IMPACT OF FEMALE MATE CHOICE ON PAIRING AND PREGNANCY IN PRAIRIE VOLES

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2014

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 2021

IMPACT OF FEMALE MATE CHOICE ON PAIRING

AND PREGNANCY IN PRAIRIE VOLES

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Date of Degree: DECEMBER, 2021

Title of Study: IMPACT OF FEMALE MATE CHOICE ON PAIRING AND PREGNANCY IN PRAIRIE VOLES

Major Field: BIOMEDICAL SCIENCES

Abstract: Female mate choice is one of the hallmark features of natural selection via sexual selection. This behavior is one of the key factors in determining what traits continue or diminish within a species. The prairie vole is a monogamous rodent often studied in the context of social behavior. While mate choice in these animals has previously been studied, whether or not choice impacts the formation of monogamous pair bonds has not yet been examined, nor have the endocrine measures associated with pregnancy and stress during this life stage.

In this study, female prairie voles were paired either with a male partner of their own choosing or with a male that was actively not chosen. Partner preference testing revealed that with few exceptions, all animals paired for 14 days formed a partner preference for their mated partner. Female mate choice did not appear to influence the incidence of pregnancy between groups, though all females in this study did have a delay in viable pregnancy establishment *vs.* results previously seen in the Curtis lab.

Offspring from females paired with preferred *vs* non-preferred partners did show differences in anxiety-like behavior in the elevated plus-maze. This indicates that some aspect of female mate choice influences the overall fitness and behavior of offspring in post-natal life.

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CHAPTER I

INTRODUCTION

Mate choice is a topic that has been of interest for hundreds of years. In the long run, mate choice drives what traits continue within a species, and what traits die out. On a much smaller scale, mate choice sets the tone for subsequent quality of life for both the mated pair and their offspring (Ihle *et al.*, 2015). In animals, important factors to consider include whether a mate resides in a territory with suitable access to food, water, and shelter. How highly predated the area is, or how much competition exists for the available resources, all play a role in the likelihood of a mother and her offspring surviving.

In prairie voles, female mate choice holds greater weight, as bi-parental care is one of the hallmark behaviors of this monogamous species. Each female of this species must choose a mate that will protect herself and her pups from predators or from conspecifics that may seek to overtake the territory in which their nest lies. Thus far, the study of female mate choice in prairie voles has shown that preferences exist for unmated males over previously mated males (Pierce and Dewsbury, 1991) and for males that come from mixed-sex litters (Curtis, 2010). Interestingly, however, female prairie voles do not use scent marking placement or frequency information as indices for mate preference (Thomas, 2002; Mech *et al.*, 2003).

Prairie voles are known for forming strong social bonds, not just with mated partners, but also within family units. These small rodents engage in alloparental care when still within the

parental nest, both males and females provide parental care, and both males and females within a mated pair engage in aggression towards unfamiliar members of the same species. These features are unusual among mammalian species, as less than 5% of mammals follow a monogamous mating system (Kleiman, 1977). The formation of these social bonds and the fact that many of the neural mechanisms underlying these features have been studies in this species make them an excellent model for social behaviors. Researchers have taken note of this and have used prairie voles to study, among other things, monogamy (Gavish *et al.*, 1981), parental behavior (Wang and Insel, 1996), alloparental behavior (Stone *et al.*, 2010), and social aspects of addiction (Ryabinin and Hostetler, 2010).

Of course, there are limiting factors to female mate choice. Available mates may be restricted due to environmental barriers, predation, or territory size. A female must choose the best mate available in whatever circumstances she finds herself. For humans, even when a female is in a position to choose a mate, she is not guaranteed that her chosen partner will be the one with which she eventually pairs. It has been found that women who are able to select their partners or have a strong influence in partner selection show higher marital satisfaction (Flicker *et al.*, 2020). Women in marriages where they are not able to select their partner have been known to suffer from subsequent mental health concerns (Rauf *et al.*, 2013).

In this dissertation, I chose to address how an "arranged marriage" of female prairie voles with either a preferred partner or non-preferred partner affects stress hormone signaling in those females. Additionally, I examined measures of fitness, such as latency to pregnancy and litter size in females with their preferred *vs* their non-preferred mate. I also examined whether a partner preference, one of the hallmarks of the formation of a monogamous pair bond in this species, formed between females or their partnered males. Finally, I used behavioral testing to assess whether maternal stress due to pairing with an unchosen partner, resulted in anxiety-like behavior in offspring.

CHAPTER II

REVIEW OF THE LITERATURE

Early studies: What is a vole?

Voles, small mouse-like rodents, were originally studied as an agricultural pest. In the late 1920's, it was estimated that in fairly low numbers, these "meadow mice" (*Microtus pennsylvanicus*) caused an overall loss of up to 30 million dollars in income to hay farmers (Bailey, 1924). In addition to being pests to grass crops, voles are harmful to trees farmed for human use, such as hardwoods and pines (Bell, 1975). Thus, an understanding of their breeding propensities was essential to proper pest management for higher agricultural yield. Voles were first studied in captivity as early as the late 1920's (Selle, 1928) for more controlled study of their breeding activities in aid in pest management. They became established as laboratory animals for behavioral testing during the 1960's (Mallory, 1985).

Prairie voles (*Microtus ochrogaster*) were first differentiated from other vole species in the 1950's (Decoursey, 1957) by differences in dental morphology. As their name implies, they can be found in the prairie region across the central United States and north into Canada (Stalling 1990, see fig 3). This species has spread via the clearing of forests, (Jones, 1983), and the railway and highway systems (Moore and Heidt, 1981).

One unusual characteristic of prairie voles as an animal model is that they engage in strong social bonds between adults, including monogamous pair bonding, which has been found to be present in 5 percent or less of mammalian species (Kleiman, 1977). The first evidence for monogamy in prairie voles came from field observations in which male and female pairs were often trapped together over several months. These pairs remained together, regardless of the female's reproductive status (Getz *et al*., 1981). The characteristics of monogamy seen in prairie voles as defined by Carter *et al* (1995) include cohabitation of mated pairs over time; aggression toward unfamiliar conspecifics; paternal care and alloparenting of pups in addition to maternal care; socially regulated estrous induction and ovulation; incest avoidance and reproductive suppression of adult individuals that provide alloparental support.

While prairie voles engage in a monogamous mating system, vole species such as the montane vole or mountain vole follow a promiscuous mating system (Lim *et al.*, 2006). Prairie voles are not the only monogamous rodent species. Pine voles, California mice, and marmosets also engage in monogamy (Lim *et al*., 2006; Gubernick and Nordby, 1993; Schorscher-Petcu, 2009). There is some variation between monogamous mating systems, with prairie voles known for forming socially monogamous pair bonds in which extra-pair copulations may occur (Ophir *et al*.,2008) while other monogamous species, such at the California mouse, engage in sexually monogamous pairings (Gubernick and Nordby, 1993).

Underlying neural mechanisms for social behavior

The underlying neurological mechanisms of pair bond formation in prairie voles have been a topic of many laboratory investigations. Three primary neurotransmitters found to be involved in the formation of these bonds are vasopressin, oxytocin, and dopamine. Each of these work within the hypothalamus and in brain regions with hypothalamic connectivity to

physiologically direct social interactions. A particularly vital neural region involved in the formation of social bonds in the prairie vole is the nucleus accumbens. This brain region is considered to be a part of the extended amygdala, a portion of the limbic system, which regulates emotion.

While oxytocin plays a role in pair bond formation for both males and females (Young *et al.*, 2001), it was found to preferentially induce partner preference by activating oxytocin receptors in the nucleus accumbens of female prairie voles (Ross and Young, 2009; Young *et al*., 2001). Consistent with this observation, females in non-monogamous vole species as well as rats, display little or no oxytocin receptor binding in the nucleus accumbens (Insel and Shapiro, 1992; Witt *et al.*, 1991).

Like oxytocin, vasopressin plays a more pronounced role in the formation of pair bonds of one sex than the other, with males expressing more vasopressin receptors in the ventral pallidum, a region connected to the nucleus accumbens, compared to females (Lim and Young, 2004). Oxytocin also plays a role in the formation of pair bonds in males, though a much larger influx of oxytocin is necessary for males than for females (Cho *et al*, 1999; Winslow *et al*., 1993). The use of antagonists to block vasopressin V1a receptors in the ventral pallidum (a region that acts as a throughway between the amygdala and other neural regions) or in the lateral septum prevents the formation of a partner preference after mating (Lim and Young, 2001; Liu *et al*., 2001). While male prairie voles show abundant binding of vasopressin in the ventral pallidum, non-monogamous meadow vole species do not (Young and Wang, 2004). Other monogamous species, such as California mice, show high densities of V1a receptors in this same region (Young, 1999).

Dopamine also acts in the nucleus accumbens to play a role in pair bond formation in prairie voles of both sexes (Liu and Wang, 2003; Aragona, *et al*., 2003). D2-type dopamine

receptor activation in the nucleus accumbens enhances initial partner preferences, while activation of D1-type DA receptors inhibits partner preference formation (Aragona *et al*., 2006). After a pair bond has been established for a 2 week period, males show an increase in the density of D1, but not of D2 receptors in the nucleus accumbens (Aragona *et al.*, 2006). McGraw and Young (2010) speculate that this D1 receptor increase may serve to prevent subsequent pair bond formation from extra pair copulations.

These neurotransmitters do not necessarily act in isolation. Particularly in the case of oxytocin, the co-activation of dopamine receptors is essential for pair bond formation. Liu and Wang (2003) found that in female prairie voles, both oxytocin receptors and dopamine D2-type receptors in the nucleus accumbens must be available for ligand binding for partner preference formation. When either an oxytocin receptor antagonist or a D2-type dopamine receptor antagonist were used, pair bond formation did not take place. However, when both receptors were available for endogenous ligand binding, pair bonds formed between females and their male partners.

Estrous induction and mating

Prairie voles are induced ovulators. Natural estrous is induced by the presence of olfactory signals found in male urine (Carter *et al.*, 1980; Dluzen *et al*., 1981; Gavish *et al*., 1983). When direct physical contact takes place between a female and an unfamiliar male, extensive mutual anogenital investigation is common (Getz and Carter, 1981). When urine from a gonadally intact male comes into contact with a female's upper lip there is a reliable increase in uterine weight within 24 hrs. (Carter *et al*., 1980; Dluzen *et al*., 1980), an indication of elevated circulating estrogen. However, Carter and Getz (1985) found that females receiving only chemical signals from urine rarely showed behavioral estrous, while females paired with an

unfamiliar male for several days typically mated within 24 to 72 hrs. after pairing. Thus, both chemosignals from male urine and physical contact with a male are necessary to induce behavioral estrous (lordosis) in prairie vole females (Carter *et al*., 1987).

Mating often takes place within 4 days of introduction. Nearly all females that do mate ovulate within 12 hrs after initial mating, and increased numbers of mating bouts increase litter size (Roberts *et al.*, 1999). The gestation period for prairie voles is roughly 21 days with a mean litter size in captivity of 3.9 pups (Nadeau, 1985). On average, females may be induced into estrous at 35 days of age, while males reach puberty by about 45 days (Gier, 1967). Females have been known to reach puberty as early as 26 days (Bailey, 1928). While these ages vary, the generally accepted age for both sexes to be paired with the goal of maximum breeding/ offspring production is 60 days (Kruckenberg, 1976).

Mate Choice

Mate choice has been a topic of discussion since before Charles Darwin's time, though his work, *On the Origin of the Species,* is easily the best-known reference. As a result of Darwin's work, two major themes have emerged in mating systems. The first is based on the genetic aspects of maternal and paternal contributions, while the second focuses on the type of mating system a species typically engages with (reviewed in Shuster, 2009). As mating systems in prairie voles have already been discussed, this section will focus on female mate selection with regards to genetic contribution. One of the primary potential advantages of mate choice is based on fitness, or reproductive success. Fitness may be direct, enhancing the female's personal reproductive status, or indirect by conveying a genetic reproductive advantage to her offspring. Generally, fitness advantages fall into three categories: genetic factors, territory quality or, for monogamous species, potential for parental care (Andersson 1994).

One major factor in female mate choice is the quality of genetic material that a male may provide to her offspring. Females often inspect a male's health status via scent. Klein *et al.* (1999) showed that female prairie voles prefer the odor of uninfected *vs.* males infected with roundworms. Scent is also a primary factor in the detection of major histocompatibility complexes found within the immune system. Yamazaki *et al.* (1976) found that male lab mice preferred to mate with females carrying dissimilar MHC genes. In mice, peptides bound to MHC molecules release odorants (Leinder-Zufall *et al*., 2004). The detection of MHC variation is highly sensitive, with mice being able to discern differences based on very few amino acid differences at a locus (Carroll *et al*., 2002). While MHC mating preferences have been exhibited in mice (Penn and Potts 1999), laboratory studies have shown varying results (Manning *et al.*, 1992). In one study, roughly 20% of mating strains of mice preferred a mate with similar, rather than a different MHC makeup (Jordan and Bruford 1998). While more thoroughly researched in *Mus* species, MHC-based mate preferences have been seen in bank voles, in which females traveled toward an unfamiliar male with an MHC profile less similar to their own in a T-maze (Radwan et al., 2008).

Another scent-driven mate choice factor is inbreeding avoidance, as inbreeding is known to cause undesirable reproductive results (Ralls *et al*., 1986). One means of such avoidance is to determine one's relatedness to conspecifics of the opposite sex, as is accomplished by female recognition of familiar scent. This is not determined by genetic makeup, as fostered females avoid males with the scent of their fostering units, rather than that of their biological unit (Mino and Tang-Martinez, 1999). When paired with a closely related male, female prairie voles will often not mate (McGuire and Getz, 1981). In white-footed mice, mating between closely related animals produce smaller litters or smaller pups at weaning (Keane, 1990), or, in the case of wild house mice, fewer litters overall (Krackow and Matuschak, 1991).

Female rodents are also known to select mates based on cues that do not have an olfactory origin. Dominance status, which offers a variety of benefits, is another potential aspect of mate choice. Dominant males are better able to defend a territory (Wolff, R. J. 1985; Hurst 1988). Dominant male partners in monogamous pairings may also provide better protection for their offspring and the dams themselves (Agrell *et al.,* 1998). In species where dominance features are heritable, a male's dominance status may extend to a female's offspring (Dewsbury, 1982). Prairie vole females have been shown to prefer dominant males over subordinate males, while promiscuous montane voles show no such preference (Shapiro and Dewsbury, 1986).

Female mate choice may also be driven by body size of the male (Solomon, 1993). In house mice, heavier individuals reach sexual maturity sooner than lighter ones (Fuchs, 1982). Under optimal cooperative breeding conditions, in which alloparental care is available, prairie vole pups are known to be larger (Solomon, 1991; Ahyes and Solomon 2004). This implies that a larger body mass is an indicator of a nurturing nest environment.

Prairie vole females often prefer unmated males to mated mates (Pierce and Dewsbury, 1991), while promiscuous meadow voles show no such preference (Salo and Dewsbury, 1995). Salo and Dewsbury (1995) suggested that females of monogamous species may be more choosy than females of polygamous species, as the males are responsible for providing parental care as well as genetic material.

Mate choice does not end with mating and possible fertilization. Via the Bruce Effect, a phenomenon in which female rodents of certain species may terminate a pregnancy in response to detection of a strange male's odor (reviewed in Mahady and Wolff, 2002), mate choice can take place after copulation. This has been recorded in multiple vole species, including prairie voles, montane voles, and meadow voles (Clulow and Langford, 1971; Stehn and Richmond 1975; and Jannett 1980). Among these vole species, some are able to terminate a pregnancy fairly early after

implantation, while others may do so within less than a week prior to parturition. Meadow voles show reduced pregnancy rates up to 5 days after mating (Mallory and Clulow, 1977). In prairie voles, this phenomenon has been recorded up to 15 days after mating (Stehn and Richmond 1975; Kenney *et al*., 1977).

Stress and the HPA axis

The stress axis includes the limbic system and the hypothalamic pituitary adrenocortical axis (HPA). This axis is involved in daily maintenance activities including exploratory behavior, appetite and food seeking behavior (Reviewed in Wingfield and Romero, 2001). The HPA axis mediates the effects of stressors. Growth of this axis begins during fetal development and becomes sexually dimorphic due to gonadal hormones at puberty. Neurons in the paraventricular nucleus (PVN) regulate the release of corticotropin releasing hormone from the hypothalamus, which controls the release of anterior pituitary hormones (Sheng *et al*., 2021). Also in the PVN are neurosecretory neurons that regulate the secretion of oxytocin and vasopressin (Vandensande and Dierickx, 1975; Swanson and Sanchenko, 1983). From the anterior pituitary, a host of hormones involved in the mediation of the stress response are released into general circulation, including, among others, adrenocorticotrophic hormone (Scanes, 2015). The adrenal gland releases, among other hormones, glucocorticoids, including corticosterone, from the middle layer of the adrenal cortex (Longcope, 1986) and catecholamines from the adrenal medulla (Vinson *et al*., 1994).

Activation of the HPA axis is often identified by the presence of glucocorticoids secreted from the adrenal cortex. Circulating glucocorticoids, among other mechanisms intended to induce the “fight or flight” response, suppress reproduction (Papadimitriou and Priftis, 2009). Prolonged

activation of this axis may cause harmful effects to multiple systemic functions and may be detrimental to neurons and glia (McEwen, 1998; Heck *et al*., 2020).

The bed nucleus of the stria terminalis (BNST), another brain region considered part of the extended amygdala, expresses both androgen and estrogen receptors and plays a crucial role in the regulation of HPA function via gonadal steroids (Viau, 2002). BNST neurons inhibit corticosterone responses to stress (Herman *et al*., 1994). The hippocampus, prefrontal cortex, the central and medial amygdala, and the lateral septum connected through the PVN or BNST and aid in regulation of the HPA axis. (Dong, *et al*., 2001; McKlveen *et al*., 2015).

Testosterone is known to dampen the stress response (Viau and Meaney, 1996; Stanojevic et al, 2018). Conversely, estradiol can either inhibit or enhance the stress response (Handa *et al*., 2009). When these sex-specific hormones surge during early development and again during puberty, they greatly impact the influence of the HPA axis. Due to the broad expression of estrogen receptors, estrogens have been shown to enhance HPA axis activity at several sites that stimulate secretion. For example, estradiol increases the response to adrenocorticotropic hormone in the adrenal gland (Patchev *et al*., 1996). At the anterior pituitary, the presence of estradiol causes a greater response to CRH, demonstrated by increased ACTH secretion (Seale *et al*., 2004). In contrast, other studies have shown no effect on HPA axis activity or ever decreased HPA axis activity in response to estradiol (Young *et al*., 2001; Ochedalski *et al*., 2007). Treatment with estradiol reduced adrenocorticotrophic hormone expression (Young *et al*., 2001), lowered neural activation in the PVN (Isgor *et al*., 2003; Figuierido *et al*., 2007), and lowered secretion of CRH (Ochedalski *et al*., 2007).

There may be different effects of estradiol due to activation of different receptor subtypes. In ovariectomized female rats, ERα receptor activation increases, while administration of an ERβ antagonist decreases, stress induced glucocorticoid secretion (Weiser and Handa,

2009). ERβ may directly alter HPA axis function as it is co-expressed with several PVN neuropeptides, including corticotrophin releasing hormone, vasopressin, and oxytocin (Lund *et al*., 2006; Oyola *et al*., 2017).

Corticotrophin releasing hormone (CRH) also plays a major role in the HPA axis, as it stimulates the release of glucocorticoids. This neurohormone is also produced in the paraventricular nucleus, as well as the amygdala and the bed nucleus of the stria terminalis (Wemsteeker-Cusulin *et al*., 2003). While CRH provides the primary regulation of ACTH secretion from the anterior pituitary, oxytocin and vasopressin also play a role. (Herman, 1992). Both oxytocin and vasopressin are also expressed by CRH containing neurons in the PVN, where they may also be released with CRH. However, vasopressin and oxytocin can stimulate the release of ACTH without the presence of CRH (Reviewed in Sheng *et al*., 2021). OT and AVP also serve as negative feedback signals. If they are delivered to the PVN via intracerebroventricular injection, they inhibit HPA activity (Neumann, 2007).

Not all corticosteroids that are released into circulation as the result of HPA activation are bioavailable. Corticosteroid binding globulin (CBG) stabilizes corticosteroids during transport to target tissues by binding to these molecules in circulation. This binding prevents corticosteroids from binding to receptors (de Kloet *et al*., 2005). Because of this, available corticosterone levels and total plasma corticosterone levels vary. Female rats are known to have higher total levels of corticosterone as well as twice the circulating CBG of males (McCormick *et al*., 2002).

Prairie voles are known to exhibit high levels of plasma corticosterone and to be corticosterone resistant (Taymans *et al*., 1996). They have up to 10 times the plasma corticosterone and twice the plasma ACTH of montane voles. When compared with rats, prairie voles show twice as much corticosterone binding globulin, while still exhibiting higher corticosterone levels. Taymans *et al*. (1996) found that, in addition to the above features of

increased glucocorticoid levels, prairie voles have a decreased affinity for binding at corticosterone receptors, which may drive this resistance.

Stress during reproduction

Activation of the HPA axis has the potential to inhibit reproduction. One surprising mechanism for this is during the release of estrogens, which can block the release of luteinizing hormone (Valsamakis *et al*., 2018). In addition, the HPA axis may be programmed during fetal development by maternal stressors, altering the developing stress axis components of offspring. Exposure to increased levels of glucocorticoid hormone during fetal development can lead to a disruption of HPA axis development via altered neuropeptide production (Sheng, 2021). Some speculate that this allows those offspring to better respond to stressors in their own lifetime (Matthews, 2002).

Acute and chronic stress have differing effects on the mother and developing offspring. In pigs, acute stress can inhibit the induction of estrous and ovulation, while chronic stress impairs reproduction in general (Turner and Tillburg, 2006). Corticotrophin releasing hormone binding at the ovaries inhibits ovarian steroid hormone production (Tarin *et al*., 2010). Pigs seem to adapt to repeated acute stress or cortisol release, as inhibition of estrous induction and ovulation ceases after multiple stressors (Turner and Tillberg, 2006). In humans, chronic stress and the associated rise in cortisol concentrations in pregnant women are associated with increased epinephrine levels. This catecholamine has been shown to negatively influence pregnancy outcome in rabbits (Padbury *et al*., 1981). In sheep, increased stress signaling from the ewe or from the fetus can result in premature parturition (Jones *et al*., 1989; Rakers *et al*.,. 2018). A woman's stress levels during pregnancy can impact not only the structure and function of the brain, but also metabolic function (Entringer, 2013) and infant microbiota makeup (Ziljmans *et*

al., 2015). In humans, increased circulating norepinephrine in pregnant women influenced fetal development and resulted in shorter telomere lengths in their children (Entringer *et al*., 2011). In rats, prenatal stress is known to impact male and female offspring in different manners. In female offspring, an increase in anxiety-like behavior is seen, while in male offspring, decreased spatial learning ability and increased overall mass occurs (Schulz *et al*., 2011).

Impacts of maternal stress on offspring

In early development, the stress response of the fetus is dependent upon input from the mother and placenta, as shown in mice (Gunn *et al*., 2013). However, by day 18 of embryonic development CRH is expressed in rats (Bugnon *et al*., 1982). By postnatal day 7, CRH is expressed at adult levels (Grino *et al*., 1989). CRH expression is present on day 13 of embryonic development in mice, with an increase to adult levels after birth (Keegan *et al.,* 1994; Schmidt *et al*., 2003). In rats and mice, vasopressin is present in neural regions of a developing fetus, whereas oxytocin is not present until 24-48 hrs. after birth (Yamashita *et al*., 1988; Laurent *et al*., 1989). During late gestation, the fetus is able to secrete CRH and ACTH in response to maternal stressors, resulting in the production of glucocorticoids (Gunn *et al*., 2013; Moisiadis and Matthews, 2014).

After birth, low maternal care has been correlated with increased glucocorticoid receptor expression and sensitivity in adult progeny (Liu *et al.,* 1997). Conversely, McGowan *et al.* (2001) showed lowered corticosterone and ACTH responses to acute stress in adult progeny that received increased maternal care. Extensive separation from the mother is associated with increased anxiety-like and depressive-like behaviors in adult descendants (Liu *et al*., 1997). In several rodent species this has been linked to developmental changes in the dopaminergic system,

often with the result of increasing anxiety-like and depression-like behaviors in descendants (Curley *et al*., 2011).

Both short-term and long-term effects of elevated maternal glucocorticoids on a fetus due to either prenatal stress or exposure to synthetic glucocorticoids depend on the length of glucocorticoid exposure and when during development such exposure takes place (Barbazanges *et al*., 1996). The duration of stress has been found to influence the development of CRH expressing paraventricular nucleus neurons, with rat fetuses showing shorter process lengths and greater apoptosis in these neurons when a stressor is applied for 4 hours. When the same stress was applied for 30 minutes, growth and development were enhanced (Fujioka *et al.,* 1999). Exposure to elevated glucocorticoids in utero has been shown to cause increased activity of the HPA axis in adults. This is seen via elevated levels of glucocorticoids and ACTH, as well as reduced CRH expression (reviewed in Kapoor *et al*., 2006). In contrast, postnatal administration of a synthetic glucocorticoid reduced stress-induced HPA activity in adults (Kamphuis *et al*., 2002). These results show that the timing of exposure to glucocorticoids influences the development of the stress response.

At puberty, the hypothalamic pituitary gonadal axis matures and begins to secrete the necessary hormonal signals for sexual maturation (Ojeda and Urbanski, 1994). HPA axis reactivity is greater before puberty than after puberty. In rats, an increased and prolonged stress-induced release of ACTH and glucocorticoids is present before puberty when compared to after puberty (Goldman *et al*., 1973; Romeo *et al*., 2004). Pre-pubescent male rats show increased HPA activation with elevated CRH secretion in response to stress when compared with adults (Romeo *et al*., 2006). After the administration of corticosterone, increased glucocorticoid receptor expression is found in the hippocampus, amygdala and prefrontal cortex in adolescents compared to adults (Romeo and McEwen, 2006). In pre-pubescent females, estradiol inhibits stress-induced HPA function, while in post-pubescent females estradiol injection stimulates HPA activity during

acute stress (Evuarherhe *et al*., 2009). In this same study, administration of estradiol increased basal and stress-induced glucocorticoid secretion regardless of whether females were ovariectomized before or after puberty. This implies that estradiol is inhibitory to the HPA axis during puberty and stimulatory post-puberty (Evuarherhe et al., 2009).

Parental care

Prairie voles have also been studied in the context of parental and alloparental care. Both pair bonding and parental behavior are inspired by common neurological pathways (McGraw and Young, 2010). Female prairie voles, whether virgin or post-partum, engage in nurturing behavior toward infants. However, virgin females may differ in their expression of this behavior with roughly 60% of adult females engaging in alloparental behavior with novel pups, while 40% attack or ignore pups. Virgin females that display alloparental care have higher densities of oxytocin receptors in the nucleus accumbens than females that do not display this behavior (Olazabal and Young, 2006a and 2006b). An oxytocin receptor antagonist in the nucleus accumbens prevents the display of maternal behavior (Olazabal and Young 2006a).

Early life experience and adult social behavior

Parental behaviors play a role in prosocial behavior. A study by Stetzik *et al.* (2018) showed that in animals crossbred from the Illinois and Kansas populations of prairie voles, paternity influences the formation of social bonds. Females sired by Illinois males were more

likely to express a partner preference than those with fathers originating from Kansas. In males, the impact of maternity was more potent, with differences seen in oxytocin expression and vasopressin expression in the PVN as well as in behavioral measures. Males with Illinois dams spent more time investigating the unfamiliar female than the familiar females in a partner preference test, and also showed more defensive aggression during testing.

Female prairie voles raised under less nurturing parental conditions were less likely to display alloparental behavior. Both male and female offspring raised under such conditions required longer cohabitation periods than offspring reared by both parents to form partner preferences (Ahern and Young, 2009). This paradigm also altered the number of oxytocin neurons in the PVN (Ophir *et al.*, 2008).

Summary

Prairie voles are a well-established animal model for the study of social behavior, both in regard to pairing between mated partners and in regard to parental behaviors. In other species, such as birds, male displays or physical characteristics play a major role in mate selection (Liu *et al.*, 2010). For prairie voles, mate selection hinges on more subtle signals, primarily olfactory cues which play a role in predicting the health status and genetic variability a male may bring to a pairing. More subtle behavioral cues may also play a role, but these are not as readily measured.

In addition to choosing the best possible mate, females must also have a safe environment in which to carry a pregnancy to term. Physical safety in regard to resource availability and reduced predation are fairly easy to assess, while more subtle emotional stressors may not be as readily measured. Investigation of fluctuations in the stress response over the course of pairing and pregnancy in female prairie voles may illuminate whether more subtle social stressors have a readily measurable impact on pregnant females. Because maternal stress is known to affect the

development of stress responses in offspring, measurements of how social stressors in female prairie voles may affect their offspring might provide supporting evidence for any HPA axis activity measured during the pregnancy itself.

CHAPTER III

GENERAL METHODOLOGY

GENERAL METHODS

Several experiments in this dissertation required adjustments and optimizations of established methods for accurate data assessment. The methods described in this chapter will provide the baseline information for those methods. More specific methods development information will be provided in the subsequent chapters.

Subjects

Female and male prairie voles bred at Oklahoma State University Center for Health Sciences with origins in southern Illinois were used for all experiments. Subjects were F3 or F4 generation animals from the last outcrossing with wild stock, weaned at 19-21 days post-natally and co-housed with a same-sex littermate. Housing consisted of 10 x 17 x 28 cm clear plastic cages with pine chip bedding in a facility at ~23˚C with a 14/10 light/dark cycle. Purina rabbit chow and sunflower seeds along with filtered water were available *ad libitum*. All animal care, handling, and experimental procedures were reviewed and approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee.

Mate choice testing

For mate choice assessment, sexually naïve female prairie voles were placed into an apparatus typically used for partner preference testing with prairie voles. The layout and use of this apparatus is described in Chapter 5 of this dissertation (Figure 3). LED motion sensors at tunnels connecting cages were used to measure time spent in each cage by the female as well as the total number of crossings between cages. Video recordings of each test allowed for manual recoding of contact time. This test lasted for two hours.

A minimum of 10 minutes total contact time, regardless of whether is was spent with one male or it was split between multiple males, was required to establish that a mate choice had been made. The chosen male and non-chosen male were determined for each female that made a choice based on which male the female shared more contact time with. The females that made a choice were randomly assigned to be placed with the male of their choosing (Preferred (P), n=13) or the unchosen male (Non-Preferred (NP), n=12). Females that did not make a choice within the 2 hour mate choice test were considered "ambivalent" (AMB) and were placed into a separate experimental group (n=18). The partners for ambivalent animals were selected between the two potential male choices via coin flip. Due to technical errors, data for cage crossings and cage time were not collected for two of the preferred females. Contact time was collected for all subjects.

A separate group of females (n=19) was not exposed to the mate choice paradigm and served as a control group (Ctrl). These subjects were placed with a sexually naïve male for the two hour duration that would have taken place in the mate choice paradigm. These same males were then the females' partner for the remainder of the experiment.

Prior to mate choice testing, male mass and anogenital distances were measured to determine whether either of these factors play a role in mate choice for the females that made a

choice, as implied by other research (Schulte – Hostedde and Miller, 2004; Ophir and Delbarco-Trillo, 2007). Both of these measures were evaluated via *t*-test.

Partner preference testing

After two weeks of cohabitation with their opposite-sex partner, either the female or male of each pair within each experimental group was exposed to a 3hr partner preference test. This test took place in the same apparatus used for mate choice testing, but rather than exposing the test subject to two unfamiliar conspecifics, the animals tested were allowed to roam freely between two cages, one containing the familiar partner, and the other an unknown and unrelated 'stranger' vole of the opposite sex. Measures collected include contact time, cage time, and total crossings between cages. Due to an equipment issue, cage crossing and cage time were not available for analysis for one Control female, one Preferred male, and one Ambivalent female. All subjects had contact time data available for analysis.

Repeated measures analysis of variance (ANOVA) was used to examine any differences between time spent in any of the available cages, number of cage crossings, and contact time between the subject and the partner or stranger in each test. Pairwise comparisons were done to assess statistically significant main effects or interactions. In cases where main effects were seen, but no interactions, a conservative Tukey's test was used. Where interactions were found between all possible factors in the comparisons, Fisher's Least Significant Difference (LSD) was used. These *a priori* criteria were used for all experiments requiring ANOVA.

Females and Pregnancy

After any paired subjects had produced a litter of pups, they were placed into a larger plastic cage (20 x 25 x 45 cm) with their partner and litter. Timothy hay was added to the pine bedding in these cages to allow for nest making and as an additional nutritional supplement. Date of parturition was recorded for all subjects and used to calculate latency to pregnancy. Litter sizes were also recorded and examined for differences between groups via 1-way ANOVA.

Fecal Collection

As a means of collecting endocrine samples for a longitudinal experimental design in a non-invasive manner, fecal samples were collected and used for ELISA analysis. Fecal collections were obtained from animals immediately prior to mate choice testing ('pre'), immediately after mate choice testing ('post'), and on days 1, 2, 3, 5, 7, 9, 12, 15, 18, 21, 24, 27, and 30 after pairing. Samples from 'pre' through day 21 were used in ELISA analysis. Samples collected after day 21 were not used for analysis as some animals had litters prior to the 24th day, making the data set no longer amenable to repeated measures ANOVA. During fecal sample collection, animals were isolated in 10 x 17 x 28 plastic cages for approximately 1 hour, with extra time granted as needed for at least 4 fecal pellets to be collected. Fecal samples were stored in 1.5 mL microfuge tubes at -80˚C until prepared for ELISA analysis.

ELISAs

After sample preparation (described in Chapter 6 of this dissertation), all ELISAs were run via commercially available kits (ENZO) according to manufacturer's recommendations. Total concentrations of hormone in fecal samples (pg/mg) was assessed using the parameters outlined

in the manufacturer's recommendation. This information was then analyzed via repeated measures ANOVA**.**

Evaluation of Anxiety-like Behavior in Offspring

Offspring from each group were weaned at 19-21 days post natally. At weaning, adult subjects and their mates were terminated along with any offspring that could not be placed into same-sex sibling pairs. Within 1 week of weaning, 1 animal from each sibling pair was exposed to both the elevated plus maze (EPM) and open field (OF) test to measure anxiety-like behavior. More recently weaned subjects completed the OF test (Ctrl n=19, P n=14, NP n=10, AMB n=26) than completed the EPM (Ctrl n=11, P n=10, NP n=8, AMB n=25), as some animals did not meet the criteria for completion of that test (described below). Both animals in any given cage were then shaved on opposite hips for identification purposes at a future testing date. Animals were re-shaved for identification as needed.

Within 1 week of 60 days of age, all animals were tested in the elevated plus maze and open field apparatus again. This testing served as a re-test for animals tested at weaning , or an initial test for those not tested at weaning. As occurred at weaning, not all subjects completed the EPM test. For those animals that were tested as both weanlings and adults, 53 animals completed the open field test, while only 38 completed the elevated plus maze (EPM: Ctrl n=5, P n=8, NP n=6, AMB n=23; OF: Ctrl n=11, P n=10, NP n=9, AMB n=17). For those animals that received their first and only testing sessions as adults, 60 completed the OF test, while only 54 completed the EPM test (EPM: Ctrl n=8, P n=10, NP n=7, AMB n=25; OF: Ctrl n=10, P n=10, NP n=9, AMB n=31).

Elevated plus maze

An elevated plus maze consisting of two 35 x 6.5 cm open arms and two 35 x 6.5 cm closed arms with 15 cm high walls elevated 45 cm from the floor of the testing area. Subjects were placed at the center of the maze and allowed to roam freely for 5 minutes. Each EPM test lasted for 5 minutes and was video recorded via Ethovision XT (Noldus) for analysis of time spent in open and closed arms, and locomotor activity (distance traveled and velocity within each test). Animals that did not remain on the maze for ≥ 2.5 min were re-tested after a minimum 20-minute interval up to a maximum of 3 total tests. Any animal that did not complete an of the three 5-minute tests was noted as untestable and excluded from analysis.

Open Field

An open field apparatus with dimensions of 56 cm x 56 cm with 20 cm high walls was used and video recordings and analysis were done via Ethovision XT (Noldus). Analysis was based on time spent in each zone, where zones were determined as edge and central zones and were made up of sixteen 14 x 14 cm evenly placed squares that divided the entire open field arena. The edge zones were further divided into corner zones for statistical analysis. In addition to the measures specific to each test, total distance traveled and average velocity were recorded for each animal via 1-way ANOVA or repeated measures ANOVA, where appropriate. Each Open field test was 10 minutes in duration.

CHAPTER IV

EFFECTIVE MEASUREMENT OF CHANGES IN ESTROGEN OVER TIME VIA FECAL EXTRACTION

INTRODUCTION

Several experiments in this dissertation required the detection of changing hormonal levels as measured from fecal pellets through ELISA. In order to establish that hormonal changes can be adequately measured from prairie vole fecal pellets, and that commercially available ELISA kits would be adequate for examining these measures, some efforts were spent on developing an appropriate protocol for sample preparation prior to ELISA. 17 –β estradiol, a hormone of interest for this study, was examined in these tests.

Determination of whether methanol interferes with accurate hormonal measurement in ELISA

An indication that methanol, the solvent used in our hormonal extraction protocol, may interfere with immunoassay readings was found in the literature (von Maltzan and Pruett, 2011). To determine if samples still in methanol may be used in ELISA, or if any methanol present in sample dilutions would interfere with accurate hormonal measurements, samples extracted in 90% methanol from fecal pellets from female prairie voles and expected to have high and low levels of circulating estrogen were prepared with no dilution, 1:5 dilution in assay buffer, or 1:10

dilution in assay buffer. Additionally, estrogen standards were prepared in 90% methanol and diluted in assay buffer to establish whether the presence of methanol in the sample may prevent accurate quantitation of estrogen with fecal sample extractions. The dilutions used were: no dilution of samples in 90% methanol, 1:5 dilution of 90% methanol: assay buffer, 1:10 methanol: assay buffer, and 1:25 methanol: assay buffer. Calculations were completed to establish concentrations for different dilutions of each sample, but results were extremely inconsistent. We surmised that methanol in the samples may be a confounding factor and altered our sample preparation protocol to include the removal of methanol from the sample.

Does removal of methanol from samples via dehydration and rehydration in assay buffer alter the detection of β-estradiol?

We evaporated fecal extractions to remove the methanol, then rehydrated in equal amounts of assay buffer (Figure 2). Samples from animals expected to have either low or high fecal estrogen (n=3/group) were assayed in a series of dilutions in assay buffer to determine consistency of results. Dilutions used include no dilution, 1:5, 1:10, 1:25, 1:50, and 1:100 of sample reconstituted and diluted in assay buffer. The results of this experiment showed consistent measures of β-estradiol present within each sample, regardless of dilution factor, implying that dehydration of samples in 90% methanol and rehydration in assay buffer does not negatively impact the readings for those samples and confirming that removal of methanol from samples is necessary for reliable readings.

Are standards prepared in assay buffer appropriate to use with this sample preparation protocol?

To determine whether our protocol for hormonal extractions from feces required that the quantitation standards for estrogen supplied with the ELISA kits be prepared in a similar manner, estrogen standards prepared in assay buffer per ELISA kit protocol were run alongside and compared with estrogen standards prepared in methanol, and then dehydrated and rehydrated in the same manner described above. This also allowed for a determination of whether our sample preparation altered the measureable amounts of estrogen present in each sample, as the estrogen standards contain a known amount of estrogen at initial preparation. We found that the measurement of known estrogen amounts using standards from the ELISA kit was not affected by preparation in 90% methanol, dehydration, or rehydration. We also found that a standard curve prepared in assay buffer per kit protocol provides reliable readings for estrogen samples rehydrated from a dehydrated methanol preparation.

Serial Extractions: How effective is our fecal estrogen extraction protocol?

To establish that estrogen can be successfully extracted from fecal pellets in prairie voles, fecal samples collected 1 hour after prairie voles were injected with 10mg/kg β–estradiol were dried via SpeedVac then homogenized in 90% methanol and centrifuged. The supernatant was collected and an additional volume of 90% methanol was added, homogenized into the fecal sample, centrifuged and the supernatant collected as a separate aliquot. This was done for a total of 4 supernatant collections. Initial extractions from each sample yielded roughly 60% of the available estrogen in fecal pellets. This decreased by roughly half with each subsequent extraction, showing that the extraction protocol is effective for removing an adequate amount of estrogen from fecal pellets to observe overall trends in fecal estrogen over a time course (Fig. 1). Based on these results, we determined that a combination of the first two extraction aliquots should be used for ELISAs.

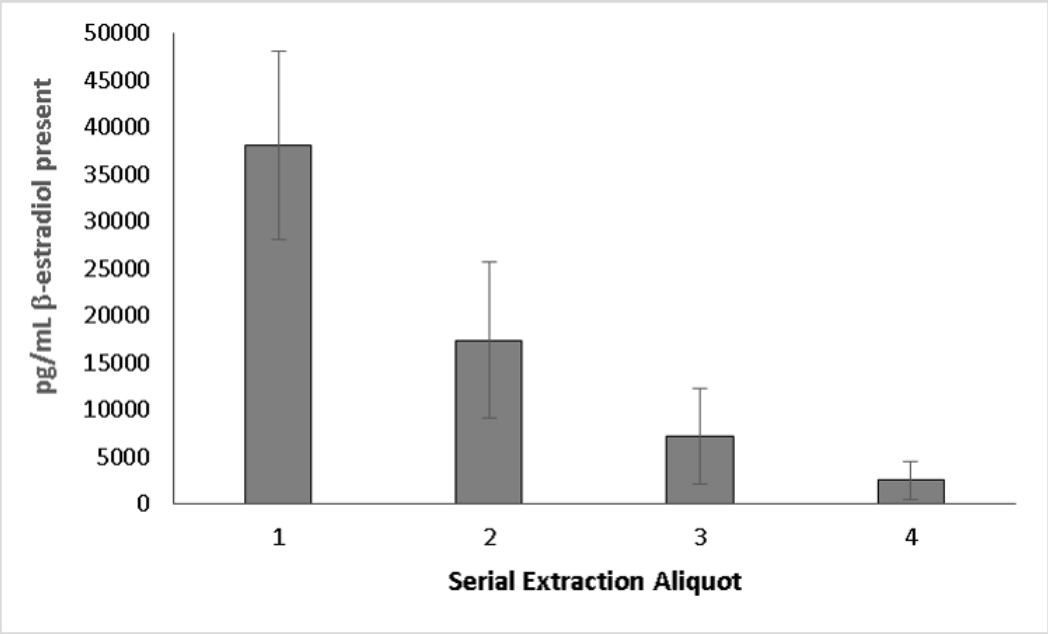

Figure 1: Estrogen concentrations of serial aliquots from fecal samples.

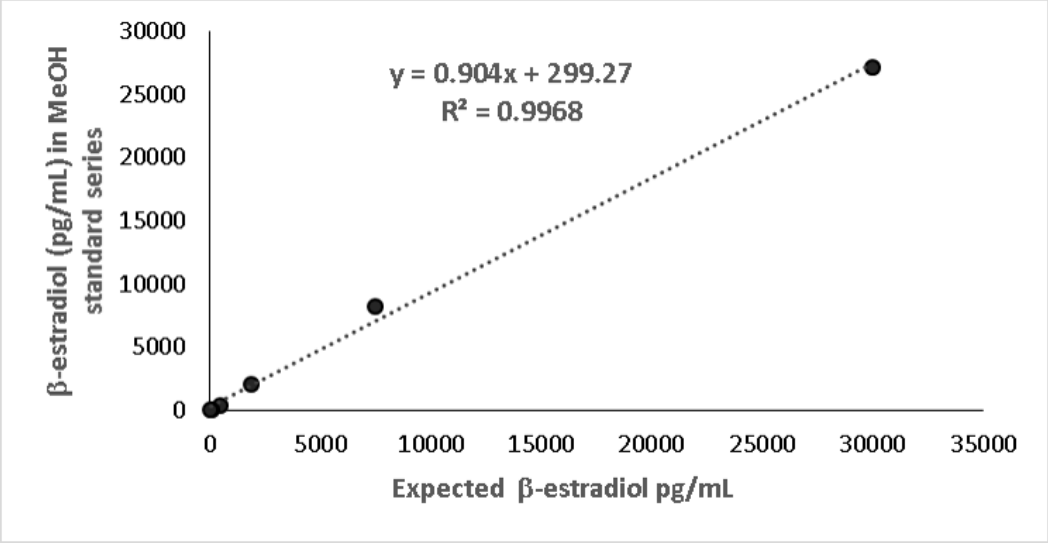

Figure 2: Expected β-estradiol concentrations *vs.* measured β-estradiol concentrations. 17 β-estradiol concentrations measured in a standard series prepared in 90% methanol, dehydrated, and then resuspended in assay buffer.

The results shown in figures 1 and 2 indicate that estrogen in the feces of prairie voles can be reliably extracted and quantified for estrogen content from fecal pellets when analyzed via

ELISA after removal of the methanol used for extraction followed by reconstitution in assay buffer from the ELISA kits being used.

CHAPTER V

FEMALE MATE CHOICE AND SUBSEQUENT PARTNER PREFERENCE

INTRODUCTION

Female mate choice is an important feature of any reproductive strategy. Each potential mate brings characteristics that may hinder or help with the ultimate fitness of that female or her offspring. Many physical factors come into play such as the size and health of the male, or the diversity the genetic material he may provide for any young produced by a pairing. In monogamous species, such as prairie voles, aspects beyond genetic factors are of importance, as the male will become a lifelong partner for the female as well as the sire of her pups.

The formation of a partner preference is well established in prairie voles, but how female mate choice affects pair bond formation and any pregnancies that may result from that pairing have not been studied. In this chapter, I describe how female mate choice affects pair bond formation in both females and males. Additionally, I examined whether females paired with their preferred mate became pregnant earlier, or produced larger litters than females paired with a partner they did not choose.

METHODS

Mate Choice Paradigm development

While female mate choice has been examined in prairie voles by other investigators, those studies did not involve subsequent experiments that measured changes in hormone levels. As female prairie voles are induced into sexual receptivity (Richmond & Conaway, 1969; Carter, *et. al.,* 1987), it was desirable to determine the minimum length of testing within which a choice was likely to be made, thus reducing the female's exposure to estrous-inducing stimuli prior to pairing with a partner.

To establish a minimal amount of time needed for a female prairie vole to choose a mate, sexually naïve females were placed into an apparatus in which two, sexually naïve, unrelated males were tethered into cages attached by small tunnels to either side of a centrally located cage (Figure 3). An untethered female subject was placed in to the central cage and allowed to roam freely for the duration of the three-hour test.

Contact time, defined as physical contact between the subject and either stimulus animal that extended at least 1 minute or more at each episode, was used to determine a preference, described hereafter as a choice, for either of the potential mates. The 3-hour test was binned into 30-minute sections and cumulative contact time was assessed with the addition of each new bin. Statistical analysis via *t*-tests was applied to compare the contact time female subjects spent with each potential mate. The point at which a statistically significant difference was seen in contact time between the two stranger animals was determined to be the minimum amount of time needed for a female prairie vole to make a choice within this paradigm.

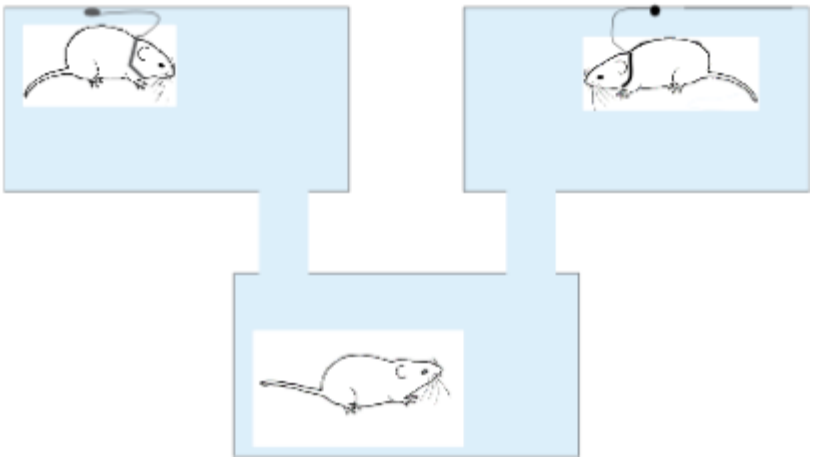

Figure 3: Mate choice and partner preference testing apparatus

Partner preference testing

After two weeks of cohabitation, either the female or the male from each mated pair were exposed to a 3-hour partner preference test with a novel stranger of the opposite sex and their partner of two weeks. Data collected included total contact time with either the partner or the stranger, total crossings into each cage, and total time spent in each cage. Statistical analysis of partner preference data was analyzed via repeated measures ANOVA.

RESULTS

How much time is needed for a female prairie vole to select a mate?

Exposure to a potential mate induces estrous in female prairie voles. Thus, minimizing the length of time each female spent in contact with males during the mate choice test was an essential component of experimental design. t-tests of cumulative contact time at 30-minute

intervals revealed that a statistically significant difference between time spent with the preferred male and the non-preferred male in the mate choice paradigm became evident at the 120 minute time point ($t_{(17)}$=2.59;p<0.01). Based on these results, it was determined that a 2-hour mate choice test would be sufficient for further experiments. Mate choice selection testing was then conducted in the manner described in Chapter 3 of this dissertation.

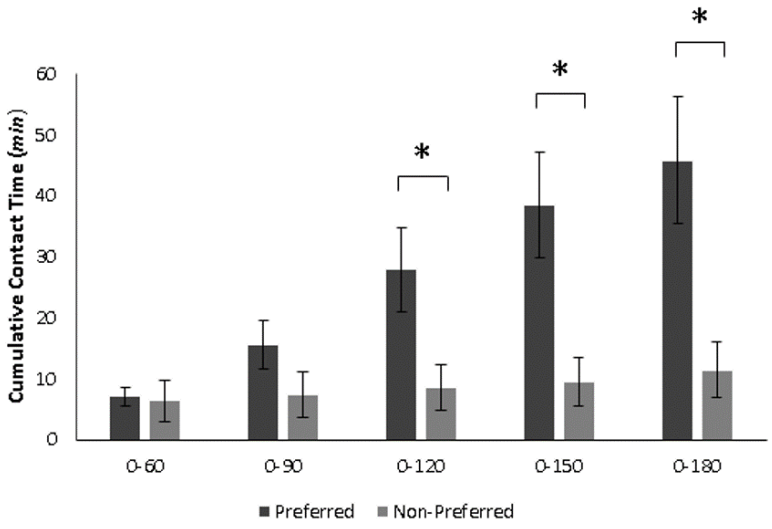

Figure 4: Cumulative contact time with preferred *vs.* non-preferred male in mate choice. A clear choice is made by 120 minutes during mate choice testing. Data are expressed as means ± SEM with * p<0.01.

Mate choice testing and group assignments

Females were allowed two hours to choose between one of two unrelated males in a mate choice test. The expectation was that all females would make a clear choice. However, during the choice testing an unexpected grouping emerged – females that did ***not*** make a clear choice between the potential male partners. This group of subjects was termed "ambivalent' and was

included in subsequent experiments. Ambivalent females were characterized by spending a total of less than 10 minutes combined contact time with the two males during the 2-hour mate choice test.

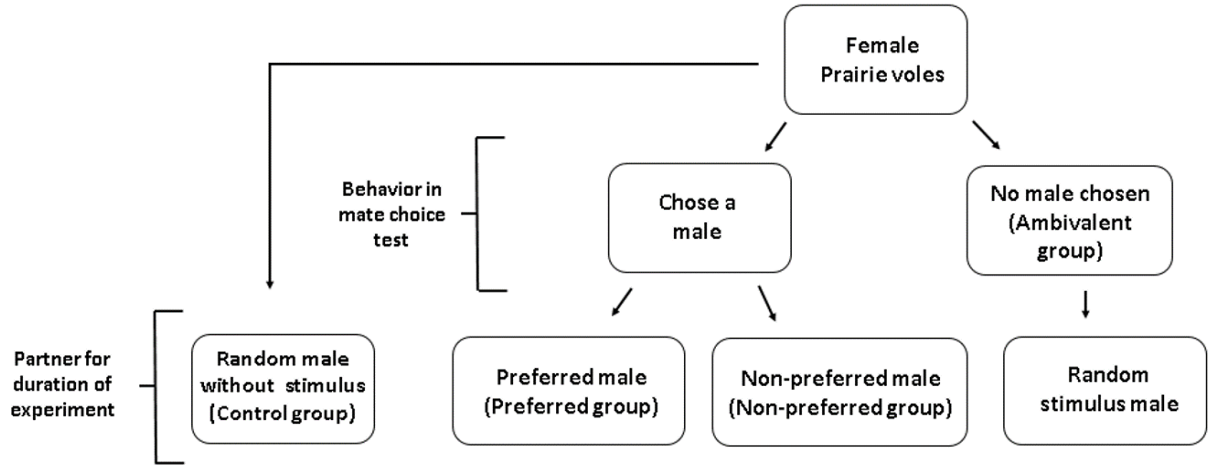

Figure 5: Female mate choice groups. Female groupings were based on their behavior during mate choice testing (not applicable for control group) and the male they were paired with after the test.

All females used in mate choice testing were of a similar age (p=0.23). All of the adult males that females were allowed to choose between were of similar ages and masses at testing. Anogenital distance (AGD) likewise was similar across all males used in this study (Table 1). For Ambivalent females, the preferred male and non-preferred male are the male the female was paired with and not paired with, respectively.

Table 1: Mate choice animal demographics

	Female		Preferred Male			Non-preferred Male		
Group	Age at Test	Mass at Test (g)	Age at Test (d)	Mass at Test (g)	AGD at Test (mm)	Age at Test (d)	Mass at Test (g)	AGD at Test (mm)
Preferred	109 ± 1.8	35.0 ± 1.0	103 ± 6.8	41.1 ± 2.7	11.3 ± 0.6	94 ± 2.6	42.1 ± 1.6	11.1 ± 0.5
Non-preferred	101 ± 5.2	32.1 ± 1.4	83 ± 2.8	43.6 ±2.1	11.9 ± 0.6	86 ± 5.7	41.7 ± 2.0	11.2 ± 0.4
Ambivalent	98 ± 2.8	36.6 ± 1.1	89 ± 5.0	40.8 ± 1.6	11.8 ± 0.4	91 ± 3.2	41.4 ± 1.6	11.6 ± 0.3

Females that chose a male during the 2-hour mate choice test spent significantly more time with the preferred male than with the non-preferred male ($t_{(24)}$=8.63, p<0.01; Figure 6).

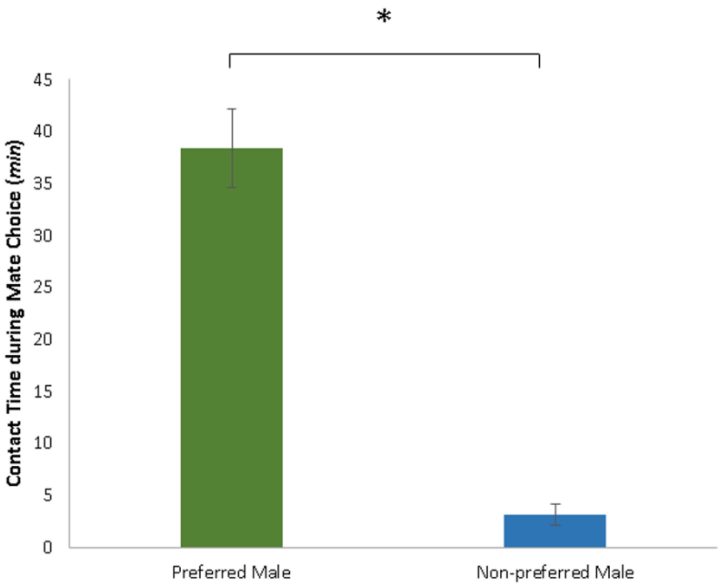

Figure 6: Contact time with preferred *vs.* non-preferred male during mate choice testing. All females that made a choice spent significantly more contact time with the preferred male than with the non-preferred male. Data are expressed as means ± SEM with * $p<0.01$.

To investigate the factors that influence mate choice, we tested whether or not females ultimately chose the first male they came into physical contact with. Females that made a choice had a shorter latency to contact with the preferred male as compared to that with the non-preferred male ($t_{(14)}=3.69$, $p<0.01$; Figure 7) revealing that females tended to prefer the first male that they encountered in the mate choice paradigm.

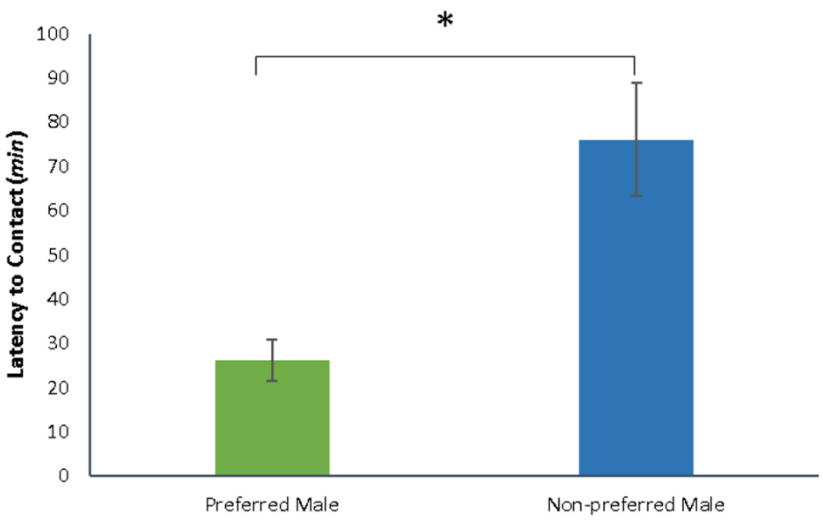

Figure 7: Latency to contact with preferred and non-preferred male. All females that chose a mate tended to choose the male they first came into physical contact with. Data in this figure are limited to those animals that made a clear choice. Data are expressed as means ± SEM with * $p<0.01$.

To clarify the behavior of ambivalent females *vs* those that chose a male, cage time (Figure 8) and cage crossings (Figure 9) were examined. For this comparison, cage time spent with the preferred male for the ambivalent females represents that of the male the ambivalent female was randomly paired with. Also, for this comparison, the non-preferred male cage time for ambivalent females is the time spent in the cage of the male the ambivalent female was ***not*** paired with. Females that chose a male spent more time in the cage of their preferred male than in either the center cage or in the cage of the non-preferred male ($p<0.01$; Figure 8). Ambivalent females spent more time in the center cage than in the cages of either male ($p<0.01$; Figure 11). While no effect of group ($F_{(1,41)}=2.78$, $p=0.10$) was evident for cage time, effect of location ($F_{(2,82)}=3.34$, $p<0.05$) and an interaction between location and time were present ($F_{(2,82)}=29.42$, $p<0.01$).

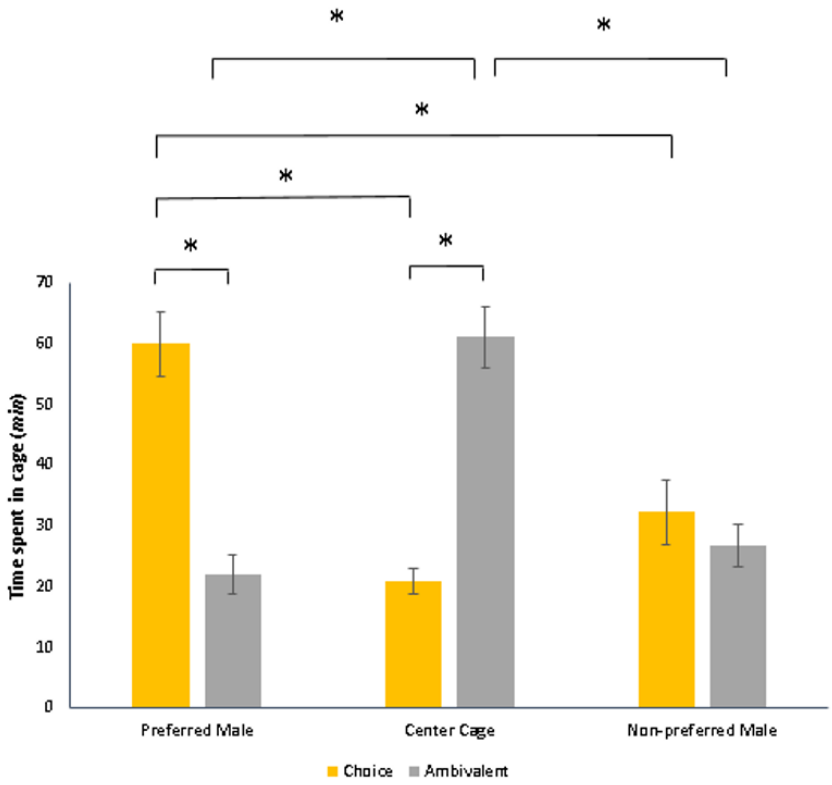

Figure 8: Cage time during mate choice testing. The 'Choice' label in this figure refers to females that made a choice during mate choice testing (includes both preferred and non-preferred groups of females, see Figure 5 for clarification). Females that made a choice spent significantly more time in the cage of their preferred male than in any other cage. Ambivalent females spent significantly more time in the center cage than in the cage of either male. For ambivalent females, the preferred male is the male the female was randomly partnered with, while the non-preferred male is the male that the female was not partnered with. Data are expressed as means ± SEM with * $p<0.01$.

While cage time indicates time spent in the proximity of a potential mate (either right or left cage) or alone (center cage), the number of crossings between cages provides an index of locomotor activity. During mate choice testing, all females entered the center cage more frequently than the cage of either male ($F_{(2,82)}=27.23$, $p<0.01$; Figure 11). No effect of group ($F_{(1,41)}=3.62$, $p=0.06$), nor an interaction between group and location were present ($F_{(2,82)}=1.58$, $p=0.21$;Figure 12).

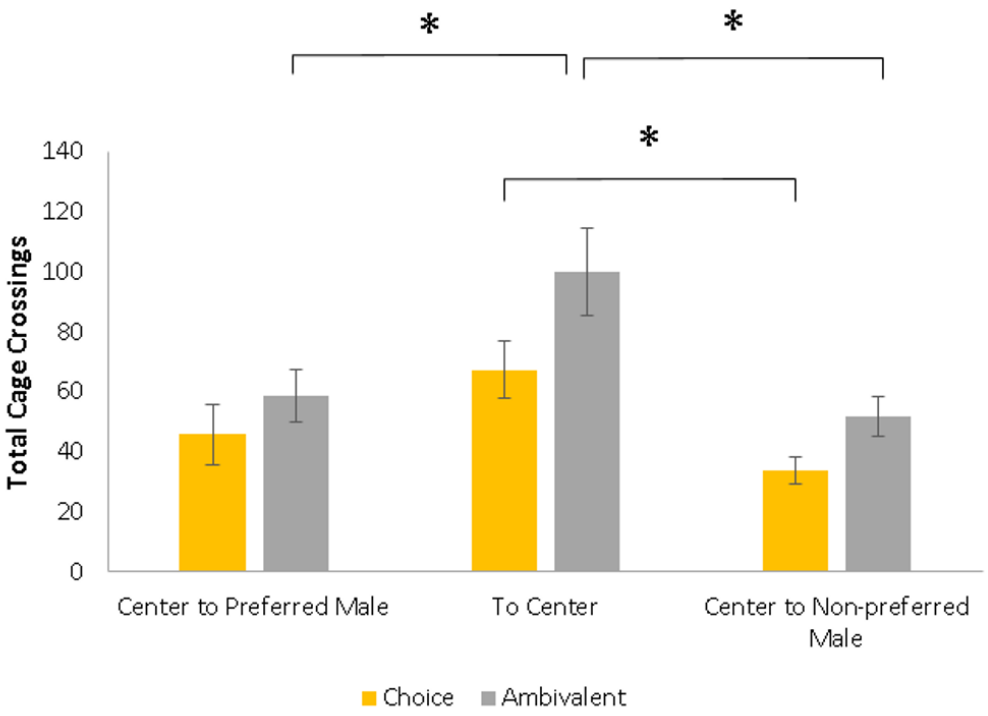

Figure 9: Cage crossings during mate choice testing. The 'Choice' label in this figure refers to females that made a choice during mate choice testing (includes both preferred and non-preferred groups of females, see Figure 5 for clarification). Data are expressed as means ± SEM with * $p<0.01$.

To further examine the behavior of the ambivalent females, the latency to contact, including that for the ambivalent females, was examined. For ambivalent females that did not make physical contact with either male during testing, this point was noted as 120 minutes (11/20 ambivalent females never made physical contact with a male), the maximum amount of time possible in the two-hour test. A main effect of group was present ($F_{(2,42)}=12.95$, $p<0.01$). When compared with females that made a choice (regardless of whether or not they ultimately were paired with their preferred male), ambivalent females took at least twice as long to engage in physical contact with either of the available males ($p<0.01$; Figure 10).

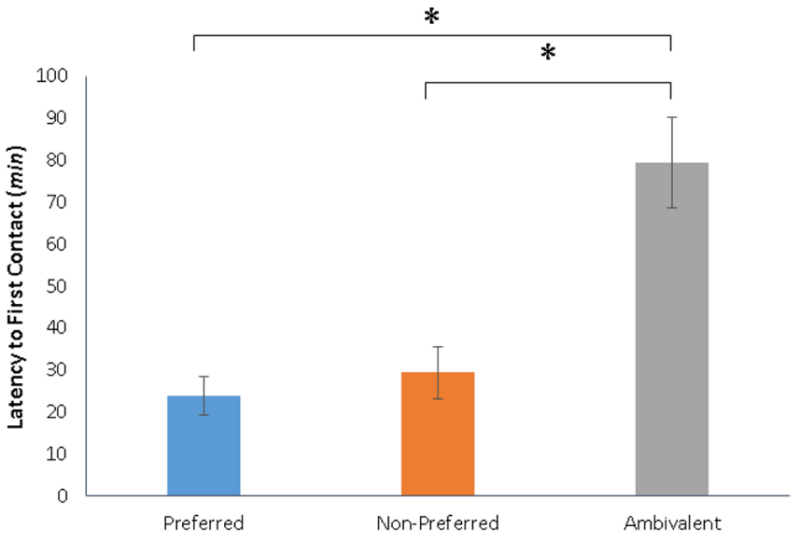

Figure 10: Latency to first contact in mate choice test by group. Ambivalent females took significantly longer than females that expressed a preference to engage in physical contact with either potential mate. Data are expressed as means ± SEM with * $p<0.01$.

While ambivalent females did not spend much time in physical contact with either male, entering the cage of either male allowed for investigation of their potential mates. Thus, the time spent in each cage of the mate choice apparatus during testing was analyzed. Ambivalent females spent more time in the unoccupied center cage than either of the cages containing males ($p<0.01$; Figure 11).

Between groups, preferred females spent more time in the left cage than did ambivalent females; non-preferred females spent more time in the right cage than did ambivalent females, and ambivalent females spent less time in the left cage than preferred females. In spite of the above differences, no main effect of group ($F_{(2,40)}=1.76$, $p=0.19$) was evident for cage time during mate choice testing.

Females that ultimately would be paired with their preferred male appeared to have a bias towards spending time in the left cage of the mate choice apparatus (effect of location ($F_{(2,80)}$=4.37, p<0.05); interaction between location and group ($F_{(4,80)}$=12.07, p<0.01)). This preference for the left cage was not attributed to either this cage lining up with the wall of the room in which the apparatus was located, nor was it associated with the left cage lining up with open space between the subject's testing apparatus and another subject's testing location. Half of the preferred males located in the left cage were in cages along the wall of the testing room, and the other half were in cages that did not line up with the wall.

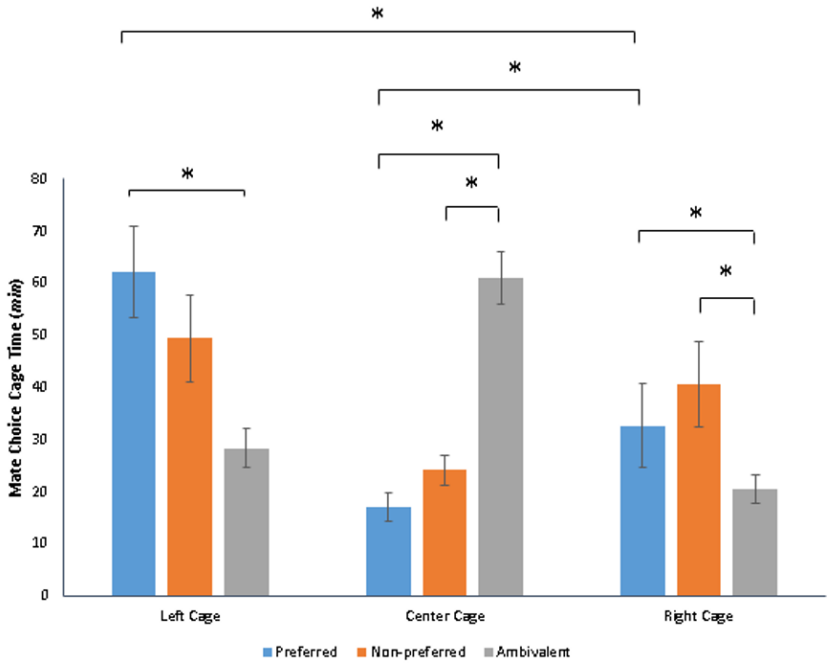

Figure 11: Time females spent in each cage during the mate choice test. Ambivalent females spent more time in the center cage than did preferred or non-preferred females, and less time in the right and left cages than did the preferred and non-preferred females. Data are expressed as means ± SEM with * p<0.01.

All experimental females had similar locomotor activity overall ($F_{(2,40)}$=2.53. p=0.09) and no interaction between group and location was evident ($F_{(4,80)}$=1.50, p=0.21). Ambivalent females made more crossings into the center cage in the mate choice apparatus during the two-hour test (Figure 12) than did their preferred and non-preferred counterparts, as revealed by a Tukey's HSD analysis of the effect of location found via ANOVA ($F_{(2,80)}$=20.25. p<0.01). This revealed that the increased time the ambivalent females spent in the center cage was not the result of any locomotor challenge.

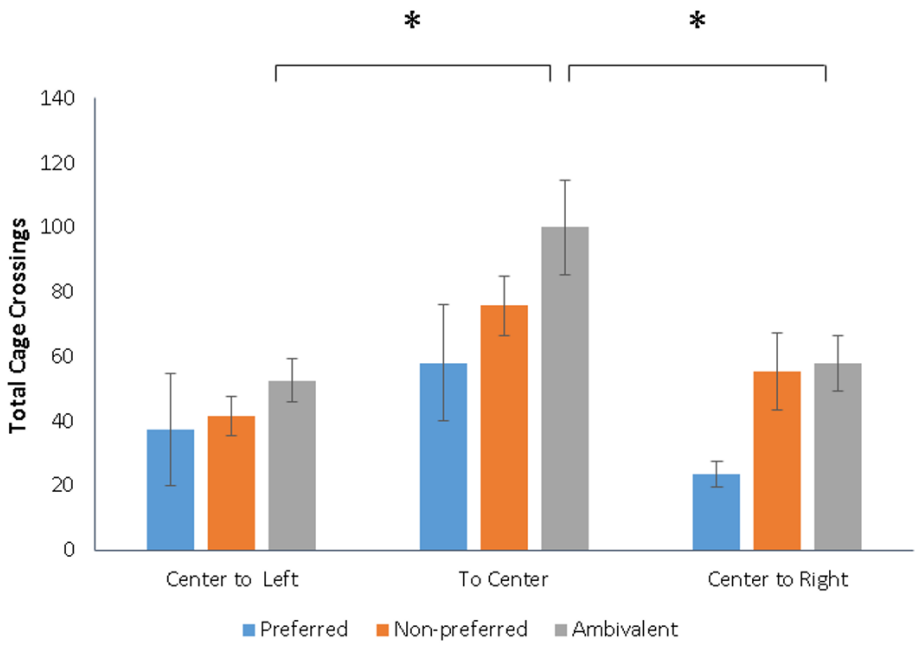

Figure 12: Total cage crossings during mate choice test by group. Ambivalent females crossed into the center cage more than into the right or left cages, while preferred and non-preferred females made similar numbers of crossings across all cages. Data are expressed as means ± SEM with * p<0.01.

Partner Preference Testing

14 days after placement with their mates, either the male or female of each pair was exposed to a 3-hour partner preference test. Overall, regardless of sex ($F_{(1,57)}$=1.09, p=0.30) or group ($F_{(3,57)}$=0.95, p=0.42), subjects spent significantly more time with their partner than with the stranger, as revealed by an effect of location ($F_{(1,57)}$=123.04, p<0.01). No interaction between group and sex ($F_{(3,57)}$=1.21, p=0.31) was evident in contact time during partner preference testing. No effect of location and group ($F_{(3,57)}$=1.54, p=0.21), location and sex ($F_{(1,57)}$=0.69, p=0.41), or any interaction between location, group and sex ($F_{(3,57)}$=2.01, p=0.12) was evident. Males paired with the ambivalent females were an exception, spending roughly the same amount of contact time with both the partner and stranger animal (p=0.88; Figure 13).

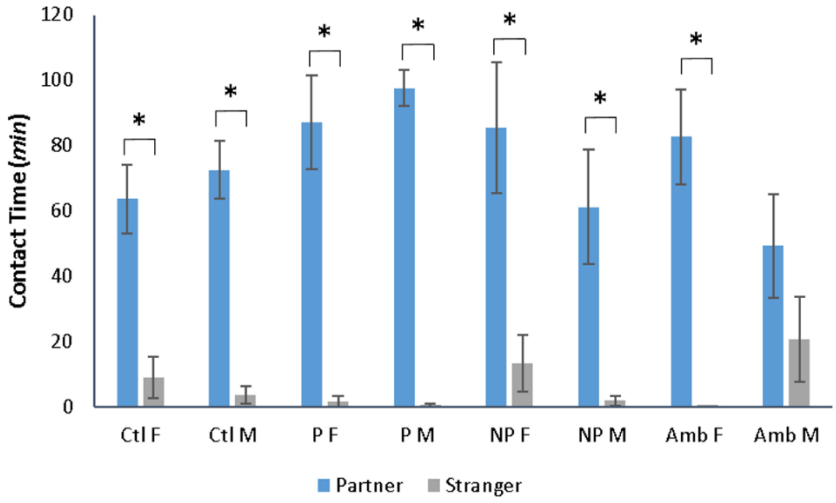

Figure 13: Contact time during partner preference testing. With the exception of ambivalent males, all animals, regardless of sex or group, spent more time with the partner than with the stranger. Groups include control females (Ctl F), control males (Ctl M), preferred females (P F), preferred males (P M), non-preferred females (NP F), non-preferred males (NP M), ambivalent

females (Amb F), and ambivalent males (Amb M). Data are expressed as means ± SEM with * $p<0.01$.

In addition to contact time with the partner or stranger animal in the partner preference test, the amount of time spent in each cage was examined. A main effect of location was present ($F_{(2,108)} = 133.21$, $p<0.01$). Tukey's post-hoc analysis revealed that during this test, all animals spent more time in the partner cage than in the center or stranger cages ($p<0.01$). The exceptions to this observation were the non-preferred and ambivalent males, which spent similar amounts of time in all cages. The preference for the center cage by ambivalent females during mate choice testing was no longer present 14 days after pairing (Figure 14).

An interaction of location and sex was present ($F_{(2,108)}=5.46$, $p<0.05$). No interaction between location and group was present ($F_{(6,108)}=0.59$, $p=0.73$), nor any interaction between location, sex, and group ($F_{(6,108)}=1.08$, $p=0.38$). No effect of sex ($F_{(1,54)}<0.01$, $p=0.99$) or group($F_{(3,54)}=0.52$, $p=0.67$) was seen, nor an interaction between the two ($F_{(3,54)}=1.90$, $p=0.14$).

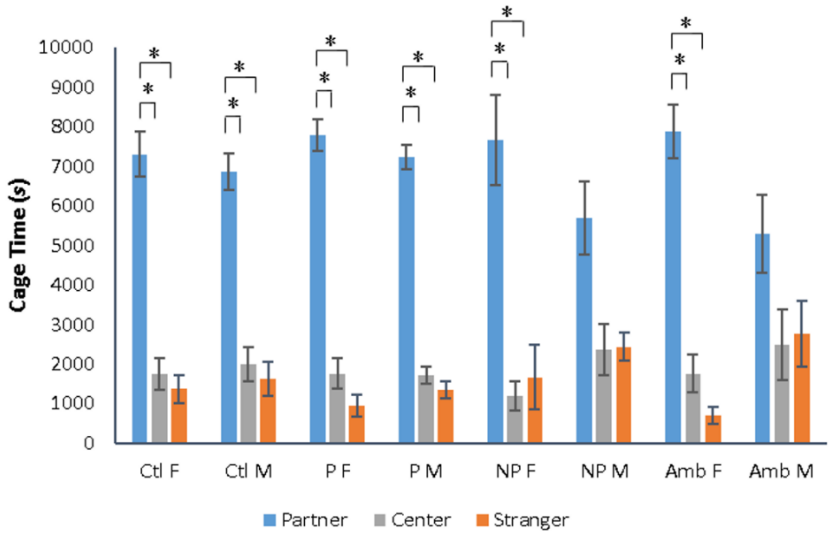

Figure 14: Cage time during partner preference testing. All groups, except for non-choice males and ambivalent females, spent more time in the cage of their partner than in the center or stranger cage. Groups include control females (Ctl F), control males (Ctl M), preferred females (P F), preferred males (P M), non-preferred females (NP F), non-preferred males (NP M), ambivalent females (Amb F), and ambivalent males (Amb M). Data are expressed as means ± SEM with * $p<0.01$.

Cage Crossings

During the partner preference test, ambivalent females crossed into the center cage more than into the stranger cage (Figure 15). Otherwise no differences in cage crossings were seen, regardless of group ($F_{(3,53)}=0.48$,$p=0.70$) or sex($F_{(1,53)}=0.03$, $p=0.87$). An effect of location was present ($F_{(2,106)}=27.5$, $p<0.01$). No interaction between group and sex, ($F_{(3,53)}=1.24$, $p=0.30$), location and group ($F_{(6,106)}=0.77$, p=0.59), location and sex ($F_{(2,106)}=0.22$, $p=0.81$), or between location, sex, and group ($F_{(6,106)}=0.81$, $p=0.56$) were evident.

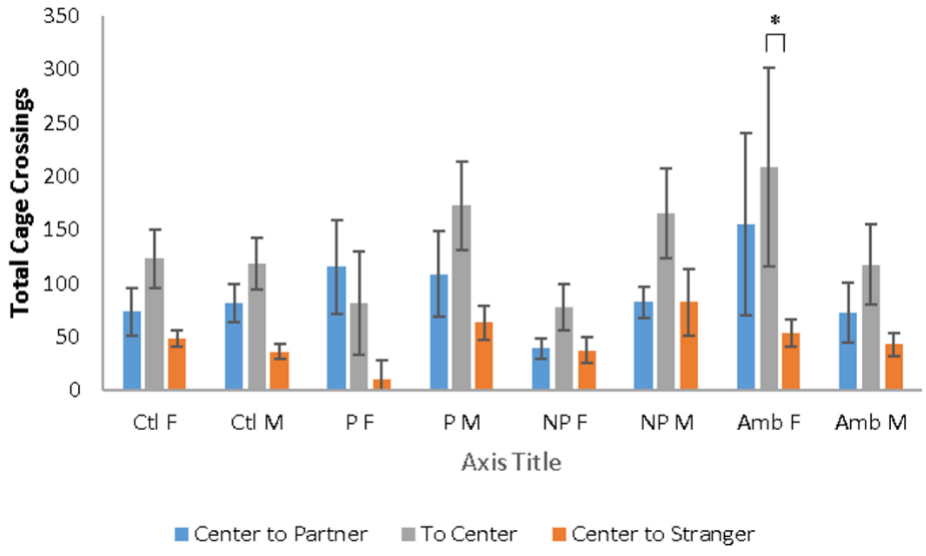

Figure 15: Cage crossings during partner preference testing. Ambivalent females crossed into the center cage more than into the stranger cage during partner preference testing. Groups include control females (Ctl F), control males (Ctl M), preferred females (P F), preferred males (P M),

non-non-preferred females (NP F), non-preferred males (NP M), ambivalent females (Amb F), and ambivalent males (Amb M). Data are expressed as means ± SEM with * $p<0.01$.

Latency to Pregnancy and Litter Size

All groups had a similar latency to pregnancy ($F_{(3,56)} = 0.61$, $p = 0.61$), with most litters being born between 27-29 days after pairing. All litters consisted of 3-4 pups. No significant differences between groups in regard to overall litter size ($F_{(3,53)}=2.66$, $p=0.06$) were found.

DISCUSSION

Mate Choice Behavior

Female prairie voles are naturally induced to estrous by exposure to an unrelated conspecific male, specifically exposure to chemosignals in urine of a male (Richmond and Conaway, 1969). Other researchers have noted that 1 hour of exposure to bedding soiled by an unrelated male is sufficient to double uterine mass, an indication that sexual receptivity has been induced (Carter *et al.*, 1980). In order to avoid prematurely inducing estrous by overexposing the females to potential mates, it was necessary to determine the minimal amount of time a female requires to choose a mate in the mate choice paradigm. Females that made a choice were likely to do so between 90 and 120 minutes of testing (Figure 4).

Physical indices such as body mass and anogenital distance were assessed for each male in the mate choice paradigm. No differences were found for the anogenital distances or masses of males within or between groups. This implies that females used some other factor to select a partner.

Most females in this experiment spent more time in physical contact with, and thus "choosing", the male they first came into physical contact with (Figure 7). This was not surprising as these animals often follow an opportunistic mating strategy, partnering with the first non-related conspecific they encounter (Getz *et al*., 2004).

Ambivalent females spent time in all available cages, but spent more time in the central cage (Figure 11) and displayed a greater latency to engage in contact with one of the males than did females that selected a partner during mate choice testing (Figure 10). The total number of cage crossings for ambivalent females was similar to that of females that made a choice, indicating that these animals were not less active (Figure 12).

Partner Preference behavior

After two weeks of pairing, almost all animals, regardless of mate choice grouping or sex, showed a preference for their partner over a stranger animal. The exception to this was males paired with females that were ambivalent during mate choice testing. Instead of showing a preference for their partner, these males did not show a preference for either the stranger or partner (Figure 13). These results were unexpected, as previous studies have shown that pregnancy status has an impact on partner preference formation in males. All females used in partner preference testing were pregnant by the 14-day partner preference testing time point. Curtis (2010) showed that male prairie voles with partners that became pregnant within 48-72 hours after pairing expressed a partner preference, whereas males with partners that became pregnant after this timeframe did not. In these experiments, all of the females achieved pregnancy at roughly 6-8 days post pairing, almost twice the timeframe found to be necessary for males to form a pair bond in the Curtis 2010 experiments. With this in mind, it is surprising that the males paired with ambivalent females were the only males to lack a partner preference at 14 days post-

pairing. In light of this information, the results imply that some aspect other than pregnancy status is the impetus for this lack of social bonding.

While the males partnered with ambivalent females did not show a partner preference, the previously ambivalent females showed a preference for their male partners. This may be due to pregnancy status, as pregnant prairie vole females are known to prefer their mate over a stranger male (Getz *et al*. 1981).

CHAPTER VI

ENDOCRINE MEASURES OF MATERNAL STRESSORS BASED ON PREGNANCY STATUS

INTRODUCTION

Pregnancy is known to be a physiological stressor to the mother (Geller, 2004) and stress activates the HPA axis. A commonly used indicator of HPA activation is the measurement of glucocorticoids, such as corticosterone, in circulation. Rising estrogen levels are an indication of estrous induction in virgin female prairie voles. Both the onset of estrous after pairing and any post-pairing pregnancy that might result were points of interest in this study. To better track progress of sex specific hormonal changes resulting from pairing and pregnancy, 17-β estradiol measurements were taken via ELISA from fecal samples collected at time points throughout pairing and pregnancy. Because collection of blood plasma samples and excessive handling may be stressful to animal subjects, the less invasive method of analyzing endocrine changes in fecal samples was optimized for use in these experiments (described in Chapter 4 of this dissertation).

It was expected that as pregnancy progressed, so would estrogen levels, and, as an indicator of the anticipated increase of stress during this time, corticosterone levels would also rise.

METHODS

Female fecal samples collected before and immediately after mate choice testing, as well as on days 1,2,3,5,7,9,12,15,18,2 and 21 after pairing, were prepared as described in the general methods section of this dissertation. Those fecal extracts were used in commercially available ELISA immunoassays designed to measure 17-β estradiol or corticosterone quantities respectively per manufacturer instructions. Resulting data were analyzed via repeated measures ANOVA using Statistica software.

Fecal Extraction Protocol

To efficiently extract the hormones of interest for these experiments, a methanol extraction protocol was used (Larson, et. al., 2013). Approximately 0.1g of fecal pellet was dried via vacuum for 1hr at ~43˚C in a SpeedVac (Savant Instruments). After drying, each sample was weighed to assess dry mass, then 0.5mL of 90% methanol was added to each sample. Samples were then placed on a shaker at medium speed for 1 hour. Next, samples were sonicated on the lowest available setting, followed by the addition of another 0.5mL methanol and ~ 15 seconds of vortexing. The resulting slurry was centrifuged at 2.5 x 1000 RPM for 20 minutes and 600uL of the supernatant was collected into a new Eppendorf tube. An additional 1 mL of methanol was added to the remaining sample residue, vortexed, and centrifuged at 2.5 x 1000 RPM for 20 minutes. Again, 600uL of supernatant was collected and combined with the previous aliquot collected, for a total sample of ~1200uL (1.2 mL) from two collections for each fecal sample. These methanol solutions were stored at -80˚C until needed for ELISA.

ELISA preparation and analysis

Results from methods development confirmed that the presence of methanol in the sample can confound ELISA results. To avoid this concern, methanol was removed prior to ELISA analysis by drying under heated vacuum, followed by reconstitution in a volume of assay buffer either equal to the total sample volume originally dried (17-β estradiol), or at a 1:10 dilution (corticosterone). Samples examined for corticosterone were diluted to allow samples to fall within the standard curve of the ELISA plate. After reconstitution in assay buffer, each sample was stored at 4˚C overnight prior to ELISA assay.

RESULTS

17 β–estradiol ELISAs

All females, regardless of group, showed an increase in fecal estrogen levels over time ($F_{(11,484)}$=69.60, p<0.01), with some groups showing a marked increase at specific time points ($F_{(33,484)}$=1.60, p<0.05). Ambivalent animals showed a significant increase in estrogen relative to baseline by 5 days after pairing. Preferred and non-preferred animals differed from their respective baselines by 9 days after pairing, and control animals showed a significant increase by the 18th day after pairing. Despite these interactions between group and time, no significant effect of group alone was present ($F_{(3,44)}$=2.43, p=0.08; Figure 16).

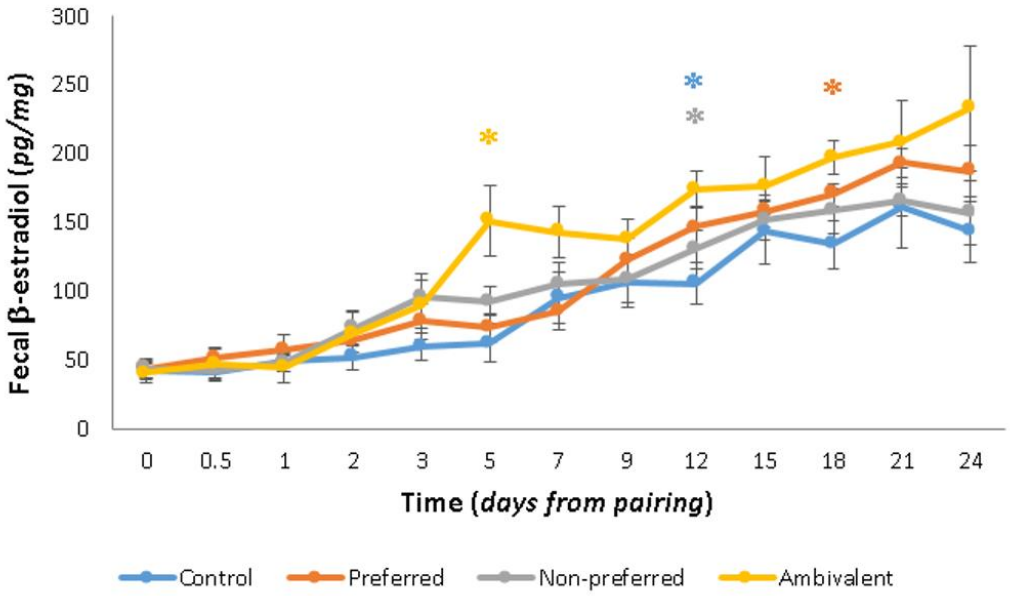

Figure 16: Changes in 17 β-estradiol over the time course of the study. Fecal 17 □-estradiol measured via ELISA from samples taken before, immediately after, and on days 1, 2,3,5,7,9,12,15,18,21, and 24 after either mate choice test (preferred, non-preferred and ambivalent) or pairing with randomly selected male (control). All groups showed a significant increase in fecal 17 β-estradiol from baseline. Significance notations (*) indicate the *first* point of change from baseline. For all groups, significant differences from baseline continued from the first point of significance to the end of the timeline. Data are expressed as means ± SEM with*p<0.05 when compared to first sample taken within the same group.

Corticosterone ELISAs

Samples used for fecal estrogen measurements were also used for corticosterone analysis. All animals showed elevated levels of this stress hormone for the pre- and post- samples collected at the beginning of the time course ($F_{(11,484)}$=6.43, p<0.01;Figure 17). Corticosterone levels quickly decreased with subsequent samples. While no differences were seen between groups ($F_{(3,44)}$=1.98, p=0.13), all samples except for those on day 1 and day 9 differ significantly in fecal corticosterone than those collected immediately after mate choice testing ('post' samples). No interaction between time and group was present ($F_{(33,484)}$=1.26, p=0.16).

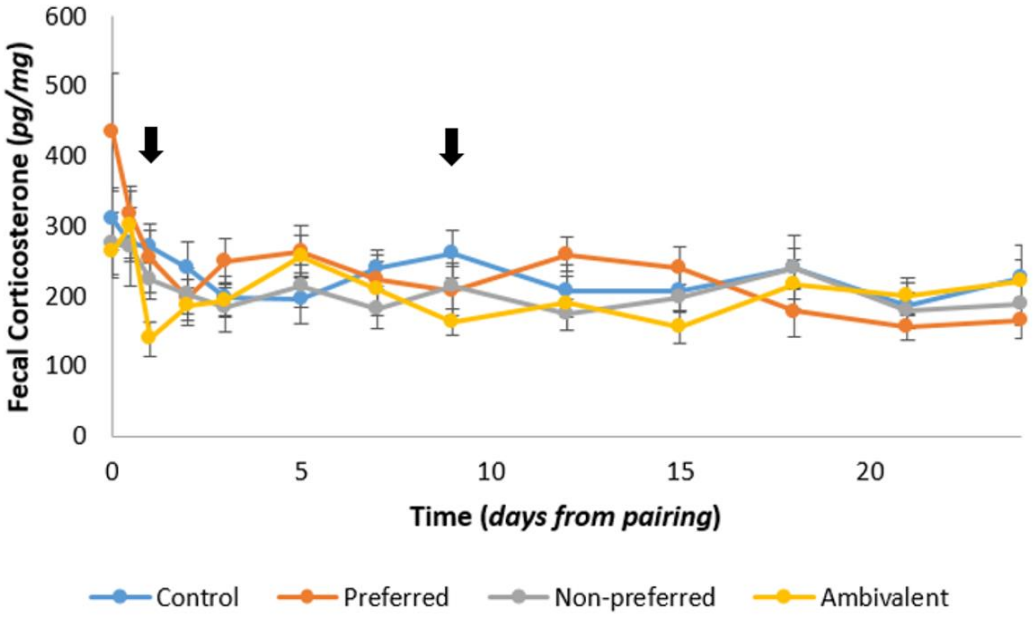

Figure 17: Changes in fecal corticosterone over the time course of the study. Fecal corticosterone measured via ELISA from samples taken before, immediately after, and on days 1, 2,3,5,7,9,12,15,18,21, and 24 after either mate choice test (preferred, non-preferred and ambivalent) or pairing with randomly selected partner (control). All females showed a significant decrease in fecal corticosterone after pairing with a male. Arrows indicate the only time points that do *not* show a significant change ($p<0.05$) from immediately after mate choice testing (or random pairing, as appropriate per group).

Table 2: Correlations between fecal 17 β-estradiol and fecal corticosterone levels

group	F-stat	df	*p*-value
Control	7.88	152	0.01
Preferred	12.88	152	<0.01
Non-preferred	3.5	149	0.06
Ambivalent	0.04	151	0.84

To parse out potential correlations between stress measures and fecal estrogen concentrations, regressions were run for these measures from each set of samples. Significant correlations were found between corticosterone and 17 β-estradiol levels for the control and preferred females, with the non-preferred females showing a strong trend (p=0.06). Ambivalent females however, showed no correlation between corticosterone and estrogen levels (Table 2).

DISCUSSION

All females, regardless of group, showed increased levels of fecal estrogen over time. These results were as expected as all animals used for hormonal analysis became pregnant and gave birth over the course of the study. The ambivalent females showed fecal estrogen levels differing from baseline 5 days after pairing, while the females paired with their preferred or non-preferred partner did not display a significant increase in EB from baseline until 12 days post-pairing. The control animals, which were not exposed to the mate-choice paradigm, did not show a statistically significant change from baseline until 18 days after pairing. Delayed increase from baseline estrogen levels in all groups except ambivalent females is surprising as ovulation, a period associated with increased estrogen levels, takes place roughly 12 hours after first copulation (Roberts *et al.*, 1999). Behavioral estrous occurs roughly within 2 days after initial introduction to a possible mate (Roberts *et al.*, 1998).

Despite time differences for the rise of estrogen levels above baseline, no significant differences were found in latency to parturition, or in litter size. The ambivalent females did, however show a trend of producing larger litters (p=0.06), which may account for their qmore rapidincrease in estrogen levels. Higher estrogen levels during pregnancy are associated with multiple births in other species such as humans (Póvoa *et al.*, 2018), pigs (Edgerton *et al.*, 1971), and small tail han sheep (Bi *et al.*, 2005).

CORT levels were similar for all groups at baseline measurements taken prior to mate choice testing. By day two of pairing, CORT levels for all paired females had decreased significantly, regardless of mate choice grouping. This result was the reverse of what was expected. Pregnancy is known to be a stressor (Roesch *et al*., 2004) and increases in CORT are associated with the stress response (Denenberg, 1969). Therefore, it was anticipated that CORT levels would increase over the course of the study, along with estrogen levels.

The decreased CORT levels after baseline shown in this experiment may be the result of habituation, as repeated exposure to a stressor such as electric shock often results in a decreased response to that stressor (Bassett *et al.*, 1973; Pittman *et al*., 1990). One opportunity for habituation in this experiment would be the fecal collection method. Initial separation from the female's home cage for baseline fecal collections may have been more stressful than subsequent fecal collections, as up until that time, animals had experienced minimal handling and virtually no isolation. Subsequent CORT levels may have dropped as subjects became more familiar with the protocol. This may have been avoided by habituating the females to the fecal collection protocol for roughly 3 days prior to mate choice testing.

While examination of fecal estrogen and CORT separately did not shed much light on possible stress differences between groups, correlations between these two hormonal measures did reveal differences between the mate pairing groups. Females paired with the preferred partner and females from the control group showed a positive correlation between estrogen and CORT levels, while the females paired with their non-preferred partners showed a strong trend (p=0.06) towards having a positive correlation. These correlations indicate that as pairing and pregnancy progress in female prairie voles, so does their endogenous estrogen and stress-hormone levels. This is supported by Ochedalski *et al*. (2007) who found that plasma CORT increased with elevated estradiol levels. Females that were ambivalent during mate choice testing exhibited no correlation between estrogen and CORT numbers. This lack of correlation is likely driven by the

earlier spike in estrogen seen in these animals, which may be linked to their slightly, but not significantly, higher number of pups per litter.

Overall, the trends in estrogen levels for these animals were consistent with pregnancy status throughout the course of the experiment. While the CORT levels were not as initially expected, they followed a reasonable pattern that may reflect habituation to handling throughout the experiment.

CHAPTER VII

EFFECTS OF FEMALE MATE CHOICE ON ANXIETY-LIKE BEHAVIOR IN OFFSPRING

INTRODUCTION

External and internal events that cause stress for the mother may impact development of her offspring. Maternal illness during certain states of embryological development is known to impact fetal development (Capra *et al.*, 20103). One known impact of maternal stress is alteration in HPA axis development of her offspring, with many developing anxiety-like behaviors (reviewed in Maniam *et al.*, 2014). Adolescence is a time of particular sensitivity to these physiological challenges (Niehaus *et al.*, 2019), with adolescent offspring of mothers who experienced stressful events during pregnancy being more likely to develop and exhibit anxiety and depression.

In this chapter, I examined anxiety-like behavior of the offspring of dams used in the mate choice testing paradigm. The elevated plus maze and the open field were used for these assessments, both of which are well-established behavioral tests for the assessment of anxiety-like behaviors in laboratory rodents.

METHODS

Offspring of females from each group used in the mate choice paradigm were exposed to behavioral testing in both the elevated plus maze and in the open field apparatus. Offspring were tested as juveniles only, adults only, or as juveniles then again as adults. Details of testing and specific age ranges for each group were as described in the general methods of this dissertation. Data collected was analyzed via 1-way or repeated measures ANOVA, as appropriate.

RESULTS

Elevated Plus Maze

Weaned offspring from each experimental pairing were co-housed with a same-sex sibling at weaning and used in behavioral experiments to measure anxiety-like behavior. Both an open field test (OF) and an elevated plus maze test (EPM) were performed on offspring at each testing session. From each sibling pair, one animal was tested both within a week of weaning (weanling), and again at roughly 60 days of age (adult). The remaining sibling was tested only as an adult. For analysis, the results of all of these tests were grouped by: 1) Results for weanling animals, 2) Adults exposed to the testing paradigm for the first time, 3) As subjects that were tested both as weanlings and again as adults (twice-tested animals).

Pups tested as weanlings

A main effect of location was present for time spent by weanlings in the EPM ($F_{(1,44)}$=68.23, p<0.01). Control, non-preferred, and ambivalent weanling offspring spent more time in the open arms than in the closed arms of the maze (p<0.01; Figure 18). This behavior was

not seen from preferred pups. No effect of group ($F_{(3,44)}$=1.46, p=0.24; Figure 18) or any interaction between location and group ($F_{(3,44)}$= 0.86, p=0.47) was present. No groups displayed evidence of impairments in locomotor activity in the elevated plus maze, as no effect of group was found for distance traveled ($F_{(3,44)}$=1.27, p=0.30) nor for velocity ($F_{(3,44)}$=1.22, p=0.32).

When weanlings were analyzed for possible sex differences, both male and female offspring showed similar measures of locomotor activity ($F_{(1,46)}$=0.05, p=0.82 and $F_{(1,46)}$=0.08, p=0.77 for distance traveled and velocity, respectively). There was no effect of sex on time spent in the open *vs* closed arms of the maze ($F_{(1,46)}$=0.01, p=0.91). Both male and female weanlings spent more time in the open than the closed arms of the EPM ($F_{(1,46)}$=75.90, p<0.01). No interaction between sex and location was found ($F_{(1,46)}$= 1.07, p=0.31).

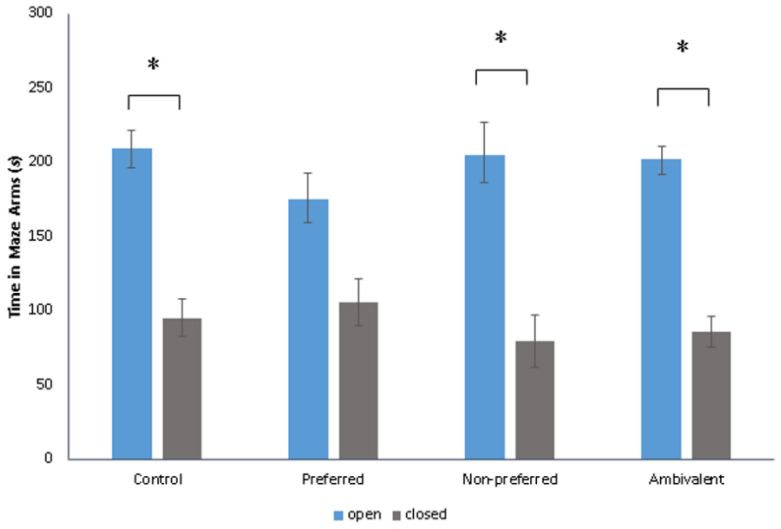

Figure 18: Time in open *vs.* closed arms of EPM for weanling offspring. All groups, *except* the preferred weanlings spent significantly more time in the open arms than the closed arms of the EPM. Data are expressed as means ± SEM with * p<0.01.

Pups tested as adults

When tested as adults, pups from ambivalent females spent more time in the open arms than in the closed arms of the EPM ($p<0.01$), while other groups did not (Figure 19). An effect of location was present both when analyzed by group ($F_{(1,45)}=16.05$, $p<0.01$) and by sex ($F_{(1,47)}=25.85$, $p<0.01$) revealing that both males and females spent more time in the open than the closed arms of the maze. As seen with their weanling counterparts, adult offspring newly exposed to EPM testing showed no effect of group ($F_{(3,45)}=0.24$, $p=0.87$), nor of sex ($F_{(1,47)}=3.21$, $p=0.08$) for time spent in the open vs closed arms. No interaction between location and group ($F_{(3,45)}=0.80$, $p=0.50$) or location and sex ($F_{(1,47)}=0.11$, $p=0.74$) was present.

Adult offspring showed no group differences for locomotive measures ($F_{(3,45)=}1.25$, $p=0.30$ and $F_{(3,45)}= 1.19$, $p=0.32$ for distance traveled and velocity, respectively). Additionally, no differences for these measures were found between the sexes ($F_{(1,47)}=0.02$, $p=0.90$ for distance traveled and $F_{(1,47)}=0.03$, $p=0.86$ for velocity).

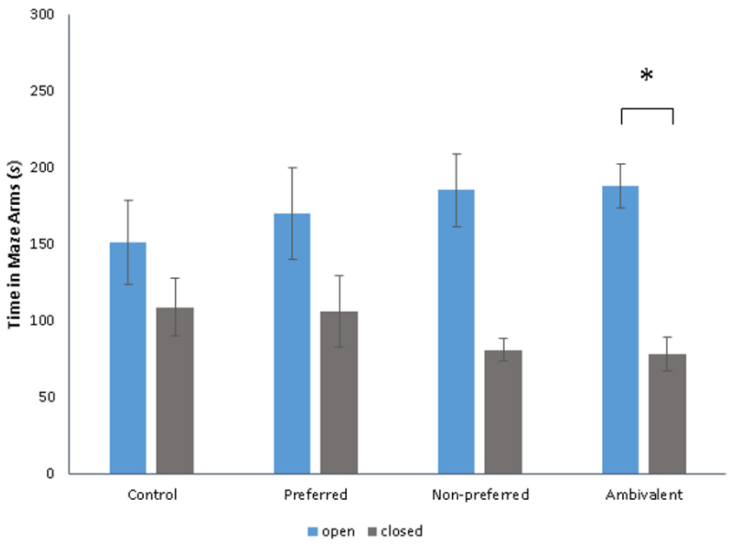

Figure 19: Time in open *vs* closed arms of EPM for pups tested as adults. Only adult offspring of ambivalent females spent more time in the open arms of the EPM, while the adult offspring of control, preferred, and non-preferred females did not exhibit any significant

differences in time spent in the open *vs* closed arms. Data are expressed as means ± SEM with * $p<0.01$.

Offspring tested as both weanlings and as adults

Adult offspring from control, preferred, and ambivalent females tested both as weanlings and again as adults spent more time in the open than the closed arms of the EPM during testing both as weanlings and as adults ($F_{(1,32)}=18.48$, $p<0.01$; Figure 20). This was true regardless of sex ($F_{(1,34)}=30.06$, $p<0.01$). Offspring of females paired with the non-preferred males spent more time in the open than the closed arms as weanlings, but not as adults. The offspring of control females transitioned from spending more time in the open than the closed arms as weanlings, to spending similar amounts of time in both arms as adults. Adult offspring of control females also spent less time in the open arms and more time in the closed arms than the adult offspring of choice females did. Additionally, the adult offspring of control females spent more time in the closed arms than adult offspring of ambivalent females did. Adult offspring of preferred females spent less time in the closed arms than the adult offspring of non-preferred females, and the adult offspring of non-preferred females spent more time in the closed arms than the adult offspring of ambivalent females did (interaction of time and location($F_{(1,32)}=12.28$, $p<0.01$),interaction between time, location and group ($F_{(3,32)}=3.05$, $p<0.05$)). There was no effect of group ($F_{(3,32)}=0.34$, $p=0.80$), nor of time ($F_{(1,32)}=1.02$, $p=0.32$), nor an interaction between group and time($F_{(3,32)}=0.61$, $p=0.61$) for time spent in the open *vs* closed arms of the EPM by animals tested both as weanlings and again as adults.

Both male and female weanling offspring spent more time in the open than the closed arms, but no differences were present for time spent in the open rather than closed arms as adults for either sex ($F_{(1,34)}=4.51$, $p<0.05$). No interaction between time, location, and sex was found ($F_{(1,34)}=0.65$, $p=0.43$). No effect of sex ($F_{(1,34)}=0.11$, $p=0.74$), time ($F_{(1,34)}=2.20$, $p=0.15$) or

interaction of time by sex ($F_{(1,34)}$=0.06, p=0.81) was present. No interaction between location and group was seen ($F_{(3,32)}$=1.02, p=0.40), or any interaction between location and sex ($F_{(1,34)}$=0.42, p=0.52).

As for locomotor behavior in the EPM, the offspring of preferred and ambivalent females traveled a greater distance as weanlings than as adults. The adult offspring of non-preferred females that were tested both as weanlings and again as adults traveled a greater distance in the EPM than the adult offspring of ambivalent females that were tested both as weanlings and again as adults. An interaction between time and group ($F_{(3,34)}$ = 3.39, p=0<0.05) was present. No effect of group ($F_{(3,34)}$=0.29, p=0.83), nor of time ($F_{(1,34)}$ = 2.51, p= 0.122) was present. Further analysis did not reveal an effect of sex ($F_{(1,36)}$ = 0.11, p= 0.74), nor an interaction between sex and time ($F_{(1,36)}$ = 0.89, p = 0.35), while an effect of time was evident($F_{(1,36)}$ = 5.51, p<0.02).

Regarding velocity, no effect of group ($F_{(3,34)}$ = 0.56, p=0.65) was seen, nor an effect of sex ($F_{(1,36)}$ < 0.01; p= 0.99). An effect of time was present when animals were viewed by group ($F_{(1,34)}$= 4.25, p<0.05) and again when viewed by sex ($F_{(1,36)}$ = 8.29, p<0.01), but no interaction between time and group($F_{(3,34)}$ = 1.64, p=0.20), nor one of time by sex ($F_{(1,36)}$ = 1.09, p=0.30).

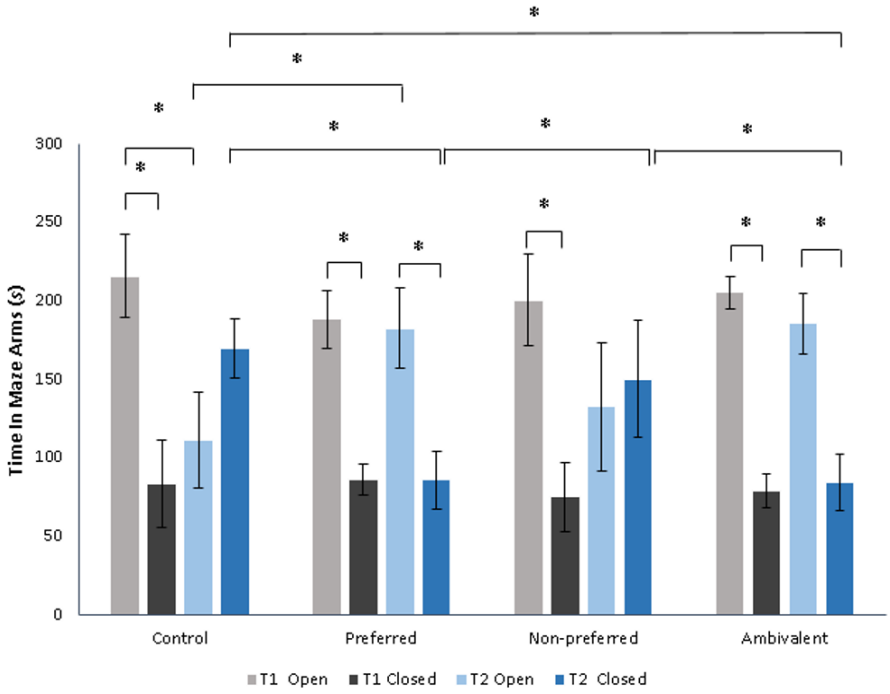

Figure 20: Time in open *vs* closed arms of EPM between groups across time. Time spent by animals as weanlings (T1) and adults (T2) in the open and closed arms of the EPM. Offspring of both preferred and non-preferred females spent more time in the open than the closed arms of the EPM as both weanlings and adults. Offspring of control and non-preferred females showed more variability in their behavior over time. Data are expressed as means ± SEM with * $p<0.01$.

Open Field Data

Pups tested as weanlings

All pups exposed to the open field test, regardless of maternal experimental grouping or sex, spent more time along the edges of the open field arena than they did in the center (Figure 21). An effect of location was found ($F_{(1,52)} = 1878.72$, $p < 0.01$). No interaction between location and group ($F_{(3,52)} = 0.20$, $p=0.90$), between location and sex ($F_{(1,52)} = 0.45$, $p=0.50$), nor an interaction between location, group, and sex ($F_{(3,52)} = 0.23$, $p=0.87$) was found.

The corners of the open field maze were also selected as locations of interest for analysis. All weanlings, regardless of maternal grouping ($F_{(3,52)} = 0.36$, $p=0.78$) or sex ($F_{(1,52)} = 0.01$, $p =$

0.91), spent roughly the same amount of time in the corners of the open field. No interaction between group and sex was present ($F_{(3,52)} = 1.91$, p=0.14).

No differences were seen between group or sex for locomotor measures. Recently weaned offspring showed no effect of sex ($F_{(1,52)} = 0.05$, $p = 0.83$), group ($F_{(3,52)} = 0.62$; p=0.61), nor an interaction between the two ($F_{(3,52)} = 0.25$, p=0.86) for distance traveled in the OF. Likewise, no effect of sex ($F_{(1,52)} = 0.04$, p=0.85), group ($F_{(3,52)} = 0.57$, p=0.64), nor an interaction between sex and group ($F_{(3, 52)} = 0.23$, p=0.88) was seen in the velocities traveled in the OF.

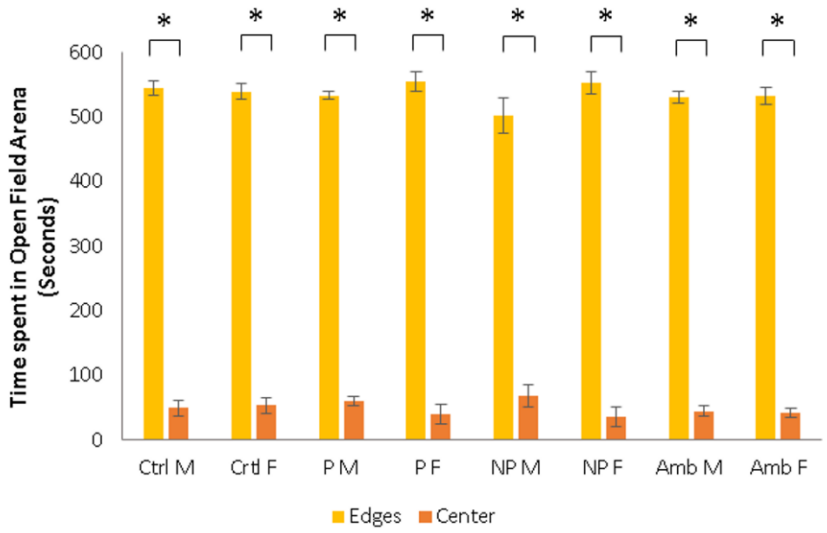

Figure 21: Time spent in center *vs.* edges of open field (OF) by weanling offspring. All groups, regardless of sex or maternal grouping, spent more time along the edges, rather than in the center of the OF. Groups represented are offspring of control females (Ctrl F), control males (Ctrl M), preferred females (P F), preferred males (P M), non-preferred females (NP F), non-preferred males (NP M), ambivalent females (Amb F), and ambivalent males (Amb M). Data are expressed as means ± SEM with * p<0.01.

Pups tested as adults

All adult offspring experiencing a first exposure to the open field spent more time along the edges of the open field than in the center (Figure 22) regardless of group ($F_{(1, 56)} = 2329.38$, $p < 0.01$) or sex ($F_{(1,58)} = 3043.15$, $p<0.01$). No interaction between group and location was found ($F_{(3, 56)} = 0.40$, $p= 0.75$), nor between sex and location ($F_{(1, 58)} = 0.42$, $p =0.52$). No effect of group ($F_{(3, 56)} = 0.98$, $p =0.41$) or of sex ($F_{(1, 58)} = 0.017$, $p=0.79$) was present for time spent along the edges rather than the center of the OF.

As seen when the offspring were tested as weanlings, no adult offspring tested for the first time in the OF showed any differences in time spent in the corners, regardless of group ($F_{(3,56)} = 1.25$, $p=0.30$) or sex ($F_{(1,58)} = 0.48$, $p=0.49$). These animals also showed no differences in locomotor measures, with no effect of group ($F_{(3, 56)} = 0.92$, $p=0.44$) or sex ($F_{(1, 56)} = 0.31$, $p=0.58$) on distance traveled in the testing apparatus. There was also no effect of group ($F_{(3, 56)} = 0.85$, $p=0.47$) or sex ($F_{(1, 56)} = 0.31$, $p= 0.58$) on velocity in the open field.

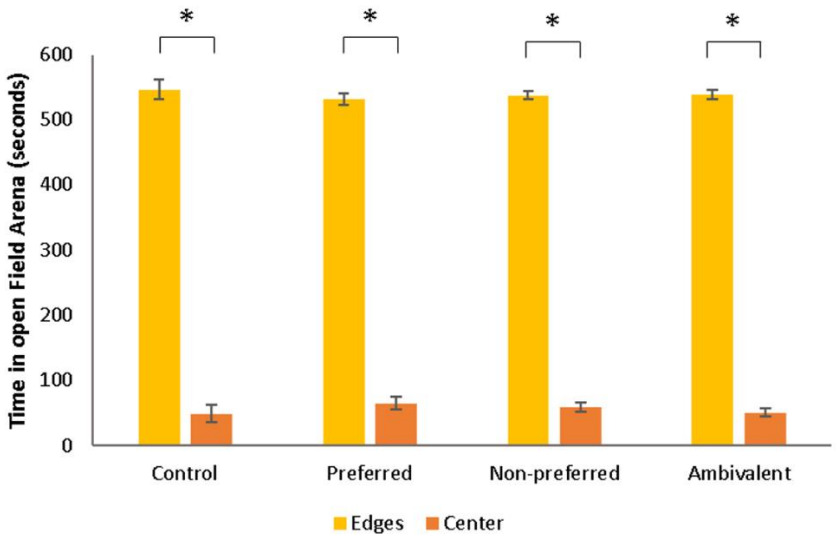

Figure 22: Adult offspring time in open field arena by group. All adults, regardless of maternal grouping, spent more time along the edges of the open field arena than in the center. Data are expressed as means ± SEM with * $p<0.01$.

Offspring tested both as weanlings and as adults

All offspring tested both as weanlings and as adults, regardless of maternal group ($F_{(1, 49)} = 2725.66$, $p<0.01$) or sex ($F_{(1, 51)} = 3387.48$, $p<0.01$), spent more time along the edges of the open field than in the center (Figure 23). A sex difference was evident, revealing that twice-tested adult females spent more time along the edges of the open field than adult males ($F_{(1,51)} = 5.61$, $p<0.05$). When viewed by group, an interaction between time and location was evident ($F_{(1,49)} = 5.68$, $p<0.05$), and again when sex was used as the categorical variable ($F_{(1,51)} =4.63$, $p<0.05$). No effect of group ($F_{(3,49)} = 0.87$, $p=0.46$) or sex (sex $F_{(1,51)} = 1.44$, $p=0.24$) was seen, nor any effect of time (group $F_{(1,49)}= 0.12$, $p=0.73$; sex $F_{(1,51)} = 1.09$, $p=0.30$). No interaction was present between time and group ($F_{(3,49)} = 1.45$, $p=0.24$), nor of time and sex ($F_{(1,51)} = 0.10$,$p=0.75$),nor any interaction between location and group ($F_{(3, 49)} = 0.85$, $p=0.47$). Lastly, no interactions between time, location and group ($F_{(3,49)} = 1.03$, $p=0.39$) were found, nor any interaction between time, location, and sex ($F_{(1,51)} = 1.63$, $p=0.21$).

All groups spent similar amounts of time in the corners of the open field ($F_{(3,49)} = 0.71$, $p=0.55$), but an effect of sex was present ($F_{(1,51)} = 6.43$, $p<0.01$). Tukey's HSD test revealed that weanling males spent more time in the open field corners than adult males. An effect of time spent in the corners of the open field was seen between groups ($F_{(1,49)} = 5.75$, $p<0.05$) and between sexes ($F_{(1,51)} = 6.63$, $p<0.01$). Overall, offspring spent more time in the corners as weanlings than as adults. No interaction was seen between time and group ($F_{(3,49)} = 0.41$, $p =0.74$) or time and sex ($F_{(1,51)} = 1.66$, $p=0.20$).

Offspring tested in the open field as both weanlings and again as adults did not show any differences between group ($F_{(3,49)} = 0.41$, $p=0.75$) or sex ($F_{(1,51)}= 0.03$, $p=0.86$) in distance traveled in the open field apparatus regardless of time (group $F_{(1,49)} = 0.74$, $p=0.39$; sex $F_{(1,51)} =$

0.92, p=0.34). Additionally, no interactions were revealed between time and group (time by group $F_{(3,49)} = 0.49$, p=0.69) or time and sex (time by sex $F_{(1,51)} = 0.02$, p=0.88). No differences were found between groups ($F_{(3,49)} = 0.40$, p=0.75) or sex ($F_{(1,51)} = 0.03$, p=0.86) across time (group $F_{(1,49)} = 0.51$, p=0.48; sex $F_{(1,51)} = 0.62$, p=0.430) for velocity in the OF. No interactions were found between group and time ($F_{(3,49)} = 0.48$, p=0.70) or sex and time ($F_{(1,51)} = 0.014$, p=0.84) for velocity in the OF.

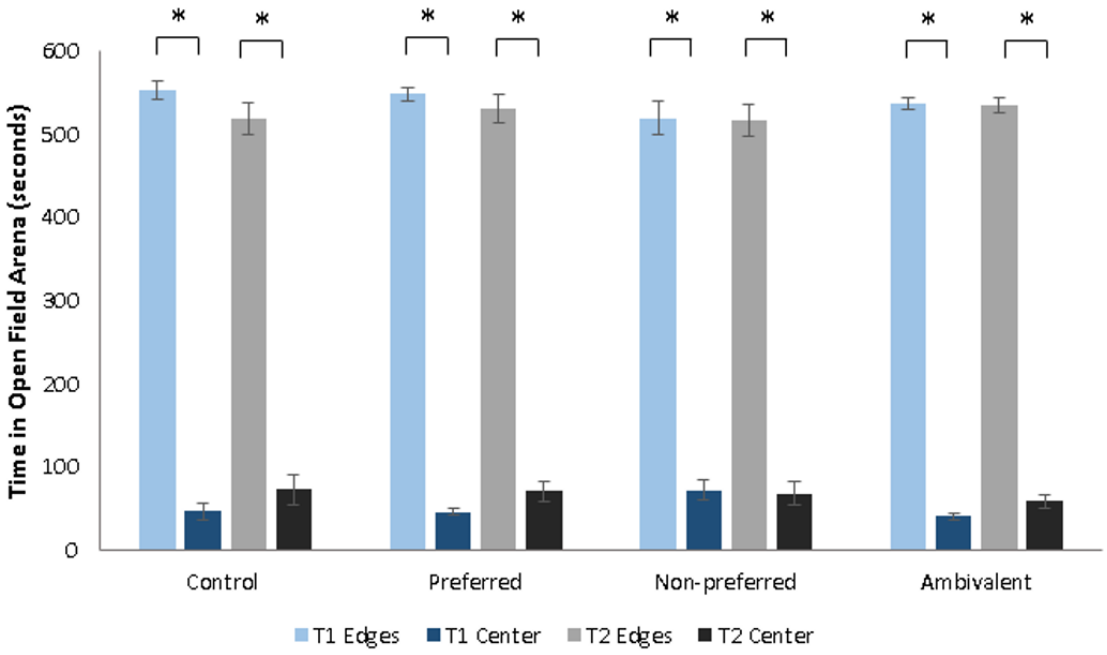

Figure 23: Time spent by offspring as weanlings (T1) and adults (T2) in the Open Field. All offspring, regardless of maternal grouping or time, spent more time along the edges than in the center of the open field. Data are expressed as means ± SEM with * p<0.01.

Regressions performed on each group of offspring comparing behavioral testing activity as weanlings and again as adults revealed few differences for EPM data, with only the offspring of ambivalent females showing a significant correlation between time spent in the open arms of the maze between testing as weanlings and again as adults (p<0.05) (Table 3).

While overall, the open field ANOVA results were less diverse, regression analyses revealed more information. Both the offspring of control females and the offspring of non-preferred females spent similar amounts of time along the edges of the OF over time while the offspring of control females spent a similar amount of time in the center of the OF arena across time. As for the corners of the OF, only the offspring of preferred females showed any significant repetition of behavior (Table 3).

Table 3: Correlations between first and second testing for twice-tested animals

test	location	group	F-stat	Df	p-value
EPM	open	control	6.77	3	0.12
EPM	open	preferred	2.56	7	0.16
EPM	open	non-preferred	0.39	5	0.56
EPM	open	ambivalent	4.84	16	<0.05
EPM	closed	control	0.04	3	0.85
EPM	closed	preferred	0.39	7	0.56
EPM	closed	non-preferred	0.67	5	0.46
EPM	closed	ambivalent	0.14	16	0.71
OF	edges	control	334.83	3	<0.01
OF	edges	preferred	1.10	7	0.33
OF	edges	non-preferred	7.73	5	<0.05
OF	edges	ambivalent	0.90	16	0.36
OF	center	control	165.53	3	<0.01
OF	center	preferred	0.67	7	0.44
OF	center	non-preferred	0.60	5	0.48
OF	center	ambivalent	0.13	16	0.73
OF	corner	control	1.84	3	0.31
OF	corner	preferred	14.19	7	<0.01
OF	corner	non-preferred	0.31	5	0.61
OF	corner	ambivalent	0.88	16	0.36

DISCUSSION

The goal of these experiments was to identify whether the mate interactions of the dams with their partners or their pups would be a sufficient environmental manipulation to merit a measureable anxiogenic reaction in their pups. From this research question, it was expected that

those offspring that had experienced a more stressful gestation period or upbringing within the nest would exhibit more anxiety-like behaviors. With this in mind, it was determined that non-invasive and well-established measures of anxiety-like behavior in small rodents would provide some insight into any stresses or anxiety that the offspring of my paired females might have endured either in utero or during their upbringing.

Both the elevated plus maze (Rodgers and Dalvi 1997; Korte and de Boer 2003) and the open field (Denenberg 1969, reviewed in Ganella and Kim 2014) are standard behavioral assays for anxiety and anxiety-like behavior in rodent models. These assays were used with pups from each maternal grouping – offspring of dams with their preferred partner, offspring of dams with the non-preferred partner, ambivalent dams, and control dams that were not exposed to the mate choice paradigm.

EPM

In general, offspring of the paired mothers tested only as juveniles exhibited a lack of anxiety-like behavior as shown by more time spent in the open arms than in the closed arms of the EPM. Exceptions to this finding were provided by offspring of dams paired with their preferred partner, which spent more time in the closed arms of the apparatus. If standard interpretations of this testing paradigm are applied, these particular offspring, unlike their counterparts, exhibited anxiety-like behavior in the elevated plus maze.

When tested only as adults, all subjects except those reared by ambivalent dams, spent more time in the closed rather than the open arms of the EPM. The behavior of these subjects again did not line up with expectations, as the offspring from the females with their preferred partner exhibited more anxiety-like behavior, while the offspring of ambivalent females exhibited a lack of anxiety behavior. The adult offspring of both control females and those females paired

with their non-preferred partner showed different behavior than did their younger within-group counterparts, spending more time in the open arms of the EPM. This, along with the results seen for offspring tested only as weanlings, is consistent with the change in EPM behavior seen by Imhof *et al.* (1993), where more mature animals exhibit more anxiety-like behavior in the EPM than do younger animals.

Offspring tested both as weanlings and as adults yielded mixed results. Those from ambivalent dams held true to the results of their group-mates tested only as weanlings and only as adults by spending more time in the open arms of the EPM during both tests suggesting a lack of anxiety. Pups of females paired with their preferred mate also spent more time in the open arms of the EPM. In contrast, the offspring of control females and of dams paired with their non-preferred partner showed a change in behavior over time, spending more time in the open arms of the EPM as weanlings and more time in the closed arms as adults. This shift may be the result of a developmental delay (Imhof *et al.*, 1993).

Open Field

Regardless of age or maternal grouping, all offspring tested in the open field apparatus (OF) spent significantly more time along the edges of the device than in the central regions. Additionally, no differences in velocity were seen between groups in this research paradigm, indicating no major differences in activity between experimental groups. The extensive time spent by my test subjects along the edge of the OF apparatus would line up well with escape seeking behavior described by other researchers, reinforcing the generally accepted interpretation that time spent along the edges of the open field are an indication of anxiety-like behavior.

Conclusion

While it is difficult to parse out the meaning of behaviors for any non-human species as many interpretations have an anthropomorphic origin, it is clear that some motivation must be present for differences in behaviors to occur. Based on my results in comparison with the literature, it is clear that age and maternal mate choice status plays a role in behavioral assay displays of anxiety-like behavior.

CHAPTER VIII

GENERAL DISCUSSION

Mate Choice

All females exposed to the mate choice paradigm that expressed a preference for one male over another spent more time with the first male that they encountered. These results are common in prairie voles. Getz *et al.* (2004) noted that prairie vole mating appeared to be opportunistic, with females pairing with the first accessible unrelated male as their mate. Even females pre-primed with estrogen spent more time in a preference test with the male they had first mated (Shapiro *et al.*, 1986). Other species also exhibit this behavior. Female *Gobimorphus breviceps*, a species of fish commonly called upland bully, are known to mate with the first male they encounter (Poulin 1994), as do termites (Mizumoto *et al*., 2020), the orangethroat darter (Pyron,1995), and male pipefish (Berglund,1993). Species that mate with the first potential partner they encounter are considered as using a random mating tactic (Jennions *et al.*, 1997). Others have described this sort of mate choice as indiscriminate *vs*. discriminate: indiscriminate females mate with the first nonrelated male encountered, while discriminate females seek out other potential partners after an initial encounter (Ah-king and Gowaty, 2016).

Perhaps the most noteworthy component of this work is that not all females made a clear choice, resulting in the ambivalent group. For many researchers, these animals might be considered outliers and any data associated with them would be discarded. Approximately 30% of

my animals self-selected into the ambivalent group. While this was unexpected, these animals produced the most interesting data. Other research on prairie voles has benefitted from including animals that might have otherwise been excluded from analysis based on social behavior. Ophir *et al.* (2007) defined partner preference in their experiments as spending more time with one particular animal over another (this is almost identical to the criteria I used). In this same paper, Ophir *et al.* mention that Insel *et al*. (1995) used more conservative criteria, in which a preference was defined as being twice as much time spent with one available animal over another. Ophir *et al.* acknowledged that if they had used this criterion, they would have omitted roughly 35% of the animals used in their experiment. Had that ~35% of animals been omitted, their results would have lined up with those reported by Insel *et al.* (1995). The experiments by Ophir *et al*. and Insel *et al.* in this case involved male partner preference rather than female mate choice, but the analytical aspect of their discussion shows that while stringent criteria allow a clear line to be drawn in one's results, they do not always relay a complete picture of a population's behavior.

Whether or not a choice was made, strong pair bonds formed within each group, as evidenced by the strong partner preference shown by both sexes at two weeks post-pairing. All females in these experiments preferred the partner animal at two weeks post-pairing. This is unsurprising, as all females used for partner preference analysis were pregnant at the time of testing, and pregnant females are known to prefer their mate (Getz *et al.*, 1981). Partner preference at the two week testing point was also strong for males with the exception of males paired with ambivalent females. This preference amongst the majority of males was somewhat surprising, given that the females did not achieve pregnancy until 3-5 days later than the time generally considered necessary for a pair bond to form for males (Curtis, 2010). While the cause of this behavior is unclear, it may be that females of this species play a strong role in establishing what mating strategy is observed by a prairie vole pair.

Identifying the mechanism of mate choice was not the focus of this study, but it was necessary to account for any clear factors that might influence choice. Beyond extended exposure to a male or male urine, multiple factors have been implicated as influencing female mate choice in prairie voles. Anogenital distance (Ophir *et al.*,2007), body mass (Solomon, 1993), dominant behavior (Shapiro and Dewsbury, 1986), and affiliative behavior (Ophir *et al.*, 2008) all have been investigated. Prairie vole females are known to prefer dominant males over submissive males, while non-monogamous Montane voles show no such preference (Shapiro and Dewsbury, 1986). Female prairie voles also prefer affiliative behavior to aggressive behavior from potential mates (Ophir *et al.*, 2008). Measures of social behavior, such as dominance status or affiliative behavior were not taken in this study, but anogenital distance and body mass at mate choice were both examined. No significant differences in these two measures were found for any group, indicating that neither of these factors played a role in female mate choice within these experiments.

One factor that may have influenced female mate choice was cage location. During the mate choice trials, females were more likely to prefer the male in the left cage of the apparatus than the right cage (Figure 11). This may have been a Type I error, or may be the result of handedness of the experimental administrator (I am right-handed and tend to pick up voles with their faces oriented towards my thumb. When the females were placed in the central cage at the beginning of testing, they may have been oriented in such a way that the left cage entrance was the first they encountered.).

The emergence of the ambivalent female group during mate choice testing was unexpected, as prairie voles are well known for their affiliative social behavior. Ambivalent females spent the majority of mate choice timeframe in the central cage. This location preference is noteworthy because the center cage was the only space in which the females could rest alone, rather than investigating or settling in with a potential partner. Another factor worth considering

is that while using this apparatus for mate choice is very reasonable in a laboratory environment, prairie voles in the wild rarely have the opportunity to choose between two mates at once (Getz, McGuire, Pizzuto, 2004).

While the monogamous behavior of prairie voles is one of the foundational reasons for their use as a model animal, references indicating that not all prairie voles exhibit this behavior can be found even from authors who are well-known for touting the monogamous behaviors of this species. Carter *et al.* (1986) notes that half or more of the prairie voles found in field studies displayed monogamous behavior, leaving roughly half the remaining animals from those studies to be classified by some other mating strategy. In that same paper, it is noted that the mating exclusivity of mated pairs is not permanent, but that in the wild, where prairie voles often live for short spans of time, the pair bond is adequate to last the length of a mated pair's shared lives.

Studies comparing prairie voles sourced from different regional populations find multiple differences in monogamy-associated behavior and physical morphology. When compared with animals derived from a population in Illinois (IL), prairie voles originating from Kansas (KS) show more exploratory behavior, are less likely to be captured in the wild with their partner, are more likely to disperse from the nest, and display more aggressive behaviors than their IL derived counterparts (Cushing *et al*. 2001). In a study comparing IL voles with those derived from a Western Tennessee (TN) population, Ophir *et al.* (2007**)** found both "resident" and "wanderer" males, where the male either stayed within the partner's nest, or went on to potentially impregnate other females. In another study, the same lab found that in open field enclosures, females often had mixed paternity litters (Ophir *et al*., 2007).

Sexual dimorphism in which males are larger than females has been linked to mating systems that favor polygamy, while animals follow a monogamous mating strategy are known for lacking sexual dimorphism (Boonstra *et al*., 1993; Dewsbury *et al*., 1980). In the present studies,

however, I found sexual dimorphism to be the case ($p<0.001$), with males tending to be roughly 6 grams larger than females at the time of mate choice testing. One possible reason for sexual dimorphism in mass is age difference. While the ages of females and males in the present studies did differ significantly ($p<0.001$), the practical difference in age for the females vs the males was not consequential (102.5 ± 2.0 vs 91.3 ± 1.9 respectively). What is noteworthy is that the males were younger than the females and still larger than the females, indicating that this sexual dimorphism was not due to a difference in age. While some researchers have associated sexual dimorphism with differences in mating strategy (Boonstra *et al.*, 1993; Heske and Ostfeld, 1990), others found no such correlation (Ophir *et al.*, 2007).

Partner Preference behavior

In the present studies, animals showed partner preference behavior consistent with other literature, as prairie voles are commonly found to have established a partner preference by two weeks post-pairing. In this case, the males paired with females that were ambivalent during mate choice testing were an exception to the rule, showing no partner preference after 14 days of pairing.

The establishment of pregnancy at 6-8 days post pairing is unusual for animals from our lab, as a previous study showed that in female-paced mating, roughly 63% of females achieved pregnancy within 48 hours, with an additional 26% achieving pregnancy between 48 and 96 hrs after pairing (McCracken, *et al.*, 2015). While the mating in the current experiments may be delayed, other researchers have reported that mating often takes place within 4 days of introduction with ovulation occurring within 12 hrs after initial mating (Roberts et al 1999). This adds at least 1 to 1&1/2 days to the timeframe previously reported in the Curtis lab. In another

study, only 50% of female prairie voles were found to ovulate within 8 days after the first mating bout (Grey *et al.*, 1973).

Multiple comparative studies have sought to parse out mating systems according to regional climate or physical characteristics (Cushing *et al.,* 2001; Ophir *et al.,* 2007; Roberts *et al.*, 1998). One physical characteristic associated with polygynous behavior is sexual dimorphism in body size. As previously noted, sexual dimorphism in body mass was present in the current experimental subjects. The behavior of the ambivalent females appears to fall in line with polygynous species, as the females exhibited drastically less social interaction at mate choice testing than their counterparts. It may be that female behavior plays a role in determining whether a pairing is more likely to result in a monogamous pair bond.

Hormonal changes throughout pairing and pregnancy

The primary focus of this project was how female mate choice affects pair bonding and offspring fitness. With that in mind, methods focused on investigation into how female mate choice in prairie voles influences both bonding and fitness during the maternal experience as inferred via hormonal levels throughout pregnancy. Of particular interest in this study was how mate choice influences a female prairie vole's anxiety over the course of pairing and pregnancy. Corticosterone (CORT), a glucocorticoid well-established as an indicator of stress response (Denenberg, 1969, DeVries *et al*. 1995), was measured at various points during the course of pairing and pregnancy. 17-β estradiol is often one of the estrogens that increase during pregnancy. Estrogen levels, along with backdating from parturition, served as an indicator of pregnancy status at different time points for each animal. As induced ovulators, prior to any pregnancy, an initial surge in estrogen levels is a key indicator that a prairie vole female is ready to mate (Dluzen and Carter, 1980). Increases in estrogen are also known to induce stress

(Ochedalski *et al*., 2007). Similarly, an increase in circulating estrogens is common during pregnancy in many mammalian species (Buckwalter *et al*, 1998; Tai and Taylor, 2021). With this in mind, a search for any correlation between estrogen and CORT within groups was undertaken. In order to reduce excessive handling and the stresses associated with blood sample collections, the non-invasive process of collecting fecal samples for hormonal analysis was used.

Estrogen

Fecal estrogen has been shown to mirror circulating levels in numerous mammalian species, including rhinoceros (Schwarzenberger *et al*., 2000), baboons (Wasser *et al*., 1994), and sea otters (Larson *et al*., 2013). Other bodily secretions, such as milk (Wang *et al*., 2012) and urine (Erb *et al*., 1968) have been used to measure estrogen levels. As for measurement of pregnancy progression, increases in fecal estrogens throughout the course of pregnancy have been found in gorillas and orangutans (estrone; Bamberg *et al*., 1991), dogs (17-β estradiol; Gudermuth *et al*., 1998), and black rhinoceros (17-β estradiol; Berkeley *et al*., 1997).

While all females in this study showed increases of fecal estrogen over time, the ambivalent females showed increased estrogen from baseline a week or more before females from other groups. This was unexpected, as all females became pregnant during the course of the experiments, and no differences were present between groups in latency to parturition. The ambivalent females did show a trend towards larger liters (p=0.06), which may account for the earlier increase in estrogen levels. These earlier increased estrogen levels without earlier parturition may also be indicative of aborted initial pregnancies. All females showed progressively increasing levels of estrogen throughout the sample collection time course, as would be expected with pregnancy status.

While increases in estrogen are expected, the type of estrogen that increases over the course of pregnancy may differ between species. For example, some species do not show increased secretion of 17-β estradiol over the course of pregnancy. In pregnant bovines 17-β estradiol does not significantly increase during pregnancy while other estrogens, such as 17-α estradiol, do increase (Erb *et al*., 1968). In cats, estradiol levels decrease shortly after the onset of pregnancy and then rise shortly before parturition (Verhage *et al.* 1976). In other species, no changes in in common estrogens are found during gestation. Female rats have shown consistent levels of estradiol, estrone, and estriol in ovarian venous blood over the course of pregnancy (Yoshinaga *et al*., 1969).

Corticosterone

All females showed high initial CORT levels that quickly abated. This initial peak was likely the result of the stress or the novelty of the handling associated with the fecal collection and initial pairing with a male partner. High initial CORT levels were unexpected, as pregnancy is a known stressor for females (Roesch *et al.*, 2004). In spite of this, rodents have been found by other researchers to exhibit decreased CORT levels during pregnancy (Neumann and Bosch, 2007). Consolation behavior in prairie voles has been shown to decrease CORT levels in their cage mates (Burkett *et al*., 2016). Whether the CORT decrease seen in these experiments was driven by habituation or by comforting behaviors provided by their new partners is unknown.

Of particular note is that CORT is known to facilitate partner preferences in males, but inhibits them in females (DeVries *et al*., 1996). This may account for the consistently seen partner preferences in these experiments, despite the delayed pregnancy seen in the females across groups. Of additional note is that this sexual dimorphism in CORT response is another example of unexpected sexual dimorphism in a monogamous species.

Correlations between Estrogen and Corticosterone

Positive correlations were found between CORT and estrogen levels (or, in the case of the non-preferred group, nearing a correlation at p=0.06) in all groups except ambivalent females. While the lack of correlation for the ambivalent group may have been driven by the earlier rise of estrogen levels, the lack of correlation reinforces the uniqueness of the ambivalent group. While stress hormones and the progression of pregnancy are correlated for the other animals, ambivalent females appear to be free of this phenomenon.

Anxiety-like behavior in juvenile offspring

Nearly all juvenile-only tested animals showed a lack of anxiety-like behavior in both the elevated plus maze and during open field testing. Unlike juveniles of the other groups, offspring of dams paired with their preferred partner, spent more time in the closed arms of the EPM. These results were the reverse of what was anticipated, as the group with what might be assumed to be the most stability showed anxiety-like behavior, while the groups expected to have the more stressful development and early life showed a lack of anxiety-like behaviors.

While an unexpected result, this was surprisingly in line with similar literature, indicating developmentally appropriate behavior. Imhof *et al.,* (1993) found that adolescent rats preferred the open arms of the EPM to the closed arms. This behavior shifted to a preference for the closed arms in animals tested at later post-natal dates. In my study, the offspring of females paired with their preferred mate exhibited behavior patterns more commonly associated with more mature animals by spending more time in the open *vs.* the closed arms of the EPM. Conversely, researchers working with male mice found that 4 week-olds exposed to a stressor showed fewer open arm entries and weighed less than same-aged control animals when compared to 8-week old mice, indicating that more mature animals were better able to handle stress (Stone and

Quartermain, 1997). Age itself may also be a factor. In rats, age differences were found between 4 month and 22-month-old rats in a water maze, where the younger rats moved more slowly and traveled shorter distances in the maze than did their older counterparts (Sirvio *et al* 1991).

The increased time spent in the open arms of the EPM by the juvenile prairie voles may be an indication that these animals received more nurturing maternal care. While maternal behavior was not measured in this study, nurturing behavior towards pups from dams has been shown to positively alter neurological development in rats (Weaver *et al*., 2004).

Anxiety-like behavior in adult offspring

With the exception of adult offspring of ambivalent dams, all offspring tested in the EPM only as adults exhibited anxiety-like behavior by spending more time in the closed, rather than the open arms of the maze. For the offspring of ambivalent dams and those of dams paired with their preferred partner, these results mimicked those seen in offspring tested only as juveniles. The adult offspring of control females and of dams paired with the non-preferred partner exhibited different behavior than their juvenile counterparts by shifting from a lack of anxiety-like behavior to exhibiting more anxiety-like behavior. This shift in behavior with increased age lines up with the results mentioned earlier from Imhof *et al.* (1993) in which more mature rodents engage in more safety-seeking behavior in the EPM.

Other researchers have also seen inconsistent results in the EPM between rodents within the same species of different ages. Some studies have shown no differences between older and younger animals (Walker *et al*, 2004), or that younger animals exhibit more anxiety-like behavior (Doremus *et al*, 2006). Whether these differences are species or sex specific, or are the result of the stressors and assays used for measurement remains to be seen.

Anxiety-like behavior in offspring tested both as juveniles and again as adults

Interestingly, the offspring of females paired with their preferred mate that were exposed to the EPM more than once showed decreased anxiety-like behavior as adults when compared with their counterparts. Animals tested more than once in the EPM test may have habituated to the testing apparatus. On one hand, habituation apparently is not an issue in other species, such as rats, if testing is spaced at least 3 weeks apart (Adamec and Shallow, 2000). This 3-week period was exceeded in my protocol. Another factor associated with increased activity and open arm entries in the EPM is the exposure to a novel environment, including another testing apparatus prior to EPM testing (File et al, 1975; Pellow et al 1985). Even with this possible confounding factor, it is very common to administer behavioral testing in a series or battery of tests without any indications of altered behavior in the EPM. This has been found to be true in both rats and mice (Walf and Frye 2007). Conversely, other researchers found that experimental handling influenced EPM behavior. A study of Swiss mice showed a shorter latency to enter the closed arms of an EPM upon second exposure to the apparatus This latency was shortened with more intense handling, such as a saline injection, immediately prior to EPM testing (Lapin, 1995). To avoid this, minimal handling was used in my own experiments.

Prairie voles are not as common in lab studies as rats and mice and are not commercially available. As such, prairie voles are often bred in-house for experiments and are only a few generations removed from wild-caught animals. Studies in other outbred rodent species close to the wild, such as wild mice, noted exploratory behavior that would, according to traditional interpretations for EPM results, indicate a lack of anxiety-like behavior. Some researchers have noted differences between wild and lab-bred mouse species (Hendrie *et al* 1996), or that wild mouse species spend more time in the open arms of the EPM (Holmes, *et al* 2000; Hendrie *et al* 1996). Behavioral differences have also been seen between vole species, where male prairie voles spent more time in the open arms of an EPM than do male meadow voles after extended social

isolation – a condition considered to be a chronic stressor for the social prairie vole (Stowe *et al* 2005).

Multiple researchers have speculated that rather than this behavior being an indication of comfort in the EPM apparatus, it demonstrates another anxiety-like response – seeking escape. The idea that high activity in small rodents may be exploratory rather than an indicator of "low emotionality", dates back in the literature as early as the 1960's (Denenberg, 1969). If exploratory motivations drive the activity of prairie voles in the EPM, it may be that the weanlings in my own experiments that spent more time in the open arms were investigating their environment with the hope of finding an escape route, rather than displaying a lack of anxiety-like behavior.

Open Field

While no differences were found between groups, ages, or sex in my own open field experiments, it may be that measurements of behaviors other than those I examined may have revealed some differences. Other behavioral measures such as freezing or number of fecal boluses left during open field testing are common with the OF test (Denenberg, 1969). I did not check for freezing during OF testing, which may have allowed for more refined results. Bolus information was collected for each behavioral subject (results not shown); but, given the brief nature of the test, some voles failed to provide samples and analysis of what data were available from these animals was not feasible for statistical analysis. Some authors note that the OF only tests ambulatory behavior and that results from this test may be the result of "opposite exploratory and emotional drives" (Lister, 1987), a point suggesting that these tests fail to clearly interpret the motivation behind an animal's behavior. The fact that all of my subjects spent the bulk of their time in the OF along the edges of the apparatus does support the commonly accepted interpretation of engaging in anxiety-like behavior. However, the inconsistency of anxiety-based

interpretations for the OF test and the EPM testing results within the same animals imply that escape-seeking behavior may be a more likely explanation.

Another example of escape seeking behavior comes from the desire to manage allostatic imbalances, wherein an animal is exposed to a stressor that may require movement into another environment to acquire necessary resources. Often, this is tied to food resources (McEwen and Wingfield, 2003), whereas in the case of my animals it may be related to lack of cover in an open field test, lack of partner, or simply being involuntarily placed in a novel environment. Alternately, this may be an indication of a different mating strategy, such as vole populations that follow a less conservative model of monogamy. These animals may be "wandering" towards a potentially new territory or in search of a mate. Typically, these behaviors are associated with males, which have larger ranges in field studies (Ophir *et al*., 2007) but no sex differences were found for this behavior in either the EPM or OF tests.

Additional considerations

Circadian rhythms are also known to influence results between time points when testing within animals (Carobrez and Bertoglio, 2005). This has also been seen during or after chronic stress (Batuman *et al*, 1990), but, as prairie voles exhibit an ultradian rhythm (Lewis and Curtis 2016), it is unclear whether biological rhythms play a clear role in the changes seen here. For my own experiments, behavioral testing was done between noon and 6pm, depending on the number of subjects tested. This time span is sufficient for multiple ultradian cycles to take place.

Another potential confounding factor in these results is that the subjects tested only as adults were often housed with a sibling that experienced behavioral testing shortly after weaning. Previous studies in rats have shown that housing stressed with non-stressed animals does not influence the CORT level or stress behavior of the non-stressed cage mate (Ottenweller, 1992).

While it is difficult to parse out the meaning of behaviors for any non-human species as many interpretations have an anthropomorphic origin, it is clear that some motivation must be present for differences in behaviors to occur. Based on my results in comparison with the literature, it is clear that age and maternal mate choice status plays a role in behavioral assay displays of anxiety-like behavior.

Conclusion

Regardless of mate choice pairing group, partner preferences were present for both sexes at two weeks post pairing. The exception to this was for those pairs that had an ambivalent female. It may be that these females follow a different mating strategy or monogamy style. These animals, show sub-groups with deviations from traditionally acknowledged behavior. While these animals added an initially confusing layer to this research, they help to develop a fuller picture of monogamy in mammals. While it may be a life strategy adhered to by the majority of the *Microtus ochrogaster* species, its finer points vary among some members of that species. These differences appear to have a heritable component, as offspring from these dams exhibited less anxiety-like behavior, though whether this heritability is based in nature or nurture is as yet unclear.

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