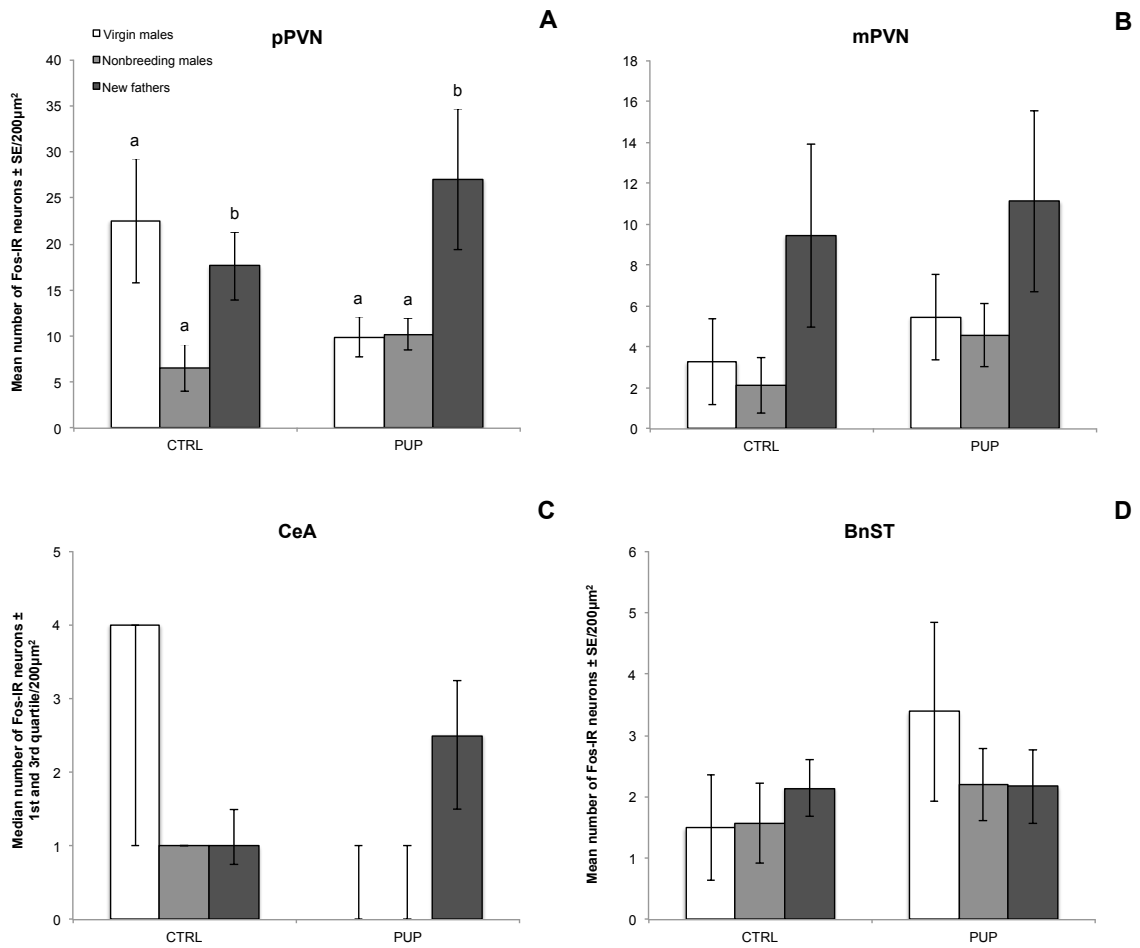


Figure 4.2: Fos-IR in the (A) parvocellular paraventricular nucleus of the hypothalamus (pPVN), (B) magnocellular paraventricular nucleus of the hypothalamus (mPVN), (C) central nucleus of the amygdala (CeA), and (D) bed nucleus of the stria terminalis (BnST) in male California mice. New fathers had higher overall (pup (PUP) and control conditions (CTRL)) Fos-IR in the pPVN than either virgin or nonbreeding males. Different letters indicate groups that are statistically different, $P < 0.01$. Note the different y-axes.

Brain region/Measure	Virgin males		Nonbreeding males		New fathers	
	CTRL (N=4-7)	PUP (N=10-17)	CTRL (N=5-8)	PUP (N=7-10)	CTRL (N=4-6)	PUP (N=4-8)
pPVN - CRH-IR ^a	5.2 ± 2.9	7.7 ± 1.8	3.8 ± 1.6	5.7 ± 2.7	3.4 ± 1.4	3.2 ± 1.1
pPVN - Fos/CRH-IR ^b	72.5 ± 13.8	61.9 ± 6.6	80.2 ± 8.3	73.5 ± 8.5	80.4 ± 7.4	82.8 ± 6.0
mPVN - AVP-IR ^a	5.6 ± 1.4	6.5 ± 1.3	6.6 ± 2.0	7.3 ± 2.2	5.7 ± 2.0	2.8 ± 0.8
mPVN - Fos/AVP-IR ^b	71.0 ± 11.5	80.5 ± 4.3	78.9 ± 7.3	77.2 ± 6.9	83.1 ± 7.4	92.6 ± 2.9
CeA - CRH-IR ^a	1.5 ± 0.6	5.6 ± 1.2	5.0 ± 1.6	5.0 ± 1.2	2.8 ± 1.3	1.0 ± 0.7
CeA - Fos/CRH-IR ^b	87.3 ± 4.8	60.0 ± 8.3	63.5 ± 9.6	61.1 ± 9.7	79.5 ± 6.9	79.6 ± 18.3
BnST - AVP-IR ^{a,c}	2.0 (1-8)	2.0 (0-10)	2.0 (0-7)	2.0 (0-6)	1.0 (0-3)	2.0 (0-3)
BnST - Fos/AVP-IR ^{b,c}	90.4 (0-92)	85.7 (29-100)	82.4 (30-100)	90.7 (40-100)	93.8 (70-100)	92.5 (40-100)

^aTotal number of immunoreactive neurons in a 0.2 x 0.2 mm square

^bPercent of neuropeptide-containing neurons that were Fos-positive in a 0.2 x 0.2 mm square

^cNon-normally distributed data represented as median, range

Table 4.3: Numbers (*mean ± SE*) of CRH and AVP-IR neurons, and percentage of double-labeled Fos/CRH and Fos/AVP neurons (i.e., Fos/neuropeptide-IR) in the parvocellular and magnocellular paraventricular nuclei of the hypothalamus (pPVN and mPVN, respectively), central nucleus of the amygdala (CeA), and bed nucleus of the stria terminalis (BnST) of California mouse virgin males, nonbreeding males, and new fathers either following 1h exposure to a pup (PUP) or under control conditions (CTRL). No effect of reproductive condition or pup-exposure was found for the measures below.

A main effect of reproductive condition was found for Fos-IR (Fos-only cells) in the pPVN ($F[2, 21] = 12.244, P = 0.001$). Tukey's post hoc tests revealed that new fathers had higher overall (PUP and CTRL conditions) numbers of Fos-IR neurons in the pPVN compared to nonbreeding males ($P = 0.001$) and virgin males ($P = 0.001$) (see Figure 4.2). Neither reproductive condition nor pup exposure affected Fos-IR in the mPVN, CeA or BnST (see Figure 4.2 and Table 4.3). Furthermore, no effect of reproductive condition or pup-exposure was found on CRH-, AVP-, Fos/CRH-, or Fos/AVP-IR in any brain area (see Table 4.3).

Mann-Whitney tests revealed that within the virgin male group, nonpaternally responsive males had higher Fos-IR in the mPVN ($Z = -2.033, P = 0.048, N = 11$ nonpaternal and 6 paternal males) and BnST ($Z = -2.723, P = 0.005, N = 7$ nonpaternal and 6

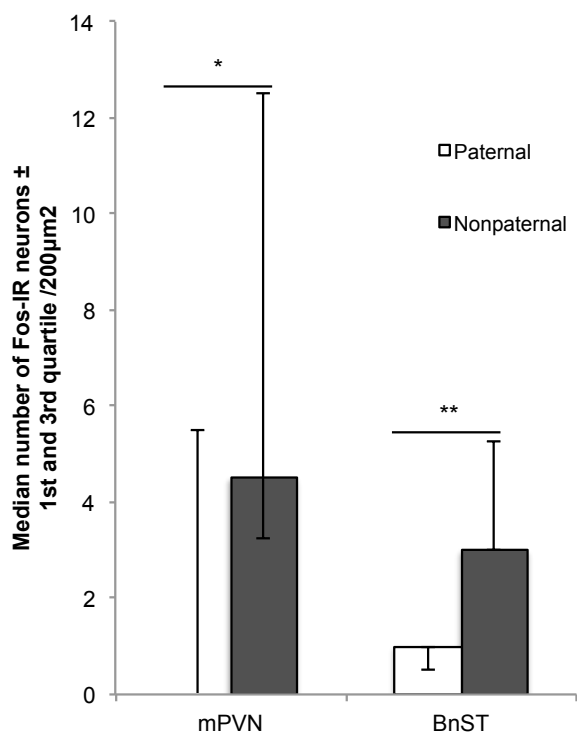


Figure 4.3: Fos-IR (*median* \pm 1^{st} and 3^{rd} quartile) in the magnocellular paraventricular nucleus of the hypothalamus (mPVN) and the bed nucleus of the stria terminalis (BnST) of virgin male California mice. Nonpaternal virgin males had higher Fos-IR in the mPVN and BnST following pup-exposure compared to paternal virgin males. * $P < 0.05$, ** $P < 0.01$.

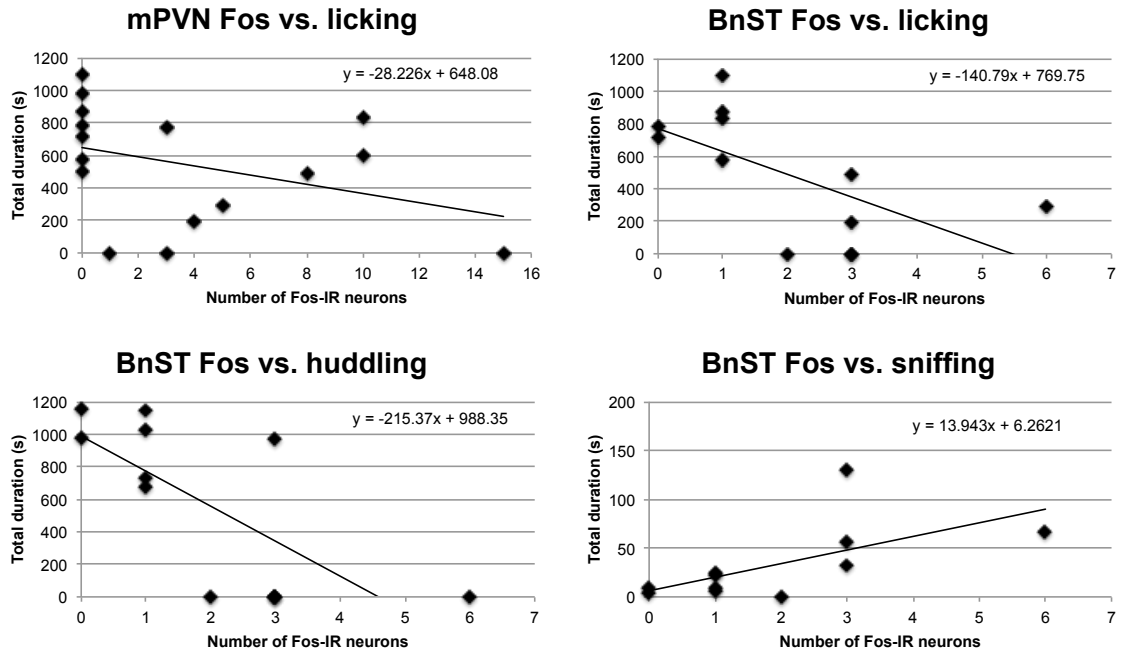


Figure 4.4: Scatter plots showing the association between time spent licking, huddling, or sniffing the pup in the first 20 min of paternal-behavior tests and Fos-IR in the magnocellular paraventricular nucleus of the hypothalamus (mPVN) and the bed nucleus of the stria terminalis (BnST) of virgin male California mice.

paternal males) compared to paternal males (See Figure 4.3).

4.4.3 Correlational analyses

Results of correlational analyses are summarized in Figure 4.4. Fos-IR in the pPVN and Fos-IR in the BnST were selected for correlations with paternal behavior, as our results indicated that these areas are differentially activated in response to pup-exposure in paternal and nonpaternal virgin males. Fos-IR in the mPVN correlated negatively with time spent licking the pup ($\rho = -0.571$, $P = 0.017$, $N = 17$). In the BnST, Fos-IR correlated negatively with time spent licking ($\rho = -0.726$, $P = 0.005$, $N = 13$) and huddling ($\rho = -0.799$, $P = 0.001$, $N = 13$) the pup, and positively with duration of time spent sniffing the pup

($\rho = 0.692$, $P = 0.018$, $N = 11$).

4.5 Discussion

New California mouse fathers engage in extensive paternal behavior immediately following the birth of their pups (Gubernick & Alberts, 1987; Ribble, 1991) without the profound peripartum hormonal changes found in females (Bridges et al., 1996, 2001; Numan, 2007; Numan & Insel, 2003). In female rats and possibly other mammals, these endocrine changes facilitate the onset of maternal behavior by inhibiting fearfulness and anxiety in response to pup stimuli (Fleming & Luebke, 1981; Numan, 2007; Numan & Insel, 2003). It is unclear whether a similar scenario occurs in males of biparental species. In this study, therefore, we attempted to determine whether the display of paternal behavior, and/or being a new father, was associated with reduced stress responses to a foster pup. Our results suggest that being a new father does not alter the neuroendocrine response to pup-exposure, but that individual differences in paternal responsiveness among virgin males are associated with differences in activation of stress- and anxiety-related brain regions in response to a pup.

4.5.1 Paternal behavior

Virgin males, nonbreeding males, and new fathers did not differ in the amount of time spent engaging in paternal behavior when exposed to a foster pup for 1 h. This result is consistent with a previous finding in our laboratory (Chauke et al., Chapter 3, In Revision), in which isolated virgin males, paired virgin males, expectant fathers (males paired with a first-time pregnant pairmate), and new fathers engaged in similar durations

of licking and huddling behavior during a 10-min paternal-behavior test. Similarly, Bardi et al. (2011) and Lambert et al. (2011) did not find an effect of reproductive condition on measures of paternal behavior in new fathers and paired virgin male California mice. These findings contrast with earlier reports (Gubernick & Laskin, 1994; Gubernick & Nelson, 1989; Gubernick et al., 1994), however, in which paired virgin males and expectant fathers were found to engage in less paternal behavior than new fathers. One possible explanation is that California mice used in prior studies by Gubernick and colleagues (Gubernick et al., 1995) were less domesticated (i.e., closer to the wild-type population) than those used in recent studies ((Bardi et al., 2011; Lambert et al., 2011; Chauke et al., Chapter 3, In Revision); the California mice used in recent studies are descended from the same populations. Alternatively, other factors such as diet, time of day, and exposure to odors and sounds of pups in the breeding colony could play a part in the greater amount of paternal behavior observed in nonfathers in this and other recent studies.

4.5.2 Neuroendocrine response to pup-exposure

We did not find an effect of reproductive condition on the neuroendocrine (CRH, AVP) response to 1 h of pup-exposure; however, new fathers displayed greater Fos-IR in the mPVN than nonfathers, regardless of test condition (PUP, CTRL). We then assessed the relationship between paternal responsiveness and the neural response to pup-exposure within the virgin male group, as this group showed the greatest variability in paternal behavior. Paternal virgin males (i.e., those that spent $\geq 30\%$ of the first 20min of the paternal-behavior test engaging in paternal behavior) exhibited lower activation of mPVN and BnST neurons than nonpaternal virgin males. Furthermore, time spent engaging in

paternal behavior was inversely correlated with Fos-IR in the mPVN and BnST, and time spent sniffing the foster pup correlated positively with Fos-IR in the BnST.

The results from the current study suggest that nonpaternal virgin males found pup stimuli more stressful and anxiogenic than did paternal virgin males. Similarly, in another study of California mice, Lambert et al. (2011) found that time spent engaging in paternal behavior was negatively associated with Fos-IR in the PVN of both paired virgin males and new fathers exposed to a foster pup for 10 min (note that Lambert et al. (2011) did not distinguish between pPVN and mPVN). The findings in our study implicate the mPVN in the differential processing of pup stimuli in paternal and nonpaternal virgin males. Neurons in this region synthesize AVP and oxytocin, and are known to be activated in response to a variety of stressors (Scott & Dinan, 1998). While the results from our study suggest that AVP-IR neurons were not differentially activated in paternal and nonpaternal virgins, as no differences in the percentage of colocalized Fos/AVP-IR neurons were found between paternal and nonpaternal virgin males, it cannot be ruled out that AVP had been cleared from some of the Fos-IR mPVN neurons. It is also possible that some of the Fos-IR cells in the mPVN were oxytocinergic neurons. Oxytocin has been implicated in paternal behavior in prairie voles and mandarin voles (*M. mandarinus*; (Bales et al., 2004; Song et al., 2010)), but not in California mice (de Jong et al., 2009; Lambert et al., 2011). Further studies on the relationship between oxytocin and paternal responsiveness need to be conducted to provide clarification.

A positive association between paternal behavior and AVP-IR in the BnST of *Peromyscus* has been described (Bester-Meredith & Marler, 2003), although these data were from a combined sample of California mice and white-footed mice (*P. leucopus*). In

a recent study, we found higher Fos-IR in the BnST of new fathers compared to virgin males in response to 5-min exposure to an unfamiliar pup confined in a stainless steel mesh ball (which permitted the male to see, hear, and smell, but not touch, the pup; (de Jong et al., 2009)). These findings have not been consistent, however, as Lambert et al. (2011) found no relationships between paternal responsiveness/reproductive condition and AVP- or Fos-IR in the BnST of male California mice. Similarly, in the present study, AVP-IR was not associated with paternal responsiveness, but paternally nonresponsive virgin males had higher Fos-IR in the BnST than paternally responsive virgin males. As high Fos-IR in the BnST has been associated with maternal behavior in female rats (Lonstein & Vries, 2000b; Numan & Numan, 1995), it is surprising that virgin males in this study showed the opposite pattern. It should be noted, however, that in the studies implicating BnST Fos-IR in parental behavior (de Jong et al., 2009; Lonstein & Vries, 2000b; Numan & Numan, 1995), total Fos-IR in the BnST was analyzed, while in the current study, data for Fos/AVP-IR and Fos-only-IR were analyzed separately. This allowed us to distinguish between activation of cells that contained CRH or AVP, and those that did not. This method, using Fos and neuropeptide colocalization as an indicator of which types of cells were activated, is well described in the literature (Carlson et al., 2007; de Jong et al., 2009, 2010).

The CRH findings in this study also contrast with those described in lactating rats. In comparison to virgin females, lactating rats have lower stress-induced activation of CRH neurons in response to a variety of stressors (da Costa et al., 1996, 2001; Lightman et al., 2001; Shanks et al., 1999). Previously, we showed that the neuroendocrine response to stress in biparental males was not mediated by reproductive condition (Chauke et al., Chapter 3, In Revision). We can now also conclude that being a father does not differentially

mediate the CRH response to pup-exposure. Furthermore, our data suggest that paternal responsiveness in virgin males is not associated with reduced CRH responses to pup stimuli.

4.5.3 Conclusion

In conclusion, our findings suggest that brief exposure to an unfamiliar pup does not consistently activate stress- or anxiety-related neuroendocrine circuits in male California mice, including new fathers, males pair-housed with a nonbreeding female, and virgin males. Furthermore, reproductive condition does not influence the neuroendocrine response to pup-exposure. Importantly, however, whether an animal behaves paternally or nonpaternally toward an unfamiliar pup appears to be associated with the level of activation of stress-related brain regions. Thus, the results of this study support the hypothesis that pup stimuli may be stressful, and that this may in turn influence the behavioral response to pups.

Interestingly, we recently found that hormonal (corticosterone) and neuroendocrine (CRH, Fos) responses to a non-pup-related stressor (predator urine), as well as neophobia and anxiety-like behavior, do not differ markedly between fathers and non-fathers or between paternally responsive and nonpaternally responsive male California mice ((Chauke et al., Chapter 3, In Revision); see also (Bardi et al., 2011)). In sum, these findings suggest that the associations between paternal behavior and neuroendocrine stress responses found in the present study do not reflect individual differences in generalized stress responsiveness or behavioral reactivity, but instead represent individual differences specifically in behavioral and neuroendocrine responses to pup stimuli. Future studies into other factors that characterize nonpaternal or paternal virgin males may further illuminate the role that activation of stress-related brain areas plays in paternal responsiveness.

4.6 Acknowledgements

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Chapter 5

Conclusions

This dissertation describes some of the first studies on the relationship between stress responsiveness and paternal behavior. The vast majority of the literature on parental behavior and its mechanistic basis, and the role of stress responsiveness in particular, focuses on lactating rats (Lightman, 1992; Numan & Insel, 2003; Slattery & Neumann, 2008). While little is known about the role stress responsiveness plays in the regulation of paternal behavior, it is well established that dampened behavioral and neuroendocrine stress responsiveness contribute to the onset and maintenance of maternal behavior.

5.1 Summary of findings

I found that reproductive condition did not strongly influence stress responsiveness. Behavioral responses to an unfamiliar pup, meanwhile, appeared to have several associations with aspects of behavioral and neuroendocrine responsiveness to stress. In the first experiment, I examined the effects of fatherhood on stress responsiveness by comparing

behavioral and corticosterone responses to predator-urine among new fathers, nonbreeding males (vasectomized and pair-housed with a female), and virgin males (housed with a male pairmate). The results of this study suggested that cohabitation with an adult female modulated the corticosterone response to a predator-urine stressor, as both breeding males and vasectomized males housed with a female showed reduced corticosterone responses to a repeated predator-urine stressor. Furthermore, fatherhood was associated with reduced behavioral responsiveness to a predator urine stressor, as both virgin males and nonbreeding males housed with a female showed an increase in stress-related behavior during predator urine exposure, whereas new fathers did not. I had expected to find similarities in postpartum stress hyporesponsiveness between fathers from biparental species and lactating females from uniparental species (Lightman, 1992; Shanks et al., 1997; Windle et al., 1997b,a). This was not the case, as fatherhood alone did not influence the corticosterone response to stress. I therefore concluded, from the results of this study, that while subtle changes in behavioral stress responsiveness occur in new California fathers, endocrine responses to stress are mostly modulated by social interactions with adult females. Furthermore, it is likely that hyporesponsiveness to stress during the lactation period does not occur in female California mice, as in this study, no differences in behavioral, or corticosterone responses to stress were found among the female reproductive conditions (breeding females, nonbreeding females, and virgin females).

To gain a clearer picture of the stress-related behavioral modifications, if any, brought about by being a father/engaging in paternal behavior, we next assessed levels of neophobia and anxiety in California mouse new fathers (housed with a pairmate and their first litter), expectant fathers (housed with a pregnant pairmate), paired virgin males

(housed with an unrelated male), and isolated virgin males (housed alone). All males were put through paternal-behavior, novel-object, and elevated-plus-maze tests at 24-48 h intervals. Results from this study showed, once again, that being a new father did not play a large role in stress responsiveness, while social housing modulated anxiety-related behavior. No differences in neophobia were found among the groups, while new fathers and paired virgins were found to exhibit lower anxiety-related behavior than expectant fathers and isolated virgins. Comparing males that were paternally responsive (i.e., paternal) and non-paternally responsive (i.e., nonpaternal) towards a foster pup, I found that paternal males exhibited more anxiety-related behavior than nonpaternal males, as assessed on the basis of the elevated-plus-maze test. Interestingly, a negative association between paternal responsiveness and neophobia was found when bivariate correlations were performed on data from all animals in the study. These findings suggested that paternal responsiveness and social housing may modulate behavioral stress responsiveness. In contrast to findings in lactating rats, new fathers showed no clear reduction in stress- and anxiety-related behavior during the postpartum period compared to non-parents. New California mouse fathers do not show changes in circulating oxytocin levels (in comparison to nonfathers; (Gubernick et al., 1995)), but have been reported to exhibit elevated circulating prolactin levels similar to those of mothers ((Gubernick & Nelson, 1989); but see (Numan & Insel, 2003) for concerns about the validity of this finding). Slattery & Neumann (2008) hypothesize that the synergistic actions of oxytocin and prolactin mediate the reduced stress- and anxiety-related behavior exhibited by lactating rats.

Reduced neuroendocrine responsiveness to stress has also been described in the postpartum period in female rats (da Costa et al., 1996, 2001; Lightman & Young, 1989).

If engaging in parental behavior modulates neuroendocrine stress responsiveness, and/or if reduced responsiveness to stress is necessary for the initial expression of paternal behavior, I would expect new fathers and paternally responsive males to exhibit reduced Fos and colocalized Fos/CRH expression in stress- and anxiety-related brain areas in response to an ethologically relevant predator-odor stressor. Neither fatherhood nor paternal responsiveness was associated with the neural response to stress exposure. Social isolation was, however, associated with a dysregulation of Fos and Fos/CRH expression in stress- and anxiety-related brain areas. Social isolation reduced overall (baseline and stress-induced) Fos- and colocalized Fos/CRH immunoreactivity (IR), and increased overall CRH-IR in the PVN. In the central nucleus of the amygdala, social isolation increased stress-induced CRH-IR and decreased stress-induced activation of CRH neurons. I concluded that the response to generalized stressors was not influenced by fatherhood or the degree of paternal responsiveness. It was unknown, however, whether nonfathers and nonpaternally responsive males found pup stimuli, in particular, more stressful than new fathers and paternally responsive males, respectively.

The final study addressed the hypothesis that a stress response to pup-exposure may result in low paternal responsiveness, and, consequently, that nonfathers and nonpaternally responsive males would find pup-exposure more stressful than fathers and paternally responsive males. My results partially supported this hypothesis; comparisons of Fos expression in the magnocellular paraventricular nucleus of the hypothalamus (mPVN) and the bed nucleus of the stria terminalis (BnST) in response to 1 h of pup-exposure revealed that paternally responsive virgin males had lower activation than nonpaternally responsive virgin males. The mPVN is a brain area associated with stress and anxiety, while the BnST

is associated with paternal behavior (Bester-Meredith & Marler, 2003; de Jong et al., 2009). I did not, however, find an effect of fatherhood on the neural response to pup stimuli, although new fathers tended to have higher overall Fos expression in the parvocellular PVN as compared to nonfathers. These results suggested that males that behave paternally may indeed differentially activate stress-, anxiety-, and paternal-behavior-related brain areas to pup-exposure in comparison to nonpaternal males. These findings were specific to virgin males, as they were the only reproductive group that showed enough variability in paternal responsiveness to provide a large enough number of paternal and nonpaternal animals for comparisons.

Together, the data from the studies described in this dissertation point to substantial differences in the mechanisms underlying parental behavior in biparental males and lactating rats. Specifically, in rats and several other mammalian species, the onset of maternal behavior is associated with reductions in fear, anxiety, and behavioral and neuroendocrine stress responsiveness, whereas in male California mice no effect of fatherhood was found on any of these measures, except for subtle reductions in the behavioral response to predator urine. These differences may be due to species differences, sex differences, or both. It is likely that paternal behavior in biparental species is controlled by different mechanisms from that of maternal behavior in uniparental species. To date, there is conflicting evidence supporting homology between the mechanisms mediating paternal and maternal behavior among species (Numan & Insel, 2003; Wynne-Edwards & Timonin, 2007). I did, however, find some support for the hypothesis that paternally behaving animals have less activation of stress-related brain areas when exposed to a pup compared to nonpaternally behaving animals. These data will contribute substantially to the current literature on the neural

basis of paternal behavior in biparental species, and will lead to a better understanding of the role stress responsiveness plays in paternal behavior.

5.2 Remaining questions and future work

It remains to be determined what role factors such as pup odors, sounds, sights, and physical contact play in the neural mechanisms mediating paternal behavior in biparental species. Further research into the relationship between the neural processing of pup stimuli and stress responsiveness in the mediation of paternal behavior will illuminate the extent of the similarities and differences between the mechanisms underlying maternal and paternal behavior.

Although paternal behavior occurs in only 5-10% of mammalian species, it is a prominent feature of parental care in humans. Human fathers and other men account for a substantial number of cases of child abuse and neglect (Francis & Wolfe, 2008; Guterman & Lee, 2005; Lee et al., 2009). Thus, understanding the biological basis of mammalian paternal care may offer new insights into parenting by human males, and may reduce the occurrence of child abuse and child neglect by men. In both men and women, child abuse has been found to be associated with parental stress (Banyard et al., 2003; Black et al., 2001; Francis & Wolfe, 2008). Thus, by elucidating relationships between stress responsiveness, fatherhood, and paternal responsiveness in California mice, these studies might shed light on the mechanistic basis of child abuse, and potentially pave the way for possible solutions this problem.

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