IMPACT OF ODORS ON PATERNAL RESPONSIVENESS AND ASSOCIATED NEURONAL ACTIVITY IN "EXPECTANT" MALE PRAIRIE VOLES (*MICROTUS OCHROGASTER*), A MONOGAMOUS SPECIES

by

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ABSTRACT

Impact of Odors on Paternal Responsiveness and Associated Neuronal Activity in "Expectant" Male Prairie Voles *(Microtus ochrogaster)* a Monogamous Species

by

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Prairie voles (*Microtus ochrogaster*) are unusual mammals because they are socially monogamous. The breeding pairs form pair bonds and jointly care for offspring. Although the duration and intensity of parental behavior in male prairie voles is similar to that observed in females, there are sex differences in the onset and pattern of infant caring in this species. The factors that contribute to sex differences in parental behavior of prairie voles are unknown. As males and females show all components of active and inactive parental behaviors, it is possible that sensory inputs from infants arouse a different pattern of parental care in each sex. Males and females may also differ in the degree of attentiveness to infant sensory cues and to other environmental stimuli. Furthermore, they may focus their attention on different aspects of infant cues or perceive the same cues differently. I conducted three experiments to test these hypotheses. In the first experiment, I tested the attentiveness and sensitivity of male and female prairie voles towards infant-related odors across the reproductive period. Males and females showed increased attentiveness to infant-related odors at different times during the reproductive period. In the second experiment, I examined the impact of female sensory cues on male responsiveness towards infant odors. The data suggest that male's exposure to the female's tactile and distal cues during the gestation period elicited indirect paternal behavior in presence of infant odors. However, infant odors alone were insufficient to stimulate direct paternal responsiveness in these males. In the third experiment, I studied the neuronal activity of brain areas that could be involved in the enhanced indirect paternal behavior that was observed in males housed with their mate through mid gestation. In response to infant-related odors, males that had stayed with their mate had higher neuronal activity in the ventral tegmental area (VTA) compared to males that had stayed with their same-sex sibling. Together, these studies indicate that female sensory cues in prairie voles play an important role in enhancing indirect paternal care by increasing the male's attentiveness to odors and activating neurons in the VTA region of his brain.

Dedicated

to

JESUS

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CHAPTER 1

GENERAL INTRODUCTION

- *Mechanisms for stimulation of parental care in rodents*

Voles are small arvicoline rodents that are classified in the genus *Microtus*. Given that most mammals display a promiscuous mating system, vole species were also thought to be promiscuous (Fitch, 1957). This was a plausible hypothesis because as prey animals for most predators, the best strategy for a male rodent to maintain its progeny is to mate with multiple females. However, research has shown that although most rodents are promiscuous, few species are monogamous (Nel, 1975). Prairie voles *(Microtus ochrogaster)* are one of those unusual rodent species that are monogamous (Thomas et al. 1979).

In promiscuous rodent species such as rats *(Rattus norvegicus)* and golden hamsters *(Mesocricetus auratus),* only the mother cares for the young (Rosenblatt et al. 1963). However, in monogamous species like California mice *(Peromyscus californicus),* both parents care for the young (Gubernick et al. 1994). In both promiscuous and monogamous rodents, the onset of parental care varies by sex. For example adult estrous-cycling female rats do not show maternal care except immediately after giving birth. The male rat is typically not paternal (Rosenblatt et al. 1963). In the monogamous mice, both the male and the female engage in parental behavior. However, they show sex differences in the onset of their parental responsiveness. The female begins to exhibit parental care after copulation, whereas the male shows paternal care after offspring birth (Gubernick et al. 1994).

Infant stimuli play a critical role in the onset and maintenance of parental responsiveness. Studies in male prairie voles have shown that chemosensory signals are important in stimulation of paternal care and other social behaviors (Kirkpatrick et al. 1994b). Similarly, in female rats, chemosensory and auditory stimuli from infants stimulate the mother to approach the young and care for them. Furthermore, maternal responsiveness is maintained through continuous somatosensory inputs that the mother receives from infants during nursing bouts (Stern et al. 1989).

There are specific neuro-endocrine factors, which influence the neural system of an animal in order to motivate parental responsiveness (Numan et al. 2003). These neuro-endocrine factors are thought to increase the attractive value of infant-related stimuli (Fleming et al. 1986), decrease the fear-arousing properties of infant stimuli, and decrease the stress reactivity responses of the parent to a variety of demanding stimuli (Rosenblatt et al. 1963). Studies in female rats have indicated that these neuro-endocrine factors, which impact parental motivation, are induced by hormonal events of late pregnancy and parturition (Numan et al. 2003). These hormonal changes include a rise in progesterone and a gradual rise in estrogen levels during the gestation period. In addition, there is an abrupt fall in progesterone levels associated with an abrupt surge in prolactin levels and a gradual rise in estrogen levels that occur at parturition (Rosenblatt et al. 1990).

In species with a monogamous mating system, the male displays a pattern of parental behavior that is similar to that shown by the female. For example, male mongolian gerbils *(Meriones unguiculatus)* shows paternal behavior a few days after the female has given birth (Clark et al. 2000). The djungarian hamster *(Phodopus campbelli)* is another monogamous and bi-parental species. The male djungarian hamster assists in the birth of his young by tearing fetal membranes from the pups. The male also exhibits paternal care later in the life of his offspring (Jones et al. 2000). Male prairie voles show paternal care throughout their adult lives (Roberts et al. 1998).

A relatively large body of evidence suggests that hormonal changes associated with pregnancy and parturition affect the way female mammals respond to infant stimuli, however the factors that affect the way male mammals respond to infant stimuli remain unknown. It is possible that sensory cues from the mate or infants stimulate the onset or maintenance of paternal behavior by influencing the hormonal levels or the neuro-endocrine factors in the male. Research performed on human males has shown that expectant men whose spouses were in late pregnancy had higher blood levels of prolactin and cortisol compared to expectant men whose spouses were in early pregnancy (Storey et al. 2000). Also, male prairie voles that had mated and cohabited with the mate were more paternal and had higher vasopressin-immunoreactive fibers in their brain than males that had not mated (Bamshad et al., 1994). On the contrary, Wynne-Edwards et al. (2007) found little evidence of hormones regulating the onset of paternal behavior in their studies on rodent animal models of bi-parental care.

- *Mechanisms of parental onset and attachment in male and female prairie voles*

Species of the *Microtus* genus show a striking difference in their mating system and parental care (Lonstein et al. 2000). Meadow voles (*Microtus pennsylvanicus*) are promiscuous and only the female shows parental care, whereas prairie voles are monogamous and both parents show parental care (McGuire et al. 1984). Prairie voles live in communal groups throughout the year. Most of the communal groups consist of an original breeding pair with additions from offspring that remain at the natal nest and a few unrelated adults (Carter et al. 1993; Getz et al. 1996). The offspring that remain with their parents are usually non-reproductive (Thomas et al. 1979). Richmond and Conaway (1969) reported that female prairie voles housed either in isolation or in groups are 98% anestrous. However, 71% of them show estrus within a week of being paired with a male. Thus, reproduction is suppressed in female prairie voles that live in groups or in isolation, but it is stimulated when females are exposed to male stimuli. A Pair bond is then established leading to a socially monogamous mating system (Thomas et al. 1979). Although both males and females show interest in pups as juveniles and young adults, the male shows more interest compared to the female (Lonstein et al. 1999a).

As adults, female prairie voles are infanticidal when they are sexually inactive (Lonstein et al. 1999a). The onset of maternal care occurs at late gestation or at parturition (Villalba et al. 1997). On the other hand, male prairie voles show paternal care whether they are sexually inactive or sexually active (Lonstein et al. 1999a). However, the level of paternal care increases gradually after the male mates and cohabits with a female (Terleph et al. 2004). Paternal behavior by male prairie voles includes all aspects of parental care except lactation. This includes defense, grooming, infant retrieval, nest building and food acquisition (Kleiman, 1977). The establishment of monogamy in prairie voles and the extensive involvement of the male in caring for their young make the male prairie vole an ideal model for understanding the neural mechanisms regulating the stimulation of paternal behavior.

- *Brain nuclei areas implicated in parental behavior*

There are species and sex differences in the neuronal activation induced by parental behavior in mammals. In maternal care, the events related to pregnancy and parturition influence the neuronal processes involved in parenting. Certain brain areas have been implicated in parental behavior in rodents including the female prairie vole (Numan et al. 1991; Kirkpatrick et al. 1994a). In males however, information on neuronal involvement in paternal behavior is scarce. In prairie voles, the male is involved in all aspect of parental care just like the female except for lactation. Therefore, looking at brain areas implicated in maternal behavior in prairie voles and other rodents, may aid in identifying the brain areas that play a role in paternal care.

Brain areas that have been implicated in maternal care include the ventral tegmental area (VTA), bed nucleus of the stria terminalis (BNST) and the lateral septum (LS). The VTA is known to be rich in dopamine, a neurotransmitter involved in reward/appetitive behavior (Wang et al. 1999; Numan et al. 2003) and social bonding in monogamous voles including the male prairie vole (Wang et al. 1999; Aragona et al., 2003; Curtis et al. 2005) and is involved in foraging tendencies that are essential for infant retrieval and nest building (Panksepp, 1998). Research has shown that electrical lesions of the VTA disrupt maternal behavior in rats (Gaffori et al. 1979). The Medial Preoptic Area (MPOA) which is involved in parental behavior in sensitized male rats (Rosenblatt et al. 1998), female rats (Numan et al. 2003) and female prairie voles (Kirkpatrick et al. 1994a) has efferent projections that terminate in the VTA (Numan et al. 1991). Thus the VTA may be a vital part of maternal behavior circuitry and may be involved in some aspects of paternal behavior.

BNST has been implicated in maternal care in rats (Numan et al. 1991). *Fos* analysis used to measure neuronal activation showed significant expression of the protein in the posterior part of BNST when rats were fully (physical contact) or partially (distal sensory cues such as auditory, visual and olfaction) exposed to pups (Kirkpatrick et al. 1994b). Thus BNST may be involved in the processing of distal sensory infant cues and triggering the onset of parental care. Also the BNST is involved in the initial onset of parental care in rodents including prairie voles (Katz et al. 1999).

The LS is shown to be involved in paternal behavior in prairie voles. Injections of a vasopressin antagonist to block the V1a receptors in the LS of sexually inactive male prairie voles decreased paternal care. However paternal care was increased when a vasopressin agonist was injected in the LS of the sexually inactive male prairie voles (Wang et al. 1994).

This study explored the effect of female sensory input on the male prairie voles' paternal responsiveness and the resulting neuronal activation, in response to infant-related sensory cues.

CHAPTER 2

ODOR PREFERENCE IN COHABITED MALE AND FEMALE PRAIRIE VOLES

- ***To compare changes in preference for infant-related odors over the reproductive period in male and female prairie voles (Published material: Yamoah et al. 2008)***

Introduction:

Parenthood is a particularly demanding stage of an adult mammal's life as infants become salient stimuli for an extended period of time and parents become attached to them (Numan et al. 2003; Depue et al. 2005). Research in laboratory rats has shown that in the process of forming attachments, mothers develop a preference for infant cues and become bolder and more vigilant towards threats to the natal nest (Fleming et al. 1981; Fleming et al. 1986; Fleming et al. 1989; Love et al. 2005). In a variety of species, hormonal and neuroendocrine changes occurring prepartum have been shown to influence the behavioral changes seen in mothers (Numan et al. 2003; Kinsley et al. 2006).

Although mammalian males do not undergo pregnancy-associated hormonal changes, males within a monogamous mating system display parental behavior (Ostermeyer et al. 1983; Dewsbury, 1988). However, little research has been conducted on understanding the behavioral and physiological changes that facilitate infant attachment and caring in males.

In this study, I compared the response of prairie vole males and females to various odors presented simultaneously at different stages of the reproductive period. The goal was to determine whether cohabited voles become selectively attentive to infant odors, and if there are any sex differences in development of a preference for infant cues. I chose to use prairie voles because they are a biparental species and as such, are ideal models for comparing sex differences in the regulation of parental attachment (Carter et al. 1993).

Prairie voles, *Microtus ochrogaster*, inhabit the grasslands of the Great Plains of North America (Anderson, 1985). They feed on grasses and use it as protective cover (Klatt et al. 1987). They build extensive underground burrows with numerous entrances where they breed throughout the year (Getz et al. 1990). In the laboratory, prairie vole breeding pairs display behaviors that are characteristic of a monogamous mating system, including selective partner preference, co-nesting, and joint parental care (Carter et al. 1993). Although grasses provide the bulk of their diet, prairie voles also feed on a variety of fruits and vegetables such as apples and lettuce (Jameson, 1947). They are considered to be pests because they chew the bark and roots of fruit trees including apple, citrus, avocado, cherries, almond, and olives and can climb the trees to destroy the shoots.

Laboratory studies of prairie voles under semi-natural conditions have shown that within the first eight days postpartum, both sexes spend a significant duration of time in the natal nest caring for infants (McGuire et al. 1984). Although both males and females are highly parental, they display subtle sex differences in caring patterns. For example, the onset of parental responsiveness occurs earlier in males than in females (Terleph et al. 2004; Hayes et al. 2007). Males also participate in more active parental care than females by engaging in more exploratory behavior and by spending more time carrying and licking their young (Solomon, 1993). Furthermore, although both sexes spend a considerable length of time in various forms of bodily contact with young, females engage in this behavior more often than males (Lonstein et al. 1999a).

I tested the hypothesis that sex differences in the onset of parental care and in the pattern of responsiveness in prairie voles are related to their sex-specific changes in response to odors. I predicted that males would display a change in preference for infant odors earlier than in females because the onset of parental responsiveness occurs earlier in males than in females (Terleph et al. 2004; Hayes et al. 2007). I also expected that changes in preference for infant odors would be more prominent in females than in males because mothers are more frequently in direct contact with young (Lonstein et al. 1999a). I exposed voles at different reproductive stages to infant odor, orange extract, and saline simultaneously and measured their responses to each odor. The purpose for giving voles a triple choice was to determine if they would become selectively attentive to infant odors at a given point during the reproductive period.

Methods:

- *Subjects*

The subjects were offspring of breeding pairs of prairie voles that we maintain as a colony in our animal facility at Lehman College. The breeding colony was established from the offspring of field animals originally captured in East-Central Illinois. We routinely wean young at 21 days and house them in same-sex groups of 2 to 4 animals. As the gestation period of voles is approximately 22 days, this procedure ensures that the first litter is weaned prior to the arrival of the next litter. We maintain litter cages in a nursery room separate from the breeding colony, and the voles remain with same sex sibling until they are selected for an experiment.

The voles in both the colony and nursery rooms were housed in 48 x 27 x 20 cm clear plastic cages. The bottom of each holding cage is covered with approximately 5 cm of bedding, consisting of a layer of moistened peat moss and a layer of wood shavings. The cage is then filled to the top with hay. We provide this type of bedding in order to simulate the underground burrows of wild prairie voles. Water and food, consisting of sunflower seeds, rabbit chow, and cracked corn, are available *ad libitum*. All vole cages are kept in rooms with fluorescent lighting and at temperatures around 20-25°C. The light:dark cycle for all rooms is set at 14:10 with lights on at 6:00 a.m. and lights off at 8:00 p.m. All animal care and experimental procedures complied with local, state and federal regulations and were approved by the Lehman College Institutional Animal Care and Use Committee.

- *Procedure*

A total of 80 adult voles was randomly assigned to four groups (N=20 per group; 10 males and 10 females). I specifically chose to use voles that were 90 days old because sex differences in parental behavior are not prominent in younger voles (Lonstein et al. 1999b). The groups were labeled as MC0, MC13, MC18 and MC26, with (M) standing for mating, (C) for cohabitation, and (0, 13, 18, 26) for the number of days males and females cohabited after their initial physical contact. The voles assigned to each of the four groups were from different litters and different parentage. In MC0, the males and females were not paired and remained sexually inactive until behavioral testing. As the gestation period in prairie voles is approximately 22 days, The MC13 group was set up to test breeding pairs in approximately mid gestation and MC18 group was set up to test breeding pairs in approximately late gestation. The MC26 pairs remained together throughout the gestation period and were tested 3 days postpartum. In order to prevent fighting during initial pairing, males and females in MC13, MC18 and MC26 were habituated to each other. Males were placed in small wire mesh enclosure (height; 10cm, diameter; 7.7cm) with some food and a piece of apple inside a regular holding cage. The male remained in this enclosure for 24 hours, while the female occupied the remainder of the larger cage. This restricted condition allowed for olfactory, auditory and visual communication between pairs. After 24 hours, the male was released into the larger cage. All behavioral tests were carried out in the afternoon by placing each vole in a specially designed apparatus for odor testing.

- *Odor collection for behavioral testing*

Each experimental animal was exposed to three chemicals simultaneously: infant odor, citrus extract and saline. The chemicals were collected in the morning and the behavioral tests were conducted in the afternoon. Solutions containing chemicals were applied to 3.2 cm diameter Watman filter papers (VWR International). Using a pair of clean forceps, three filter papers were placed on a clean piece of bench paper. A pencil was used to label the back of the filter papers as (S) for saline, (O) for orange/citrus and (P) for pups/infant. Each filter paper was then placed in a black 35 mm film container with perforated top, marked correspondingly with (S), (O), and (P). In the (S) and (O) containers, a single drop of 0.9% saline and a single drop of orange/citrus extract were placed on (S) and (O) labeled papers respectively. In the (P) container, a 2-6 day-old infant separated from its parents and littermates was placed on the filter paper for an hour and a half. The infant was then picked up, the filter paper was rubbed on its anogenital region and the infant was returned to its parents. The three odor-covered filter papers were picked up with a pair of clean forceps. Using a paper clip, each filter paper was attached to a short plastic tie that was threaded through a self-adhesive base. The bases were taped in a random order on the wall of a small cage that was part of the behavioral testing apparatus. The experimenter collecting the odors and handling the filter papers wore gloves at all times to prevent transfer of human odors and sweat.

- *Behavioral testing apparatus (Odor exposure)*

The apparatus consisted of two plastic cages (48 x 28 x 20 cm) and (27 x 16 x 12 cm) connected by a straight, yellow habitrail tube, 33 cm in length. I designed this apparatus to provide a semi-natural environment where voles would have to cross a tunnel in order to investigate odors. The larger cage was designated as the introductory cage and was used to introduce voles into the apparatus. The smaller cage was designated as the odor cage, where the odor-covered papers were mounted. The zip tie mounts holding the 3 filter papers were attached 8 cm apart on the wall of the odor cage opposite the habitrail tube. A video camera was placed at an angle in order to keep the entire apparatus in view. The test began by placing an experimental animal in the introductory cage. Most voles explored the introductory cage before entering the habitrail tube to reach the odor cage. The time to enter the habitrail tube and investigate the odors was recorded as the latency period. The five-minute behavioral sessions began once the voles entered the habitrail tube. Afterwards, I reviewed the video and analyzed the frequency and the duration of the following behaviors: movements within the odor cage and the habitrail tube, activity (combined measures of locomotion, exploring the apparatus, and sniffing the air), self-grooming, sniffing the filter papers, and manipulation of the filter papers (combined measures of nibbling and chewing the paper, or gnawing at and searching behind the mounted base). Animals who failed to enter the habitrail tube within 10 minutes were given a score of 300 seconds and were excluded from further analysis.

- *Statistical analysis*

For all statistical analysis and estimates of effect sizes, I used the NCSS statistical software program *(Kaysville, Utah).* Some of the data were not normally distributed and were transformed prior to running the statistical analysis. The type of transformation used depended on the shape of the distribution of a given variable and included square root, natural log or log (x+1). The frequency and duration of the observed behaviors were analyzed using a two-way analysis of variance (ANOVA), with cohabitation interval (groups) and sex as independent variables. Significant differences at $P \leq 0.05$ were further analyzed with the Bonferroni Multiple Comparison Test. The time spent sniffing each filter paper was compared for each sex using paired t-test. The percentage of voles that showed paper manipulation was compared using Fisher's Exact Test. The effect size was given by the Cohen's d for t-tests and a modified version of Cohen's approach for two-way ANOVAs (NCSS 2000).

Plate 2.1

Plate 2.1- Behavioral apparatus shows the odor cage with the attached odor-infused filter papers and the vole ID (279-♀IOUP-MC13) which is a female vole that cohabited with an unrelated male for 13 days (MC13) and the date of experiment. Order of odor is P (Pup odor), O (Orange/citrus) and S (Saline).

Plate 2.2

Plate 2.2- Lateral view of Behavioral apparatus. Set up shows the larger cage which is the introductory cage which is connected to the smaller cage, the odor cage, by a habitrail tube. The wall of the odor cage shows the attached odor-infused filter papers.

Plate 2.3

Plate 2.3- A closer view of the odor cage showing the odor infused filter papers attached to the wall of the odor cage.

Results:

Out of 80 voles tested, 13 did not enter the habitrail tube or did not enter the odor cage during the testing session. These non-responders (two males and one female from MC0; one male and two females from MC13; two males and two females from MC18; two males and one female from MC26) were eliminated from further analysis. The latency to enter the odor cage did not differ significantly for the remaining voles in the study.

- *Odor investigation*

Voles in all groups sniffed at least one of the odor-covered papers. Results of odor preferences in each sex are shown in Figure 2.1. Results indicate that females preferred saline to orange extract, t [34] = 3.59, P = 0.001, d = 0.94 and infant odors to orange extract, t [34] = 4.23, P = 0.0002, d = 0.98, but they showed no difference in their preference towards saline and infant odors. In contrast, males preferred infant odors to both saline, t [33] = 2.87, P = 0.007, d = 0.77 and orange extract, t [33] = 4.31, P = 0.0001, d = 0.99. Males also preferred saline to orange extract, t [33] = 2.20, P = 0.04, d = 0.47. I analyzed the duration and frequency the voles sniffed each of the three papers. For saline-covered papers, the group and sex differences for the duration of sniffing were close to significance, F [3, 59] = 2.22, P = 0.10, d = 0.33 and F [1, 59] = 2.94, P = 0.09, d = 0.21, respectively. There was a trend towards an increased duration of sniffing with increased cohabitation intervals, particularly among females. The groups displayed significant differences in frequencies of sniffing saline-covered papers, F [3, 59] = 2.90, P = 0.04, d = 0.35. However, the sex difference in frequencies of sniffing saline-covered papers was not significant. Voles in MC26 sniffed the papers more frequently in comparison to those in MC0 or MC13 but not in comparison to those in MC18. There were no significant group or sex differences in the duration and frequency of smelling orange extract. For infant odor covered papers, there were no group or sex effects for the duration of sniffing. However there was a sex difference in the frequency of sniffing, F [1, 59] = 4.42, P = 0.04, d = 0.25. Males smelled infant odor more often than females did. The group difference for frequency of sniffing infant odor was not significant. Some animals not only sniffed the papers, but they also manipulated them by biting and chewing the papers, by gnawing at the zip tie mounts, and by looking behind the mounts. Voles manipulated the papers while they were in the odor cage, but did not carry around any chewed pieces of paper. Some voles manipulated the papers frequently and vigorously; others did so only occasionally and with less intensity, or not at all. The percentage of voles that engaged in the behavior is illustrated in Figure 2.2. Results from Fisher's Exact Probability Test showed that the frequencies among the groups were significantly different for females and for males. The number of females manipulating filter papers increased abruptly at MC18 approximately a few days prior to parturition [N = 34, P = 0.01], and after the birth of young [N = 34, P = 0.007]. The number of males manipulating the papers was particularly high at MC13 approximately at mid-gestation [N = 33, P = 0.01], and after the birth of young [N = 33, P = 0.02].

I then looked at the duration and frequency of manipulating each odor-covered paper. There was a significant group effect for the duration of saline paper manipulation, F [3, 59] = 5.78, P = 0.002, d = 0.52 and for frequency of saline paper manipulation, F [3, 59] = 5.95, P = 0.001, d = 0.53. The manipulation of saline paper increased progressively with the cohabitation period, peaking after the birth of offspring. The sex effects for the duration and frequency of saline paper manipulation were close to significance, F [1, 59] = 3.24, P = 0.08, d = 0.22 and F [1, 59] = 2.89, P = 0.09, d = 0.22, respectively. There were no significant differences in the duration and frequency of manipulating orange extract paper. For the duration of infant odor paper manipulation, the group effect was close to significance and there was no sex effect. For the frequency of infant odor paper manipulation, there was a significant group effect, F [3, 59] = 3.96, P = 0.01, d = 0.42, but the sex effect was not significant. The manipulation of infant odor paper increased during late gestation and following the birth of offspring.

To determine whether the total time spent investigating odors differed by cohabitation interval or by sex, I combined the data for the duration of sniffing and the duration of manipulating the odor covered papers. The data is shown in Figure 2.3. The results showed a significant group effect, F [3, 59] = 3.15, P = 0.03, d = 0.37, but did not show a sex effect. Odor investigation increased progressively across the cohabitation period, peaking after the birth of offspring.

- *General activity*

The time spent in the habitrail tube differed by cohabitation interval, F [3, 59] = 6.53, P = 0.0007, d = 0.54, but not by sex. As the length of cohabitation with a breeding partner increased, the time spent in the habitrail tube gradually declined. Voles in MC26 spent significantly less time in the tube than those in MC0 and MC13, but not in comparison to those in MC18.

There were no significant groups or sex differences in the duration and frequency of activity, which included the combined measures of locomotion, sniffing the air and the exploration of the apparatus. However, the duration of self grooming differed among the groups, F [3, 59] = 5.46, P = 0.002, d = 0.50. Animals in MC26 spent more time grooming themselves than those in the other three groups. The sex effect for grooming was not significant.

Figure 2.1 Yamoah et al. 2008

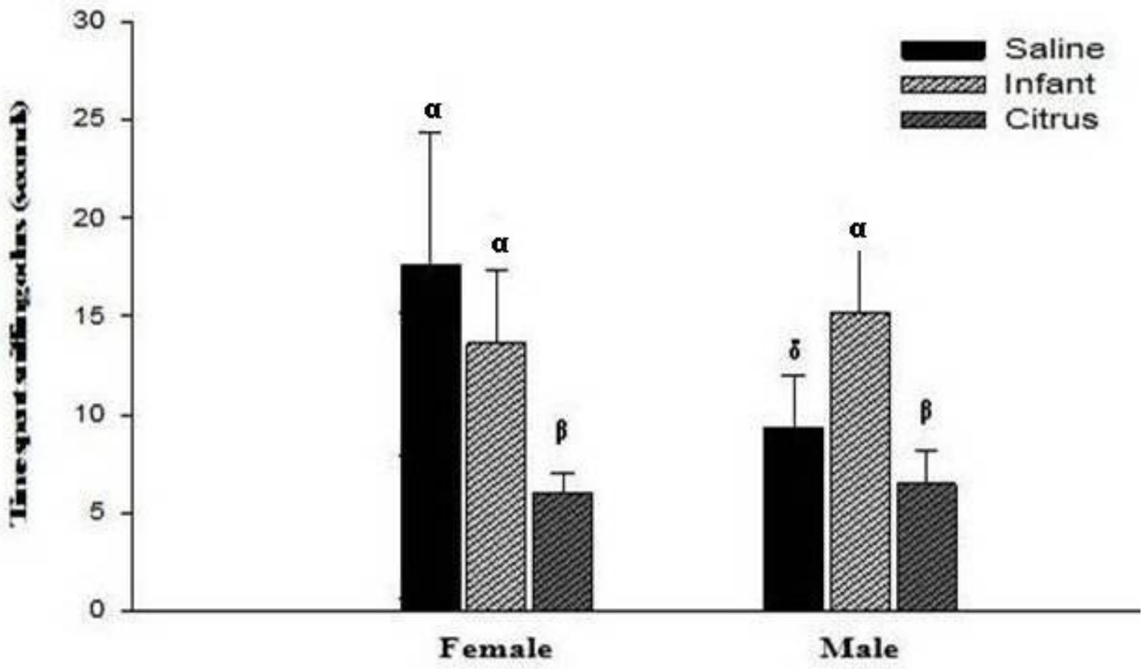


Figure2.1- Five minute analysis of both sexually inactive and active female and male prairie voles *Microtus ochrogaster*, showing preferences for filter papers covered with saline, infant odor or orange-citrus extract. Bars represent mean durations of odor sniffing and error bars show 95% confidence interval. (α, δ, β) over bars represent significant differences at $P \leq 0.05$. Females preferred infant odor and saline to citrus-orange extract. Males preferred infant odor to both saline and orange extract and preferred saline to orange extract.

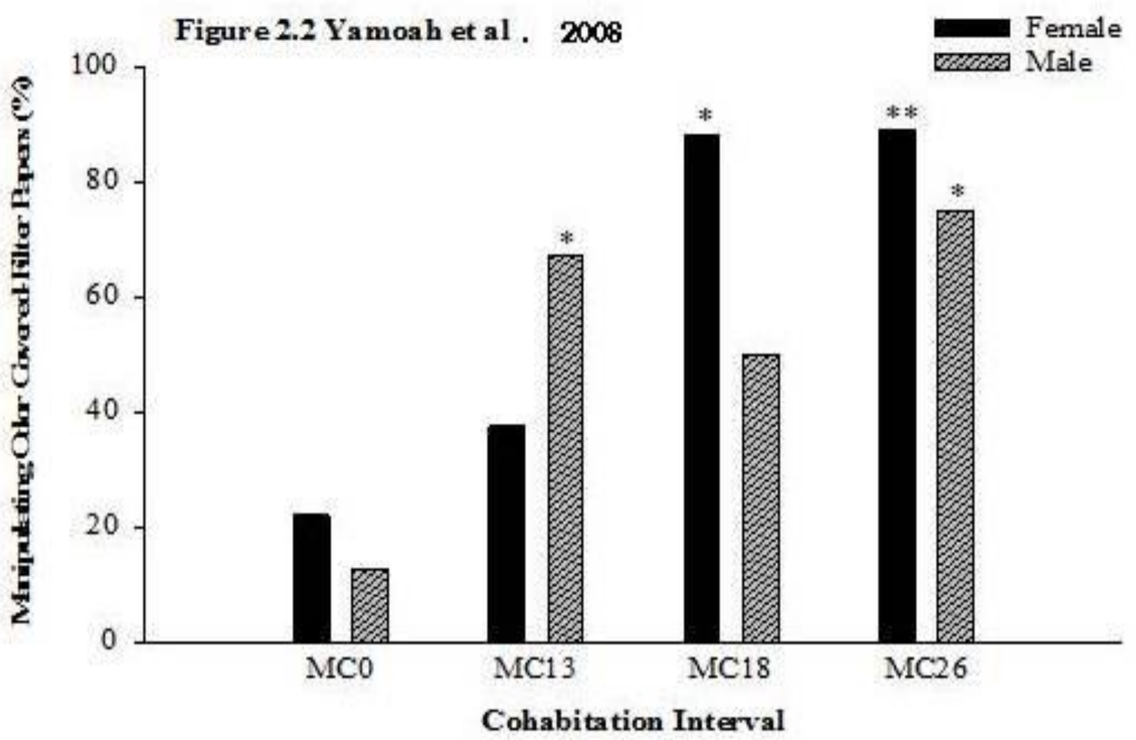


Figure2.2- Five minute analysis of the percentage in the total numbers of voles, sexually inactive and active manipulating a combination of the entire odor infused papers. Graph also shows the differences among four groups of female and male prairie voles in manipulating (combined measures of pulling, biting and chewing) the three odor-covered filter papers. The four groups of voles observed were sexually inactive (MC0), at approximately mid gestation (MC13), at approximately late gestation (MC18), and after the birth of offspring (MC26). Bars represent the percentage of animals engaged in the behavior. (*) and (**) over the bars represents significant differences at $P \leq 0.05$ and $P \leq 0.01$ respectively. A higher percentage of females manipulated odor-covered papers at approximately late gestation and after the birth of offspring. A higher percentage of males manipulated odor-covered papers at approximately mid gestation and after the birth of offspring.

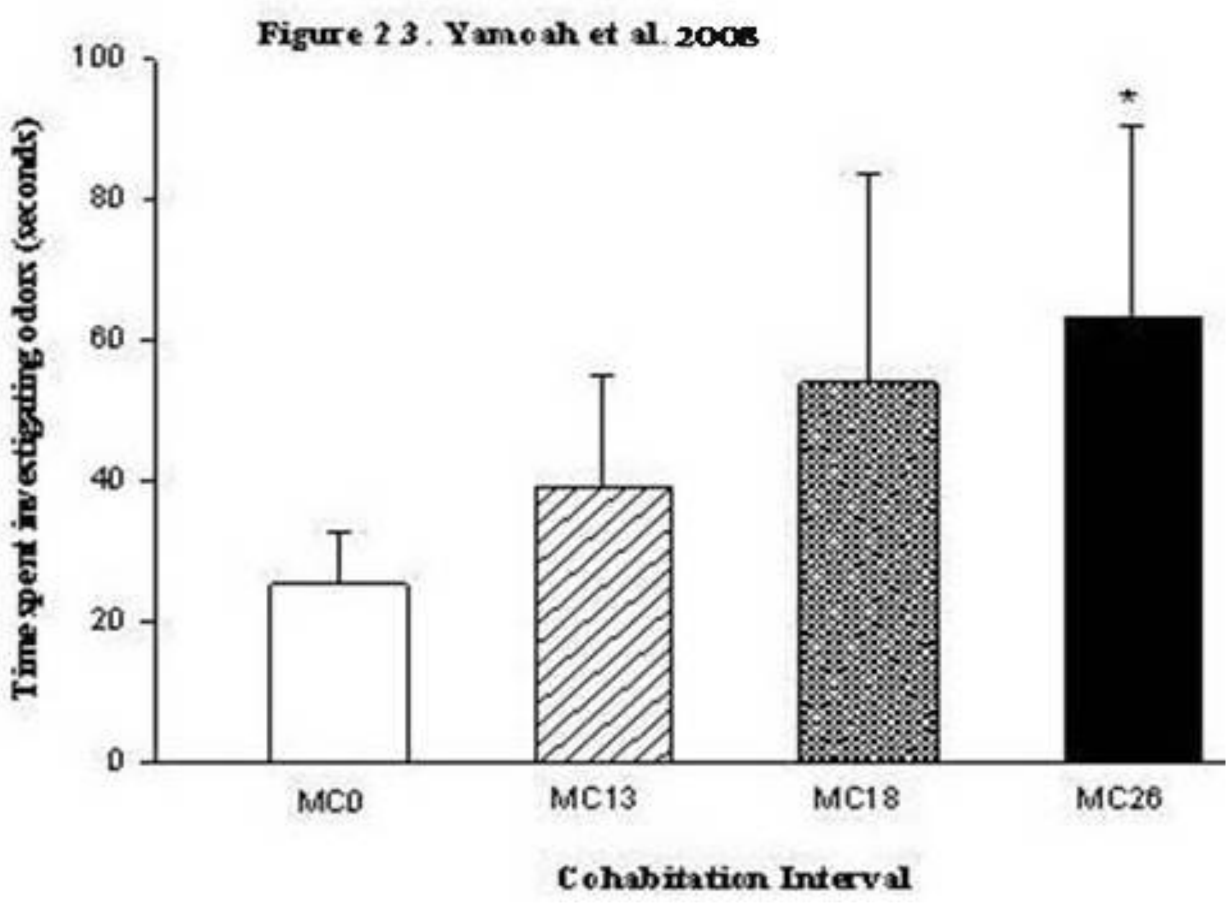


Figure2.3- Duration of time the voles spent investigating (combined measures of sniffing, pulling, biting and chewing) the three odor-covered filter papers. The four groups of voles observed were sexually inactive (MC0), at approximately mid gestation (MC13), at approximately late gestation (MC18), and after the birth of offspring (MC26). Bars represent means, and error bars show 95% confidence interval. (*) indicates significant differences at $P \leq 0.05$ among groups. The time voles spent investigating odors increased gradually during the cohabitation period. The MC26 voles spent more time investigating odors than the MC0 voles.

Discussion:

In this experiment, I studied prairie vole responses to odor cues at different phases of their reproductive cycle in order to determine if changes in odor preferences are associated with the onset of parental behavior in males and females. Additionally, I wanted to establish if voles would show selective attentiveness to infant odors at some point during their reproduction state. Based on the knowledge that the primary mode of communication between voles is olfaction (Carter et al. 1987; Wysocki et al. 1991), I predicted that infant odors would become increasingly salient for both males and females over the course of their reproductive cycle, and consequently they would spend more time sniffing infant odors over non-social odors. As the onset of parental responsiveness differs in males and females (Terleph et al. 2004; Hayes et al. 2007), I also predicted that changes in infant odor preferences would occur at different time points during the reproductive cycle in each sex.

The results indicated that male and female voles preferred infant odor to orange extract, irrespective of their reproductive status. As infant odor was a social cue and orange-extract was a non-social cue, it was not unusual for voles to spend more time sniffing papers covered with the former versus the latter odor. However, contrary to my expectations, the preference for infant odors was the same between the less parental, sexually inactive voles, and the more parental, cohabited voles (Terleph et al. 2004; Hayes et al. 2007). These data suggest that measuring the time voles spend sniffing odor-covered media may not be an appropriate indicator for parental motivation. Additional studies were still required to find sensory stimuli or approaches that would be suitable for assessing such motivations.

Although the time spent sniffing infant odor-covered media did not change during the reproductive cycle, the voles showed an unanticipated investigative behavior with a pattern that was specific to each sex. A higher percentage of females manipulated odor-covered papers a few days prepartum and postpartum, when females are most likely to engage in parental care (Hayes et al. 2007). In contrast, a higher percentage of males manipulated the papers at approximately mid-gestation and after the birth of infants, when males are most likely to show intense parental care (Bamshad et al., 1994; Terleph et al. 2004).

The significance of the paper manipulations I observed in voles remains unclear. Voles with a higher motivation to engage in active parental behaviors such as foraging, infant licking or infant retrieval may have had a tendency to manipulate objects placed in their cage. The data suggest that the manipulated objects must be of interest to the voles, since changes in paper manipulation over the reproductive period was observed only with the infant and saline odor-covered media that voles preferred. Furthermore, paper manipulation may reflect an interest in cohabited animals to search for the source of the odors. It is also possible that pair-bonded voles were more anxious or aggressive and were thus more likely to tear or chew the papers. On the other hand, paper manipulation may be related to changes in odor sensitivity or the odor detection abilities of the cohabited voles. As prairie voles cohabit with their mate and become reproductively active, the demands to detect and respond to odors increase. Although sexually inactive voles are gregarious, cohabited voles that are allowed to mate discriminate between familiar versus unfamiliar conspecifics and become aggressive towards strangers (Williams et al. 1992; Insel et al. 1995; Ross et al. 2006). In addition, mated prairie voles, forage for higher quality food and search for suitable habitats to increase their reproductive success (Cole et al. 1979; Lin et al. 2001).

Prairie voles also showed sex-specific responses to individual odor cues. Both males and females chose the odor of infants over orange extract, but females preferred to investigate saline-covered papers more than males. The female's interest in saline increased progressively during the reproductive period, with the highest levels seen after the birth of young. This change may be related to the female's enhanced salt appetite associated with the metabolic demands of pregnancy. The pregnancy-associated increase in saline preference in female voles is consistent with the increased salt appetite reported in rats, laboratory mice and rabbits, but in contrast to behaviors observed in other monogamous species such as California mice (Richter et al. 1938; Denton et al. 1978; McBurnie et al. 1988; Gubernick et al. 1983).

Male and female voles also displayed several behavioral similarities. When tested during the postpartum period, both sexes exhibited increased levels of self-grooming. Male and female voles have been reported to engage in similar increases in self grooming following licking and contact with their young (Lonstein et al. 1999a). The study showed that this enhanced self grooming is displayed by mothers and fathers in the presence of distant infant cues as well. In addition, male and female voles displayed similar changes in the amount of time spent in various compartments within the testing apparatus. As the cohabitation period progressed, both sexes spent less time in the habitrail tube, the only covered part of the testing apparatus. As prairie voles are underground burrowers and prefer to be covered (Klatt et al. 1987), the more timid individuals may have chosen to spend more time in the habitrail tube. Further studies are required to test the possibility that voles become bolder prior to the offspring birth.

Two confounding variables in my experimental design could have affected the outcomes. The first was the proximity of the three odor covered papers to each other. Voles may have reacted differently to the infant odor or the saline had the citrus-orange odor been eliminated or positioned further away. I do not consider this to be a major issue as I deliberately mounted the papers in random orders on the cage. In support of this, I observed voles vigorously manipulating papers covered with saline, whether the paper was mounted next to orange odor or next to infant odor. The second potential concern was the condition of the infant during odor collection. The infant was most likely stressed when separated from its parents and littermates. I am not aware of any studies showing that odors emitted by stressed pups are different from those emitted by unstressed pups. However, as the prairie voles that were close to parturition manipulated infant odor covered papers more frequently, it does not appear that these voles are particularly sensitive to stress-associated changes in odors.

In summary, I have shown that a shift in odor-investigative behaviors occurs with a temporal pattern that was specific to each sex. Whether this cohabitation-induced behavioral change was related to parental responsiveness was still unknown and was tested in chapter 3.

CHAPTER 3

EFFECT OF FEMALE SENSORY STIMULI ON MALE PRAIRE VOLES' BEHAVORIAL RESPONSES TOWARDS INFANT-RELATED ODORS

- ***To determine the importance of infant-related odors in eliciting direct or indirect paternal behaviors and examining the impact of female sensory signals on this process***

Introduction:

In mammals, mating system appears to be the determining factor of paternal care (Brown, 1993). Mating systems could be promiscuous, polygamous or monogamous (Fitch, 1980). In promiscuous mating systems, the female is the care-giver of the offspring whereas in monogamous mating systems, both male and female take care of their young (Dewsbury, 1981; McGuire et al. 1984). Except for lactation, behavioral characteristics of paternal care in monogamous species are similar to maternal care (Kleiman, 1977). However, the male engages in other behaviors that are also considered to be paternal behavior as these activities indirectly lead to the survival of offspring (Kleiman, 1977; Kleiman et al. 1981). Thus, male parental care is categorized as direct or indirect care. Direct care includes, hovering over offspring, licking and huddling them, whereas indirect care includes nest building, protecting nesting area and foraging for food (Kleiman, 1977; Dewsbury, 1988).

There is evidence that male rodents of monogamous species are either spontaneously paternal or their paternal behavior is stimulated. For example in prairie voles (*Microtus ochrogaster*) paternal behavior is spontaneous, males of all ages show interest in pups and show alloparental care (Wang et al. 1996; Roberts et al. 1998;

Lonstein et al. 1999a). However in California mice, (*Peromyscus californicus)* paternal care is turned on after copulation with the female (Gubernick et al. 1994). Although in spontaneously paternal species the behavior is not turned on, the incentive for infant caring might be further enhanced through exposure to sensory stimuli.

In female mammals, including humans, hormonal changes during pregnancy and the experience of parturition control the onset and enhancement of infant caring (Corter et al. 1990; Rosenblatt, 1990). In non-human primates parental care may also be induced by social experience during development (Pryce, 1996). However, factors influencing male parenting are widely speculated (Wynne-Edwards et al. 2007). Since males do not undergo the direct physiological changes associated with pregnancy and parturition, any hormonal or neural changes that may lead to initiation or enhancement of paternal care must be triggered by internal and/or external stimuli. Internal stimuli that could affect paternal care may be hormonal changes associated with growth and development. For example, research in male rats has shown that high levels of testosterone facilitate infanticidal tendencies and lower parental behavior (Rosenberg et al. 1974). These males which are usually not paternal show infant caring when they are castrated to lower the levels of testosterone and are primed with female hormones (Leon et al. 1973). In California mice, a monogamous and biparental species, paternal care is accompanied by 35% increase in blood prolactin levels (Gubernick et al. 1989). In addition Storey et al., (2000) observed an increase in the level of prolactin in human males whose spouses were in late pregnancy compared to males whose spouses were in early stages of pregnancy.

External stimuli that could influence paternal behavior may stem from the social environment in which individuals develop and live during adulthood. For instance, male house mice (*Mus musculus*) which are not usually paternal, when housed with parents and exposed to pups as juveniles become more paternal in adulthood (McCarthy, 1990). Furthermore, odors from the postpartum female are necessary to maintain paternal behavior in male California mice (Gubernick et al. 1994). In addition, the male spiny mice (*Acomys cahirinus*) spend more time caring for their own offspring than an unrelated infant due to sensory input recognition from their offspring (Makin et al. 1984).

Prairie voles are thought to be spontaneously paternal. The alloparental care by juveniles, sub-adults or sexually inactive adult prairie voles include huddling, retrieving, grooming pups and spending significant amount of time in the natal nest (Stern et al. 1988; Wang et al. 1992). There is evidence that their social environment during infancy and the experience of caring for younger siblings as juveniles influence their paternal responsiveness (Roberts et al. 1998). However, previous work in our lab has suggested that the male prairie vole's caring responses towards infants are enhanced after mating and/or cohabitation with a female (Bamshad et al., 1994; Terleph et al. 2004; Simoncelli et al. 2010). Consequently, it is plausible that external sensory cues the male experiences during cohabitation, mating or post-mating cohabitation with a female contribute to an increase in his paternal responsiveness.

Nonetheless, the mechanisms by which the male's caring responses towards infants may increase are unknown. It is possible that exposure to the female through cohabitation changes the male's perception of infant sensory stimuli. Thus, the male engages in direct paternal behaviors that are sufficiently intense and enhanced for the survival and subsequent development of the offspring. Alternatively, female cues may alter the male's attentiveness to social or other sensory stimuli in general so he can display indirect paternal responses such as guarding the nest against intruders or exploring the environment for food or nest building.

As odors are the primary sensory stimuli for rodents including prairie voles (Schultz et al. 1973; Carter et al. 1987; Wysocki et al. 1991), I tested the response of male and female prairie voles to infant odors versus non-social odors in the first study described in Chapter 2. Data from that experiment showed that male prairie voles sniffed infant-odor infused papers more frequently than females. In addition, males that had cohabited with their mate manipulated infant-infested odor papers with more intensity than males that had cohabited with their same-sex sibling (Yamoah et al. 2008). These behaviors might have resulted from an increase in exploratory behaviors typical of indirect paternal care seen in rodents (Kleiman, 1977). However, the significance of infant odors in stimulating direct paternal behavior and the impact of female cues on this process remain unclear. In this study, I conducted an experiment to determine the importance of infant-related odors in eliciting direct and/or indirect paternal behavior. I also examined the effect of female sensory signals on the male prairie vole's responsiveness towards infant-related odors in comparison to other odors. A significant difference in behavior between Female-Cohabited subjects ('expectant' fathers) compared to Male-Cohabited subjects ('non-expectant' males) will strengthen the hypothesis that sensory cues from the female during the cohabitation period increase male's attentiveness towards infant stimuli in particular and underlie his enhanced motivation to care for young.

I tested the working hypothesis that the behavioral pattern of male prairie voles differs in response to infant-related odors, depending on whether the males have cohabited with a female or not. To test this hypothesis, two groups of males were used. Males in the first group remained sexually inactive, cohabiting with their same sex sibling. Males in the second group were housed with an unrelated female and allowed to mate. The subjects were then exposed to live infants or inanimate objects (pup dolls) covered with infant odor, sub-adult odor or water. The subjects’ responses to live pups and the odor-covered inanimate objects were analyzed.

Methods:

- *Subjects*

Subjects were offspring of breeding pairs of prairie voles that we maintain as a colony in the animal facility at Lehman College. The breeding colony was established from the offspring of field animals captured in East-Central Illinois. We routinely wean young at 21 days and house them in same-sex groups of 2 to 4 animals. As the gestation period of voles is approximately 22 days (Dewsbury, 1995), this procedure ensures that the first litter is weaned prior to the arrival of the next litter. We maintain litter cages in a nursery room separate from the breeding colony, and the voles remain with their same-sex sibling until selected for an experiment.

The voles were housed in both the colony and nursery rooms in 48 x 27 x 20 cm clear plastic cages. The bottom of each holding cage is covered with approximately 5 cm of bedding, consisting of a layer of moistened peat moss and a layer of wood shavings. The cage is then filled to the top with hay. We provide this type of bedding in order to simulate the underground burrows of wild prairie voles. Water and food, consisting of sunflower seeds, rabbit chow, and cracked corn, are available ad libitum. All vole cages are kept in rooms with fluorescent lighting and at temperatures between 20-25°C. The light:dark cycle for all rooms is set at 14:10 with lights on at 6:00 a.m. and lights off at 8:00 p.m. All animal care and experimental procedures complied with local, state and federal regulations and were approved by the Lehman College Institutional Animal Care and Use Committee.

- *Procedure*

A total of 72 sexually inactive male voles were used. The males were randomly assigned to 2 groups. Males in each group were further divided into 4 sub-groups (N=9 per sub-group). The Male-Cohabited subjects in sub-groups 1-4 were housed in a new cage with their sibling brothers' and remained sexually inactive. The Female-Cohabited subjects in sub-groups 5-8 were housed in a new cage with a sexually inactive female and allowed to mate. In sub-groups 5-8, the pairs were housed together for 13 days after their initial physical contact. As the gestation period in prairie voles is approximately 22 days (Dewsbury, 1995), the groups were set up to test breeding pairs in approximately mid gestation. In addition, 13 days was chosen based on previous studies showing that male prairie vole's paternal behavior is increased after 13 days of cohabitation with a female (Bamshad et al., 1994; Terleph et al. 2004; Simoncelli et al. 2010). Furthermore, data from my previous work indicated that a higher percentage of male prairie voles engaged in manipulation of infant-odor covered filter papers after 13 days of cohabitation with a female (Yamoah et al. 2008).

Female voles assigned to males in sub-groups 5-8 were from different litters and different parentage. In order to prevent fighting during initial pairing, males and females were habituated to each other. Males were placed in small wire mesh enclosure (height; 10cm, diameter; 7.7cm) containing a small amount of food and a piece of apple. The wire mesh enclosure was placed inside a regular holding cage. The male remained in this enclosure for 24 hours, while the female occupied the remainder of the larger cage (Plate 3.1). This restricted condition allowed for olfactory, auditory and visual communication between pairs. After 24 hours, the male was released into the larger cage and remained with his partner undisturbed until behavioral testing. After 13 days, the males in all groups were placed in a specially designed apparatus and were tested for their behavior. All behavioral tests were conducted and carried out in the late afternoon in a room with the same temperature and lighting condition as the breeding room.

- *Odor collection*

For infant odor collection, two pup dolls were each placed in small bottles. All pup dolls were odorless and newly purchased. These pup dolls were similar in size to a 2 to 6 day old infant of a prairie vole (dimensions: 0.5x0.3x0.1). They were odorless and pinkish in color resembling the color of a pup. For aeration purposes, the bottles used were perforated at the top. A 2-6 day old pup was placed in the container on top of each doll. The two pups (1 pup per bottle) came from a single litter. After about 1 hour, each doll was rubbed on the anogenital and posterolateral regions of the pups, and the pups were returned to their parents. In addition, a drop of previously collected infant urine was placed on each doll. Infant urine was collected from a separate group of animals that did not participate in any experiment. For infant urine collection, 2-6 day old infants were separated from their mothers and anesthetized with isoflurane. An incision was made in the pelvic cavity of each infant to expose the bladder. The urine was withdrawn using a 1cc syringe and stored in micro-centrifuge tubes in the freezer. We have found that in using this method, we can collect large amounts of urine from a relatively small number of infants.

For sub-adult odor collection, two pup dolls were placed in a container with perforations at the top. About 2 to 3 sub-adult voles (30-45 days) were placed in the container. Sub-adult voles used for odor collection came from a single litter. After about 1 hour, each doll was rubbed on the anogenital and posterolateral regions of the sub-adult voles. The voles were then returned to their respective cages. In addition, a drop of previously collected sub-adult urine was placed on each doll. For sub-adult urine extraction, voles used were 30-45 days old. The same method used for infant urine collection was also used for sub-adult urine extraction. Selection of 30-45 day age range for collecting sub-adult odor was based on a study showing that urine of males younger than 54 days does not induce estrus in the female prairie vole (Moffatt et al. 1995). For water odor, 1ml of distilled water was sprinkled on two pup dolls.

- *Behavioral Apparatus*

Each vole was placed in a clear plastic holding cage (44x24x20 cm) that was connected to a smaller cage (27x16x12 cm) by a habitrail tube (33 cm). The larger cage (odor container) had two 5-cm diameter openings along each of its two longer sides at approximately 3 cm above the bottom surface. One of the openings was used to attach the habitrail tube in order to connect the odor container to the smaller cage (neutral container). The other opening had a cup (8.5 x 4 cm), fixed with industrial Velcro to cover the opening. The opening area of the cup was temporarily covered with a piece of Plexiglas. The neutral container contained scattered nesting material; 15 grams of hay and a flat cotton ball. The odor container contained peat moss and a round cotton ball soaked with water (Plate 3.2). Food and water was available in the odor container *ad-libitum*. The behavioral apparatus was designed to provide each subject with the choice of either remaining in the odor container or moving away from the odor source to the neutral container. The beddings (hay and peat moss) and food provided were the same as that given to the voles in their original home cage. The cages used were also clear plastic to provide a similarity to the home cage of the voles. These similarities created a more standard and familiar environment for each experimental vole.

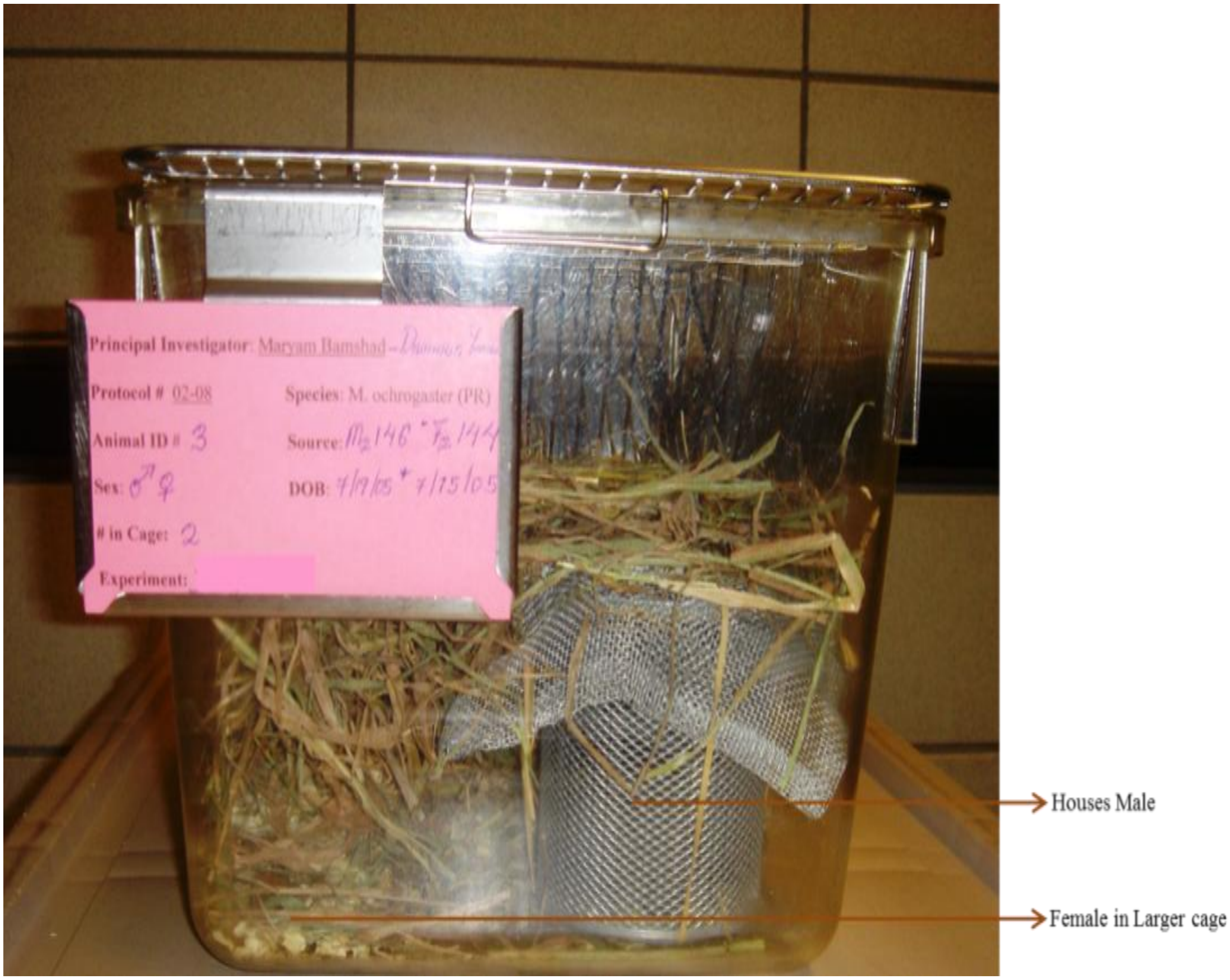
- *Behavioral Observations*

The behavior of each animal was video-taped for 120 minutes. However the video tapes were viewed and analyzed for 10 minutes. The subjects were video-taped for 120 minutes instead of 10 minutes in preparation for the subsequent brain analysis discussed in chapter 4. For behavioral analysis, the duration and frequency of the following behaviors were measured: Latency (time taken to initially approach object), locomotion (walking, running, jumping, moving around and sniffing the air), object manipulation (sniffing, licking, or chewing the objects), object cup visits, self grooming, and retrieving the object out of the odor cup. The frequency and duration of time spent in odor and neutral containers were also quantified. Previous research has shown that these behavioral activities I observed are typically performed by male prairie voles during parenting (Thomas et al. 1979). Each behavioral test began by placing the subject in the odor container. After giving the animal approximately 5 minutes to adjust to the novel cage, two live infants or two odor-infused inanimate objects (pup dolls) were introduced into the attached cup and the Plexiglas covering the cup was removed. In the control sub-groups 1 and 5, the cup contained 2 pup dolls infused with water. In sub-groups 2 and 6, the cup contained 2 pup dolls infused with infant odor. In sub-groups 3 and 7, the cup contained 2 live pups. Finally, in sub-groups 4 and 8, the cup contained 2 pup dolls infused with sub-adult odor. Following behavioral testing, each subject was anesthetized and perfused intracardially to fix the brain. The brain tissues were extracted and used for *Fos* immunocytochemistry.

- *Statistical Analysis*

For all statistical analysis I used the NCSS statistical software program (Kaysville, Utah). Some of the data were not normally distributed and were transformed by taking the square root prior to running the statistical analysis. Transformation used depended on the shape of the distribution of a given variable. The frequency and duration of the observed behaviors were analyzed using a two-way analysis of variance (ANOVA), with cohabitation and odor-conditions as between subject variables. Significant differences at $P \leq 0.05$ were further analyzed with the Tukey-Kramer Multiple-Comparison Test. Percentage of voles that showed object manipulation and object retrieval was compared using Fisher's Exact Test.

Plate 3.1



Principal Investigator: Maryam Bamshad -Dumais

Protocol # 02-08

Species: M. ochrogaster (PR)

Animal ID # 3

Source: M2 146 * F2 144

Sex: ♂ ♀

DOB: 7/7/05 * 7/15/05

in Cage: 2

Experiment: [redacted]

Plate 3.1*:* Apparatus shows the initial method of cohabitation between unrelated male and female prairie voles. The larger cage houses the female and the wire mesh container houses the male. The larger cage contains bedding consisting of hay, peat moss and wood shavings. Food and water are provided ad libitum. Wire-mesh container housing the male contains food (rabbit chow) and a piece of apple. This set up minimizes fighting between male and female voles that are initially unfamiliar to each other by allowing them to exchange their distal sensory cues.

Plate 3.2

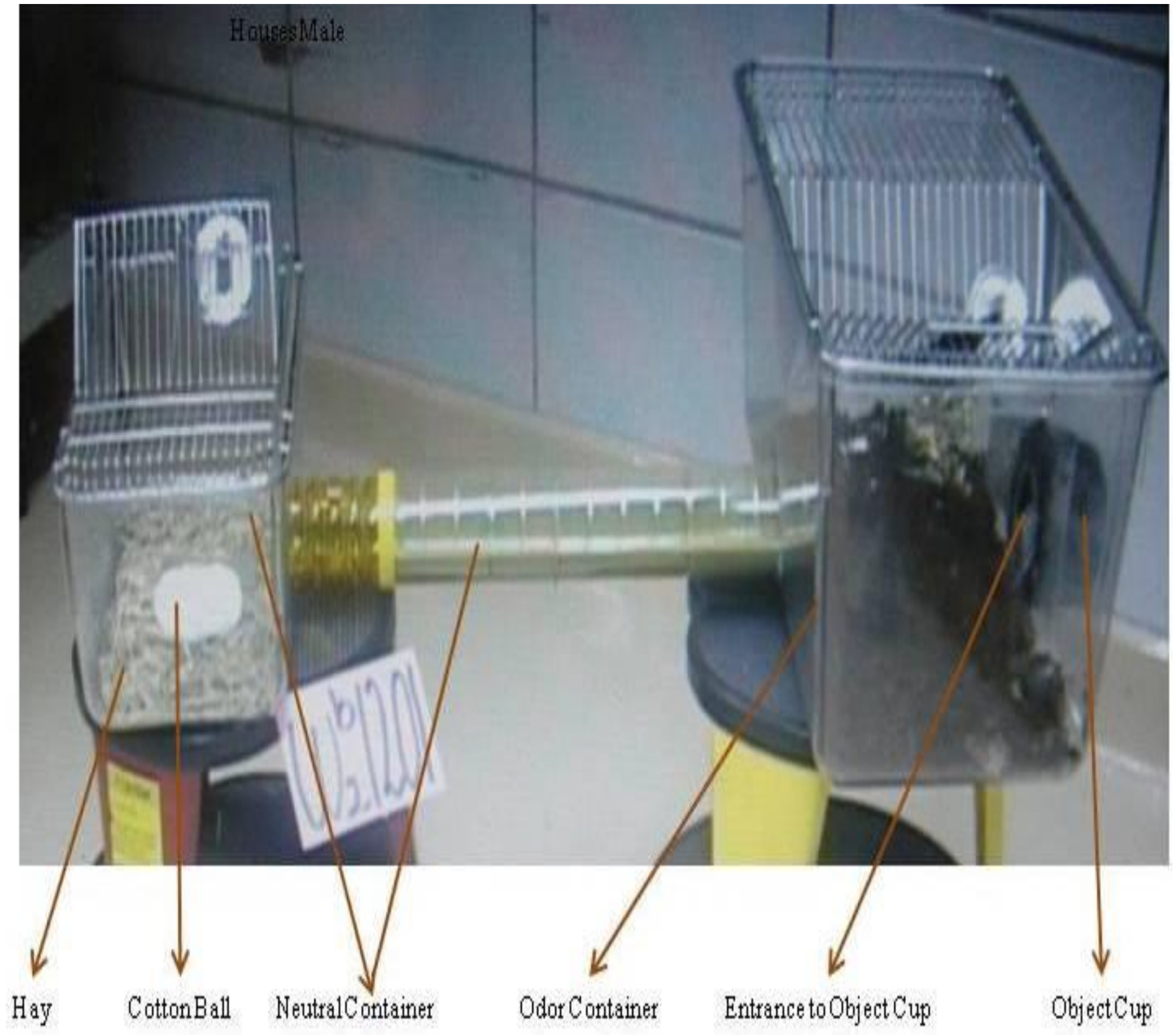


Plate 3.2*:* Behavioral apparatus shows two openings on the side of the larger cage (odor container). The object-cup is attached on one end of the opening and the opposite end of the opening is connected by a habitrail to the smaller cage (neutral container). Cages contain food, water and nesting materials. The live infants and the pup doll objects infused with their respective odors (infant, sub-adult and water) are placed separately in the object cup. The experimental male prairie vole subject is then placed in the odor container and behavioral output of the male is observed.

Results:

Out of 72 voles tested, 11 were eliminated from further analysis because their video tapes were of poor quality making it difficult to measure their behavior. Those eliminated included, 2 Male-Cohabited subjects exposed to water odor, 2 Male-Cohabited subjects exposed to infant odor, 2 Male-Cohabited subjects exposed to live pups, 1 Male-Cohabited subject exposed to sub-adult odor, 1 Female-Cohabited subject exposed to water odor, 1 Female-Cohabited subject exposed to infant odor and 2 Female-Cohabited subjects exposed to live pups.

- *Behavior Analysis*

Voles in all groups approached the object cup except one Male-Cohabited vole exposed to water. The latency data for voles to approach the object cup is shown in Figure 3.1. The results showed that males which had cohabited with an unrelated female (Female-Cohabited), had a shorter latency approaching the object cup when it contained infant odors compared to males which had cohabited with a male sibling (Male-Cohabited), F [3, 53] = 3.38. P = 0.02. Female-Cohabited subjects ('expectant' fathers) showed no significant differences in latency to approach the object cup when it contained infant odors or live infants. However, Male-Cohabited subjects ('non-expectant' males) approached the object cup faster when it contained live infants than when it had pup dolls covered with infant odors. Both Female-Cohabited and Male-Cohabited male voles showed no significant differences in approaching the object cup when the cup contained pup dolls covered with sub-adult odors or water.

The duration and frequency with which voles spent visiting and manipulating the objects were combined and analyzed. Results showed a significant difference in the duration of object visits and manipulations F [3, 53] = 9.12, P = 0.00006 (Figure 3.2), and the frequency of object visits and manipulations F [3, 53]. P = 0.002 among odor conditions (Figure 3.3). Female-Cohabited and Male-Cohabited subjects spent more time visiting and manipulating live infants compared to odor-covered pup dolls. In addition, both groups visited live infants more frequently than odor-covered pup dolls. There was however no significant differences between the Male-Cohabited and Female-Cohabited groups. The interaction effect between the groups and odor conditions for duration and frequency was not significant. Figure 3.4 shows the results of the Fisher's Exact Probability Test for the percentage of subjects that retrieved the live infants and the odor-covered pup dolls. The percentages were not significant for Female-Cohabited [N = 61, P = 0.33] and Male-Cohabited [N = 61, P = 0.17] subjects. Figure 3.5 shows the results of the Fisher's Exact Probability Test for the percentage of subjects that manipulated live infants and the odor-covered pup dolls. The percentages for Male-Cohabited subjects was significant [N = 61, P = 0.02], whereas the percentages for Female-Cohabited subjects was close to significance [N = 61, P = 0.07].

Furthermore, the data showed a significant interaction effect in the frequency of self- grooming between the groups and odor-conditions, F [3, 53] = 3.63, P = 0.02 (Figure 3.6). Female-Cohabited males spent less time self-grooming when they were exposed to sub-adult odors than when they were exposed to live infants. However there was no difference in the frequency of self-grooming among the groups or among the odor-conditions. The interaction effect for the duration of self-grooming between the groups and odor-conditions was close to significance, F [3, 53] = 2.66, P = 0.06. Female-Cohabited subjects showed a tendency to spend less time in self-grooming when they were exposed to sub-adult odors than when they were exposed to live infants. On the other hand, Male-Cohabited subjects showed no significant differences when they were exposed to live infants or to pup dolls covered with different odors.

The duration and frequency of locomotion, runway building, and time spent in odor container and neutral container were not significantly different among the groups and among the odor-conditions. Interaction effect between the groups and odor-conditions was also not significant.

Figure 3.1

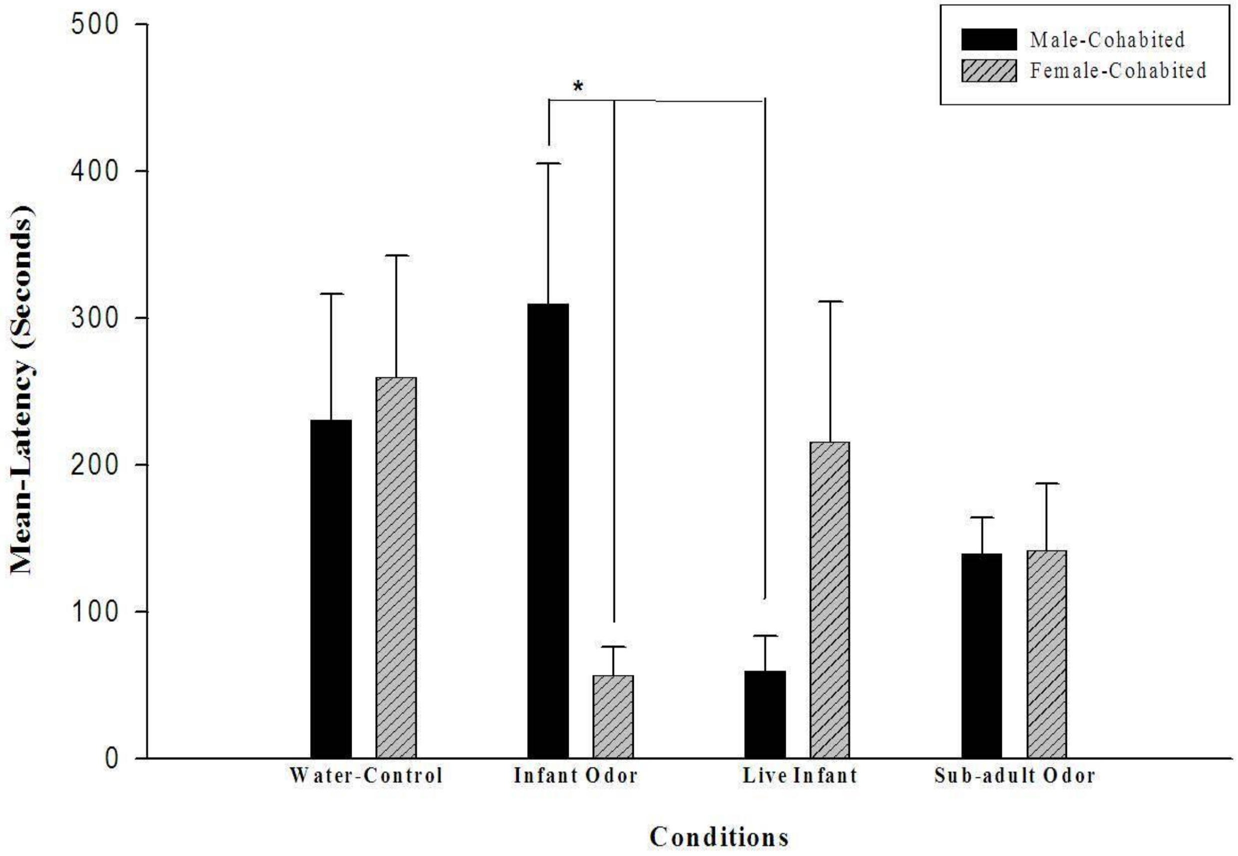


Figure 3.1: Latency to approach the object cup (object cup contained two live infants or two doll pups covered with infant odor, sub-adult odor or water) by male prairie voles that cohabited with their same-sex sibling (Male-Cohabited) or males that cohabited with an unrelated female (Female-Cohabited) for 13 days. Bars represent means (± SEM). The symbol above the bars (*) indicates significant differences at $P \leq 0.05$. When the object cup contained pup dolls covered with infant odors, Female-Cohabited males approached the cup faster than the Male-Cohabited males.

Figure 3.2

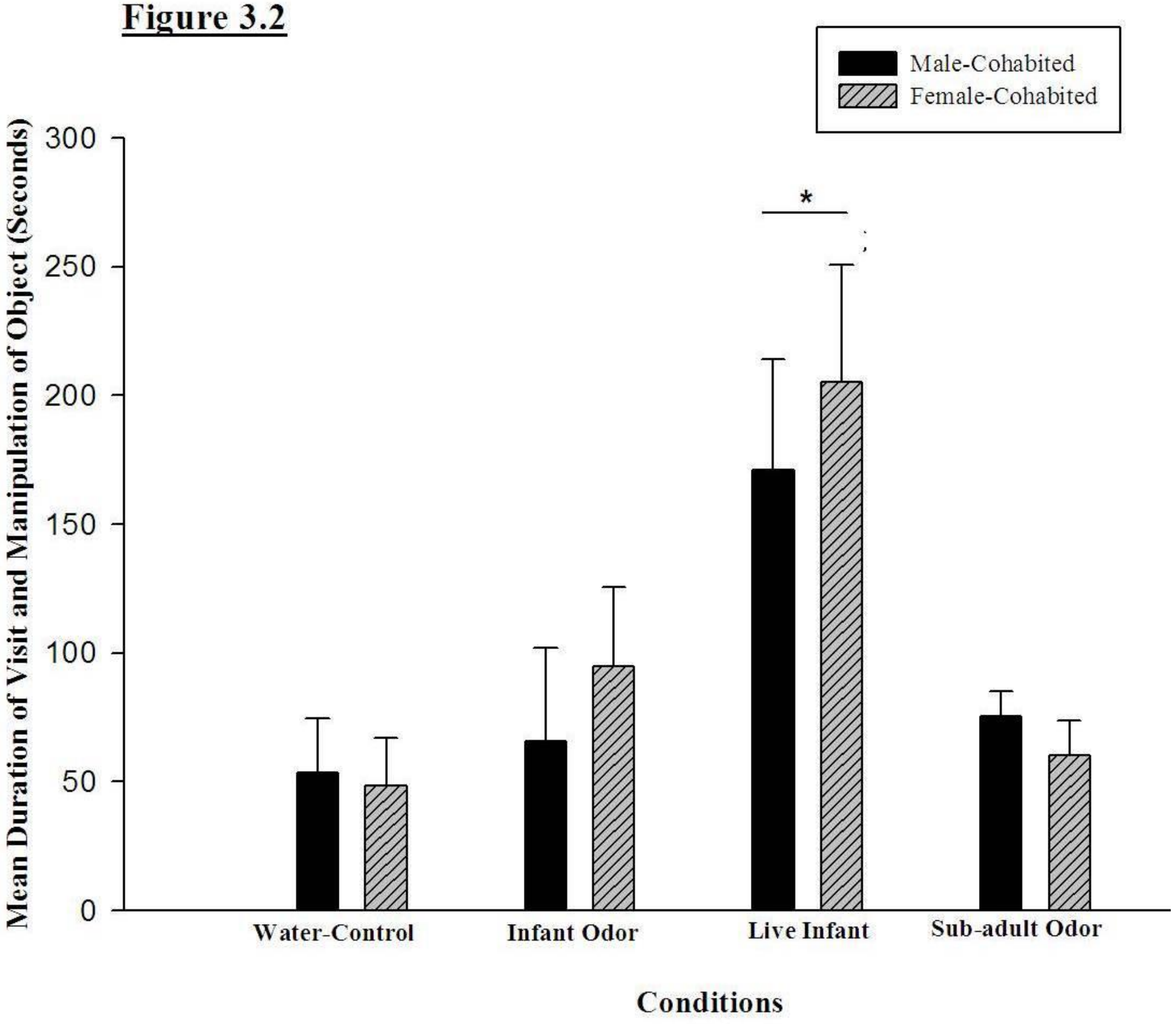


Figure 3.2: Duration of time visiting combined with manipulating objects by male prairie voles that cohabited with their same-sex sibling (Male-Cohabited) or males that cohabited with an unrelated female (Female-Cohabited) for 13 days when they were exposed to the object cup containing live infants or doll pups covered with infant odor, sub-adult odor or water. Bars represent means (± SEM). The symbol above the bars (*) indicates significant differences at $P \leq 0.05$. Male voles spent more time visiting and manipulating live infants than pup dolls covered with various odors.

Figure 3.3

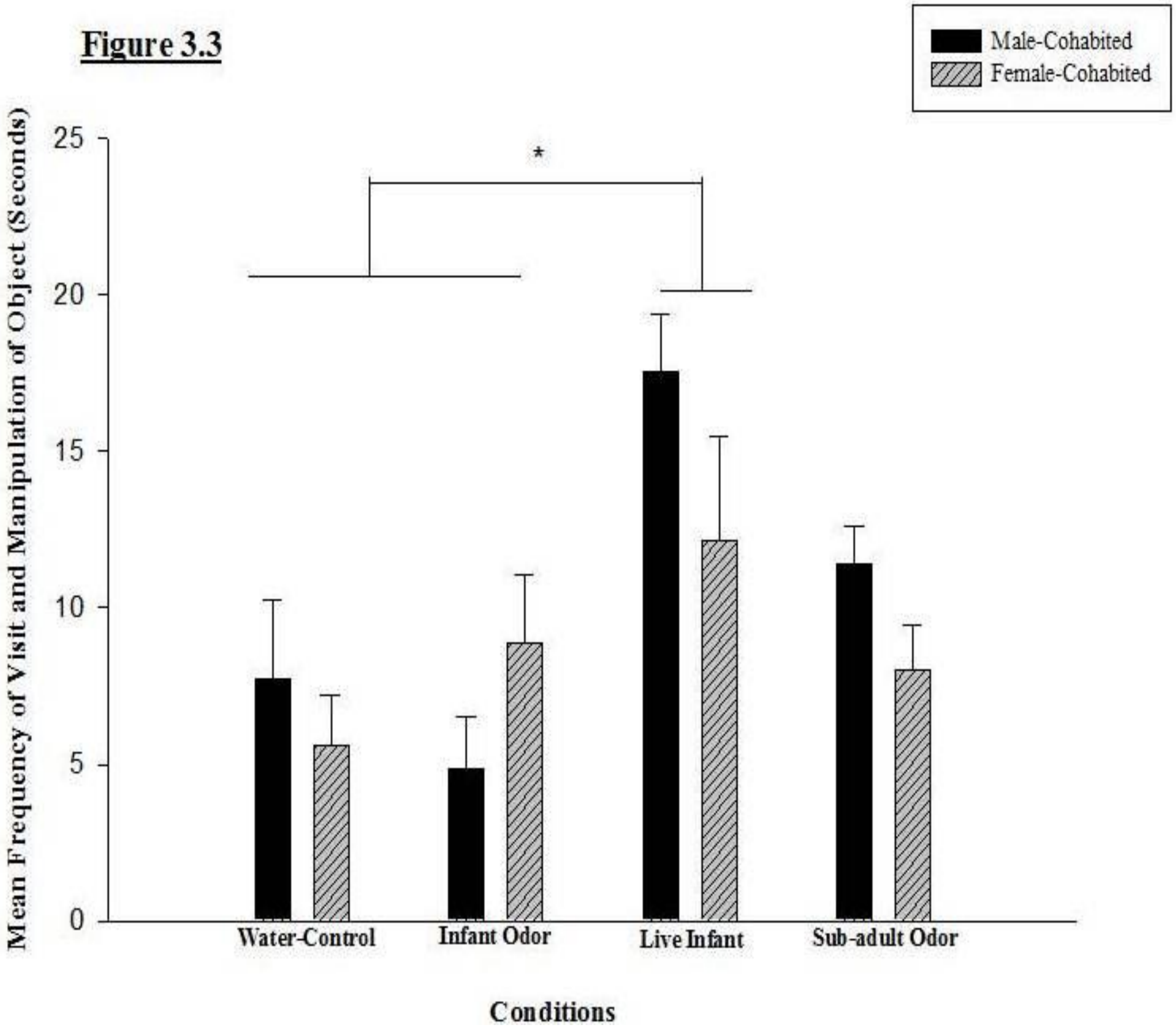


Figure 3.3: Frequency of time visiting and manipulating object by male prairie voles that cohabited with their same-sex sibling (Male-Cohabited) or males that cohabited with an unrelated female (Female-Cohabited) for 13 days when they were exposed to the object cup containing live infants or doll pups covered with infant odor, sub-adult odor or water. Bars represent means (± SEM). The symbol above the bars (*) indicates significant differences at $P \leq 0.05$. Male voles visited and manipulated live infants more frequently than pup-dolls covered with infant odors.

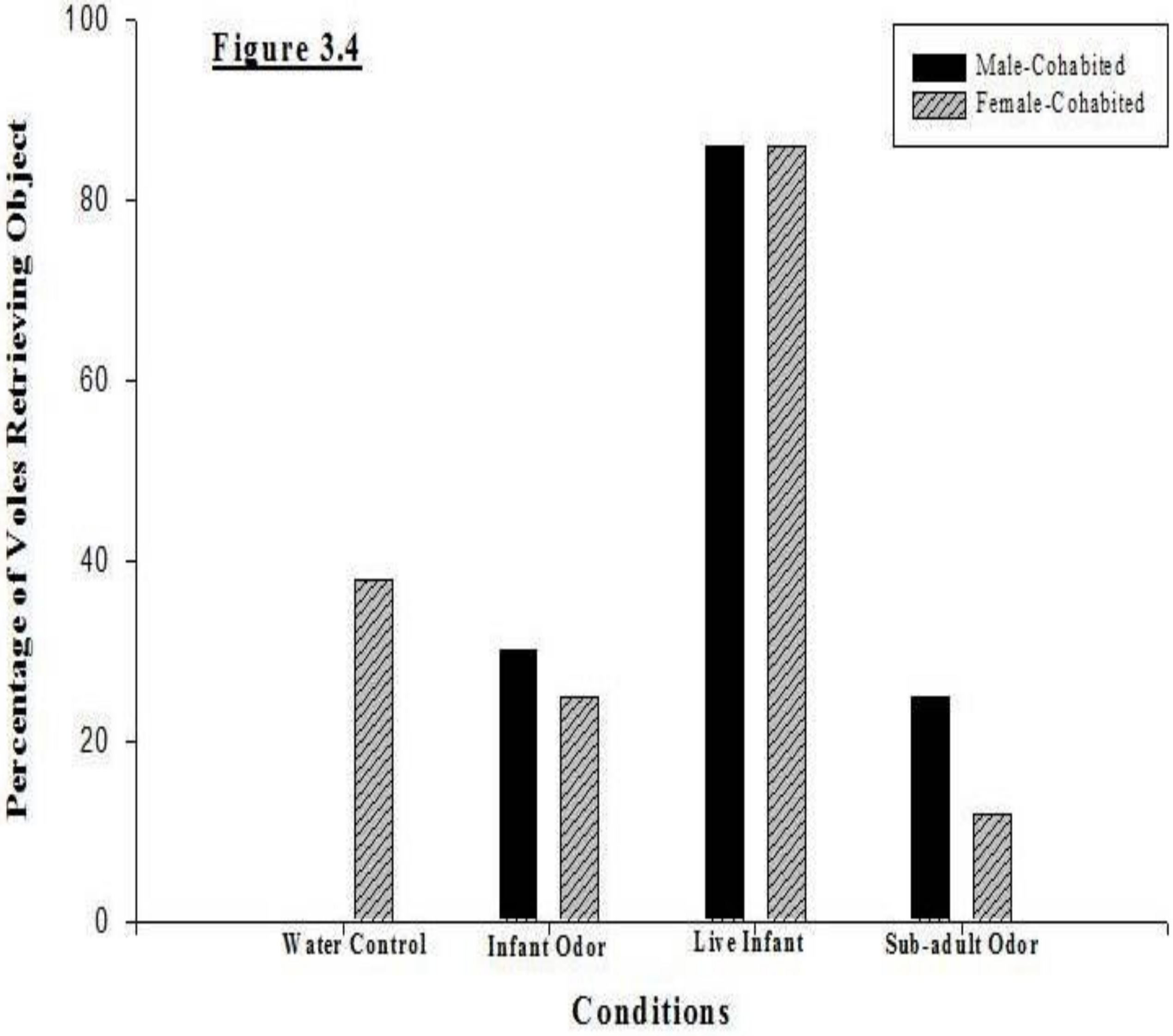


Figure 3.4: Percentage of male prairie voles that retrieved the objects out of the object cup. The object cup contained live infants or doll pups covered with infant odor, sub-adult odor or water. The subjects tested were males that cohabited with a same-sex sibling (Male-Cohabited) and males that cohabited with an unrelated female (Female-Cohabited) for 13 days. Female-Cohabited subjects retrieved all objects regardless of type and odor, but Male-cohabited subjects did not.

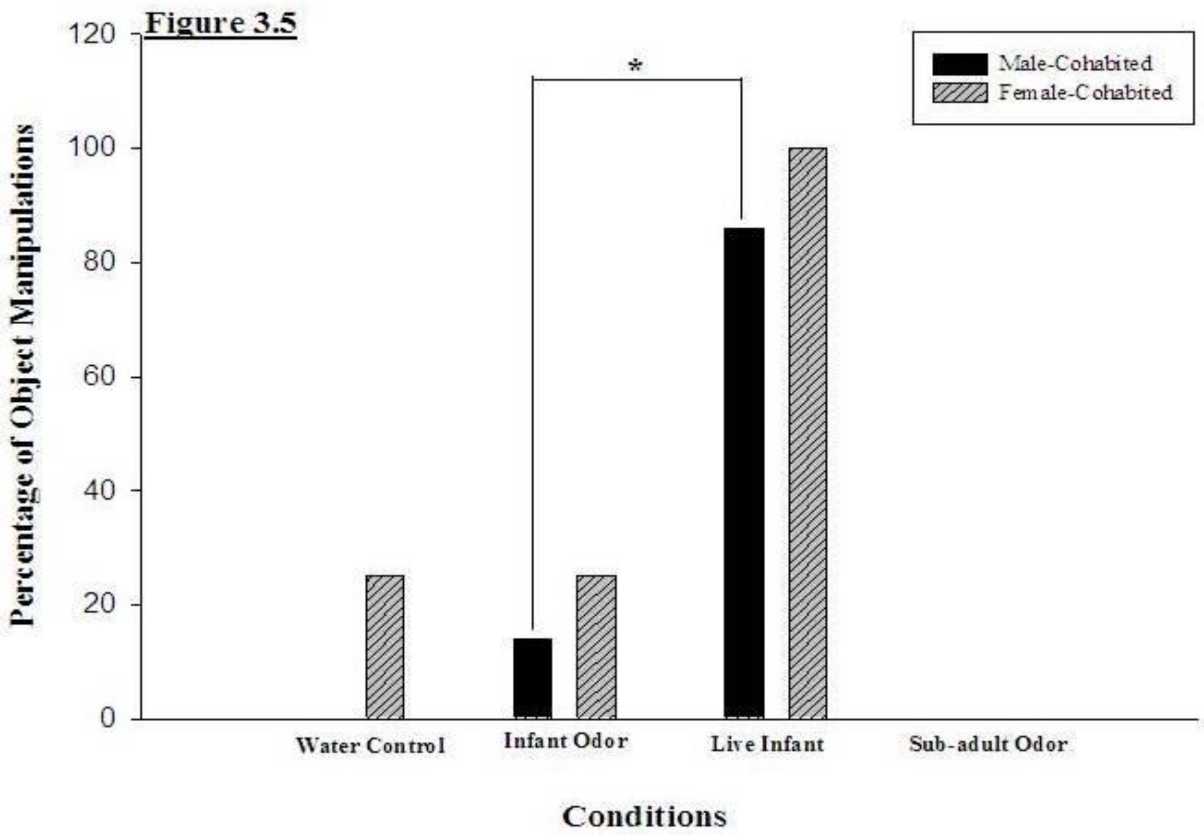


Figure 3.5: Percentage of male prairie voles that manipulated (contacting, crouching, licking or chewing) the objects retrieved from the object cup. The object cup contained live infants or doll pups covered with infant odor, sub-adult odor or water. The subjects tested were males that cohabited with a same-sex sibling (Male-Cohabited) and males that cohabited with an unrelated female (Female-Cohabited) for 13 days. The symbol above the bars (*) indicates significant differences at $P \leq 0.05$. A higher percentage of Female-Cohabited and Male-Cohabited subjects' manipulated live infants, but none of them manipulated doll pups covered with sub-adult odor. A significantly higher percentage of Male-Cohabited males manipulated live infants than doll pups covered with infant odors.

Figure 3.6

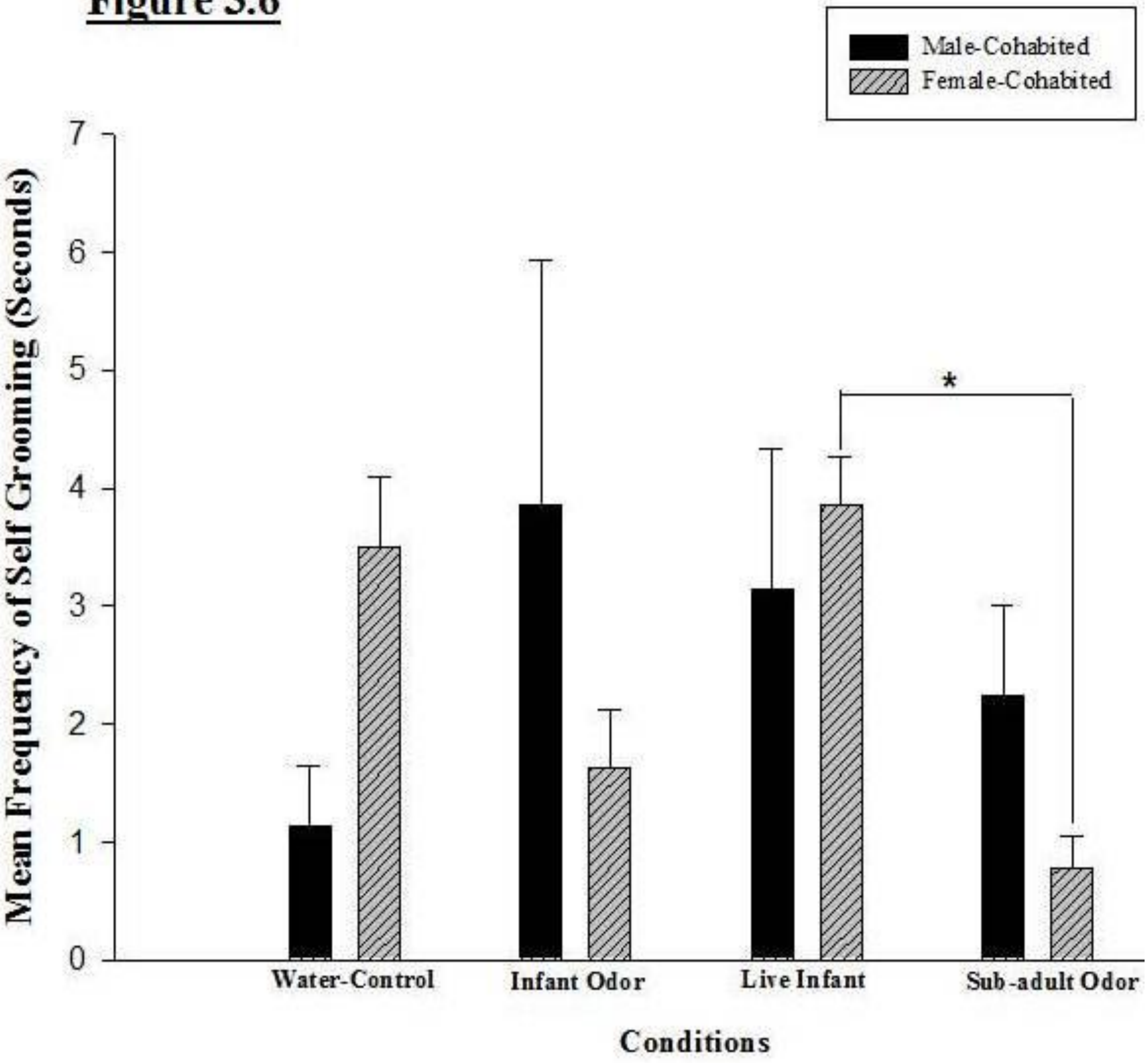


Figure 3.6: Frequency of self-grooming by male prairie voles that cohabited with their same-sex sibling (Male-Cohabited) or males that cohabited with an unrelated female (Female-Cohabited) for 13 days when they were exposed to the object cup containing live infants or doll pups covered with infant odor, sub-adult odor or water. Bars represent means (± SEM). The symbol above the bars (*) indicates significant differences at $P \leq 0.05$. Female-Cohabited voles spent more time self-grooming in the presence of live infants than in the presence of sub-adult odors.

Plate 3.3:

Male brooding over pups in nest it built.

Pups placed in nest by male vole

Plate 3.3*:* The photo depicts paternal behavior observed in male prairie voles, where the male engages in indirect caring (bottom plate) by building a nest and retrieving the pups into the built nest. In addition, males perform direct caring by brooding over the pups (top plate).

Discussion:

This experiment was designed to study the importance of infant odors in stimulating direct and/or indirect paternal care in male prairie voles and to determine if female sensory signals impact the male’s responses towards infant-related cues. Given that male prairie voles are spontaneously paternal (Roberts et al. 1998; Lonstein et al. 1999a), I had predicted that they would show a greater response towards live infants compared to other odors regardless of their reproductive status. As male prairie voles’ paternal responsiveness is enhanced following cohabitation with a female (Terleph et al. 2004, Yamoah et al. 2008), I had also predicted that Female-Cohabited subjects would be more likely to engage in direct paternal responses in the presence of infant odors but not other social odors compared to Male-Cohabited subjects.

Although male prairie voles exhibit parental tendencies throughout their reproductive cycle (Roberts et al. 1998; Lonstein et al. 1999a), the results showed that male voles that had cohabited with an unrelated female, and thus were possibly expecting to sire offspring (‘expectant’ fathers), were more attentive to and had a faster approach towards pup dolls covered with infant odors than male voles that had cohabited with a same-sex sibling (‘non-expectant’ males). In the previous study, changes in attentiveness towards infant odors was observed in male prairie voles (Yamoah et al. 2008) which is consistent with the enhanced approach to infant odors observed in ‘expectant’ fathers in this study. Given that Female-Cohabited male prairie voles’ show increased paternal care (Bamshad et al., 1994), the data suggest that one of the changes that occur when male prairie voles cohabit with their mate is increased attentiveness towards infant related sensory cues. Furthermore ‘expectant’ fathers unlike ‘non-expectant’ males, spent more time self-grooming when they were exposed to live infants than when they were exposed to pup dolls covered with sub-adult odors. These males may have groomed themselves more partly because they were more sensitive to odoriferous molecules from the live infants. Enhanced sensitivity to odors of live infants may have resulted from endocrine and neural changes that occur when males expect to sire offspring (Ferkin et al. 2005)

However, infant odors alone did not result in enhanced direct paternal responsiveness (object visits and manipulations) in the ‘expectant’ fathers or the ‘non-expectant’ males. These data suggest that olfactory cues alone are not sufficient to produce enhanced direct paternal care in male prairie voles. On the other hand, the percentage of ‘expectant’ fathers manipulating the objects was not significantly different when they were exposed to live infants or pup dolls covered with infant odors. Whereas the percentage of ‘non-expectant’ males manipulating pup dolls covered with infant odors was significantly lower than the percentage of these males manipulating live infants. These results indicate that infant odors alone were able to alert and trigger attentiveness in a higher number of ‘expectant’ fathers than in the ‘non-expectant’ males. Furthermore, neither ‘expectant fathers nor ‘non-expectant’ males investigated objects infused with sub-adult odors suggesting that both groups lacked interest in objects that looked like infants but smelled like sub adults. Therefore, male prairie voles may be able to recognize the mismatch between the odor and its source.

In addition, a higher percentage of the ‘expectant’ fathers manipulated live infants as well as inanimate objects covered with odors whereas most ‘non-expectant’ males did not manipulate the inanimate objects. The ‘expectant fathers’ may have been more attentive to all objects presented to them because they have greater exploratory tendencies, investigating their environment more so than the ‘non-expectant’ males, suggesting indirect paternal behavior in the ‘expectant’ fathers. These data are in agreement with reports of the foraging tendencies exhibited by mated male prairie voles in search of higher food quality for their offspring to maximize infant survival (Cole et al. 1979; Lin et al. 2001). ‘Expectant’ fathers retrieved all objects including those that looked like a pup, but smelled differently, whereas the ‘non-expectant’ males were not as interested in retrieving objects other than live infants. This might be because ‘expectant’ fathers assessed their environment more and were more attentive to sensory stimuli than the ‘non-expectant’ males. The result is consistent with the hypothesis of Boliver et al. (1995) who, showed that maternal rats become more caring and tend to retrieve mutant pups compared to normal litter mates, despite the reduced ultrasonic vocalization rate and compromised fitness of the mutant pups.

The fact that subjects in both groups spent the same amount of time in locomotion and visiting the odor container implies that the ‘expectant’ fathers and the ‘non-expectant’ males were able to freely move and had equal proximity to the objects irrespective of their cohabitation state. Therefore differences in behavioral output between the groups were not due to the lack of activity or access to object source.

In summary, although cohabitation with a female may induce male prairie voles’ to become aroused by infant-related odors, infant odors alone are not sufficient to stimulate attentiveness required for direct paternal care. Exposure to the female’s sensory cues during the post-mating cohabitation period appears to enhance the male’s interest in investigating sensory objects in its environment and may contribute to indirect paternal behavior. Such increased attentiveness to sensory cues might be necessary for the male to engage maximally in direct and indirect paternal behavior once his offspring are born and he is exposed to their olfactory, visual, auditory and tactile stimuli.

CHAPTER 4

PATTERN OF NEURONAL ACTIVATON IN MALE PRAIRIE VOLE'S BRAIN IN RESPONSE TO INFANT-RELATED STIMULI

- ***To identify the brain regions involved in perception of infant-related sensory stimuli associated with paternal care***

Introduction:

The precondition for parenting in most mammals is formation of a social bond between the parent and offspring (Numan et al. 2003; Broad et al. 2006). For such social bond to be established, the parent must perceive infant sensory stimuli as rewarding. Research in rodents and humans has shown that mothers or 'expectant' mothers display enhanced maternal behavior and caring associated neuronal activity when they are exposed to olfactory, visual or auditory infant cues (Beach et al., 1956; Fleming et al. 1993; Carter et al. 2001; Numan et al. 2003). For example, sexually inactive female rats are fearful of infant sensory inputs, but those in their late pregnancy and postpartum period are attracted to pup odors and such cues enhance their maternal care (Fleming et al. 1986; Kolunie et al. 1995). Also, Studies in humans have shown that early postpartum primiparous women are more attracted to infant odors than nulliparous women (Fleming et al. 1993). These results suggest that in females which are physiologically prepared for parenting, infant sensory inputs activate neural mechanisms associated with social reward, thus contributing to maternal bonding and caring (Numan et al. 2003; Broad et al. 2006).

As most mammals are promiscuous and uniparental, (Kleiman et al. 1981), research has focused on understanding the neural mechanisms of maternal behavior.

Thus, I know very little about brain regulation of paternal bonding or behavior. In biparental rodents, male and female parents perform similar activities to care for offspring such as infant huddling, brooding, licking, retrieving and nest building (Kleiman, 1977). However, the mechanisms that stimulate or enhance parental responsiveness are different in males and females. Studies in rats have shown that activation of neuronal pathways involved in the onset of maternal bonding and caring is triggered by the process of pregnancy and parturition (Rosenblatt et al. 1990; Bitran et al., 1995; Fernández-Guasti et al. 1995; Hayes et al. 2007). Males however do not undergo these physiological changes hence other factors may stimulate the neuronal pathway responsible for paternal care.

Studies performed in certain male rodents that are naturally paternal have indicated that female sensory stimuli are important in stimulation, maintenance or enhancement of paternal responsiveness (Gubernick et al. 1989). For instance the male California mice (*Peromyscus californicus*) only show parental care when the female is present (Gubernick et al. 1989). Therefore, it is possible that female sensory stimuli prepare the male brain to respond positively to infant cues in the same way that pregnancy hormones prepare the female brain to respond positively to infant cues (Rosenblatt et al. 1963). The aim of this study was to identify the brain regions involved in perception of infant-related sensory stimuli in male prairie voles that display paternal behavior under natural conditions (Roberts et al. 1998). A second focus of this study was to determine if neuronal activation in these brain areas are increased when the male prairie vole is prepared to respond to infant-related sensory stimuli through cohabitation with his mate.

A common way to map brain areas underlying behavior is to examine the expression of immediate early genes after exposing an animal to a sensory stimulus. The *c-fos* gene belongs to the *Fos* family of genes (*c-fos, fos B, fra 1 and fra 2*) referred to as immediate early genes (Sheng et al. 1990). The *c-fos* gene expresses the *Fos* protein in response to a variety of physiological and behavioral stimuli. Neurons affected by extracellular signals (e.g. hormones and neurotransmitters) undergo a biosynthetic process which usually requires the activation of immediate early genes which is then followed by late responding genes (Turner et al. 1989). There are immediate early genes other than *Fos* that can be expressed in cells that may or may not have *Fos* (Wang et al. 1992). Such immediate early genes include *c-myc and c-jun*. *Fos* protein immunocytochemistry however, is a well known technique used to identify the activated brain regions of most rodents stimulated by chemosensory cues (Moffatt et al. 1995) including the prairie vole (Fiber et al. 1993; Tubbiola et al. 1997). Immediate early genes may also be involved in transcriptional processes that can either cause an inhibitory or an excitatory effect in the cell (Sheng et al. 1990). Thus the detection of the *Fos* protein in a brain region will only suggest neuronal activity in that part of the brain. This activity could either be inhibitory or excitatory. Several brain areas have been implicated in maternal behavior (Numan et al. 2003). The areas of the brain I chose to look at were areas that have been shown to express *Fos* protein in prairie voles and have also been implicated in social behavior including parental care. These brain areas are the ventral tegmental area (VTA) (Insel et al. 2003; Febo et al. 2005), bed nucleus of the stria terminalis (BNST) (Numan et al. 2003) and the lateral septum (LS) (Wang et al. 1994; Numan et al. 2003).

The VTA is involved in strengthening of mother–infant bond formation in rats (Febo et al. 2005). The VTA regulates olfactory discrimination that underlies increased maternal motivation and leads to enhanced maternal behavior in rats (Febo et al. 2005). In addition, the VTA is involved in foraging activity like nest building, food hunting and infant retrieval in rodents which is essential for offspring survival (Kelley et al. 1985; Panksepp, 1998). Furthermore an electrical lesion of the VTA has been shown to disrupt maternal behavior in rats (Gaffori et al. 1979).

Research has also shown that *Fos* protein activation occurs in the BNST of female rats (*Rattus norvegicus*) performing maternal care (Numan et al. 2003). Furthermore, when male prairie voles cohabit with an unrelated female, *Fos* is expressed in the BNST (Cushing et al. 2003). Additional evidence for involvement of BNST in paternal behavior comes from research showing that the activity of vasopressin neurons in this region is increased in male prairie voles following post-mating cohabitation with a female (Bamshad et al. 1994; Wang et al. 1994). Several lines of research have shown that vasopressin plays a role in paternal behavior. For example, comparative studies in California mice, a monogamous and biparental species have found more vasopressin fibers in the BNST and the LS compared to their promiscuous counterparts (*Peromyscus leucopus*) (Insel et al. 1991). Also, Wang et al. (1994) showed an increase in paternal behavior when arginine vasopressin was injected into the LS of male prairie voles. Furthermore, the BNST is also involved in the onset of parental care in female rats and female prairie voles (Katz et al. 1999). Together, these data reinforce the hypothesis that the BNST is an important brain region for paternal behavior and that vasopressin activation in the BNST and the LS may be involved in stimulation or enhancement of paternal responsiveness.

In this experiment, I tested the working hypothesis that infant odors alone could activate brain areas involved in paternal responsiveness. I also predicted that activation of such brain regions would be greater in males that are ‘expectant’ fathers as they are prepared for infant care through their continuous exposure to female sensory stimuli. To test this hypothesis, males were cohabitated with females (Female-Cohabited) or with a same-sex sibling (Male-Cohabited) for 13 days. The subjects were then exposed to live infants or to doll pups covered with infant odors, sub adult odors, or water. The brains were stained immunocytochemically for *Fos*-like proteins. *Fos*-positive nuclei were counted in the VTA, BNST and LS. Differences in *Fos* expression in the brain between Female-Cohabited and Male-Cohabited subjects could reveal brain areas that are part of the paternal neuronal circuitry in male prairie voles. The activation of these brain areas may be responsible for the qualitative and enhanced behavioral output exhibited by ‘expectant’ fathers.

Methods:

- *Subjects*

The subjects were males used for behavioral testing as described in Chapter 3. Following behavioral testing, each subject was anesthetized and perfused intracardially to fix the brain. The brain tissues were extracted and used for *Fos* immunocytochemistry. Although the observation time for behavioral analysis was limited to 10 minutes, the voles were left un-interrupted for 120 minutes in their respective testing conditions. As the protein products of immediate early gene *Fos* is expressed in brain nuclei within 2 hours following sensory stimulation (Moffatt et al. 1995; Hairston et al. 2003), I was able to identify brain regions that are activated in response to infant-related sensory cues by staining the brain slices for *Fos*-like proteins.

- *Perfusion*

To fix the brain, each male was first anesthetized with 0.2ml of Ketamine cocktail. The animal was then perfused with 0.9% saline for 3 minutes at a rate of 25 ml/minute to wash out the blood, followed by 4% paraformaldehyde solutions for 5 to 6 minutes depending, on the weight of the animal, at a rate of 25ml/minute. The paraformaldehyde was used to fix proteins including *Fos* in the brain so they could be visualized by performing immunocytochemistry. The perfusion was conducted by introducing the respective solutions to the left ventricle of the heart followed by an incision on the right atrium which served as an outlet for the fluids to flow out of the body. The fixed brains were removed and post-fixed in 4% paraformaldehyde at 4ºC for 24hrs. The brains were then transferred into a 25% sucrose solution at 4ºC overnight. Each brain was blocked anterior to optic chiasm and posterior to the cerebellum. The brain tissue was sliced with a freezing microtome at 50µm thickness. Brain slices were collected into 4 vials labeled A, B, C and D containing a 0.05M Tris-NaCl buffer solution. The sliced brain sections were then transferred into vials containing crytoprotectant and stored at -20 ºC. *Fos* immunocytochemistry was performed when brains of all subjects were extracted and sectioned.

- *FOS immunocytochemistry*

Fos immunocytochemistry was performed on one of the four vials and the remaining three vials were stored in the freezer for other experiments. Sections were incubated in 3% H_2O_2 for 20 minutes at room temperature and then rinsed 3 times for 5 minutes (3x5) with Tris-NaCl. Brain sections were then placed in 20% donkey normal sera in 0.3% Tris-Triton for 10 minutes. Next, sections were placed in goat anti-*Fos* antibody diluted at 1:5000 in 2% Tritrigo and 0.1% Bovine serum albumin and left for 24 hours on a gentle shaker at room temperature. After 24hrs, sections were rinsed 3x5 minutes with 2% Tritrigo. Sections were then placed in biotinilated donkey anti-goat secondary antibody diluted at 1:200 in 2% Tritrigo for 1 hour. The sections were rinsed 2x5 minutes with 2% Tritrigo and 1x5 minutes with Tris-NaCl. Next, the sections were placed in Avidin-Biotin horseradish peroxidase (HRP) complex (ABC Elite kit) in 1:100 dilution for 1 hour. After an hour, sections were rinsed 3x5 minutes with Tris-NaCl and then stained with 3,3- diaminobenzedine (DAB substrate kit), intensified with nickel chloride for 5 to 7 minutes, followed by 3x5 minute rinse with Tris-NaCl. The stained brain sections were then mounted on gelatin-coated slides, air dried and cover-slip with DPX.

- *FOS count and analysis*

An Olympus bright-field microscope connected to a computer and equipped with a high resolution color digital camera was used to visualize and analyze *Fos* stained nuclei. Images of slide sections at 10X magnification were projected onto a computerized image analysis system (Image Pro Plus). Photographs of areas of interest were taken with the digital camera and saved on an external drive. The areas where *Fos* was to be counted were first identified on a Rat Atlas (Paxinos et al. 1980). A rat atlas was used since the anatomy of the vole brain is quite similar to the rat brain for the brain areas that were analyzed (Vandebroek et al. 1999). Each of the *Fos*-stained slides was blind-labeled. Darkly stained *Fos*-positive nuclei were then manually counted one at a time with a *cross hair* tool provided in the measurement tool box of the image analysis program. The cross-hair tool counts objects and leaves a marker on each counted object so that objects are not counted twice. *Fos* was counted on both the left and right side of the hemisphere in the VTA (bregma -6.04 and -6.30), BNST (bregma 0.20mm and -0.26mm) and LS (bregma 0.20mm and -0.26mm) and then averaged. The averaged number of *Fos* for each area was used for data analysis. The data were analyzed using a two-way analysis of variance (ANOVA), with cohabitation and odor-conditions as between subject variables. Significant differences at $P \leq 0.05$ were further analyzed with the Tukey-Kramer Multiple-Comparison Test.

Results:

Out of 72 voles initially used for the behavioral experiment described in chapter 3, I was able to analyze 53 brains for the VTA, 65 brains for the BNST and 64 brains for the LS. The remaining brains were damaged mainly during slicing and tissue preparation.

- *Fos Analysis*

Results for *Fos* expression in the brain nuclei area is shown in Figure 4.1 and Appendix C. The areas analyzed for *Fos* expression was the ventral tegmental area (VTA), bed nucleus of the stria terminalis (BNST) and the lateral septum (LS).

Results for VTA analysis showed a significant group difference, F [1, 44] = 6.3, p = 0.02. There was also a significant interaction effect between the groups and conditions, F [3, 44] = 3.99, p = 0.01(Figure 4.1). However the difference among the conditions was not significant. *Fos* expression in the VTA of Female-Cohabited males exposed to live infants was significantly higher than the VTA of Male-Cohabited males exposed to live infants. Expression of *Fos* in the BNST was not significantly different among the groups and among the conditions. The interaction effect between the group and conditions for *Fos* expression was close to significance, F [3, 57] = 2.42, p = 0.08. In the LS, *Fos* expression showed a significant interaction effect between the groups and conditions, F [3, 56] = 3.07, p = 0.04. However *Fos* expression in the LS, among the groups F [1, 56] = 3.75, p = 0.06 and among the conditions, F [3, 56] = 2.28, p = 0.09 was close to significance.

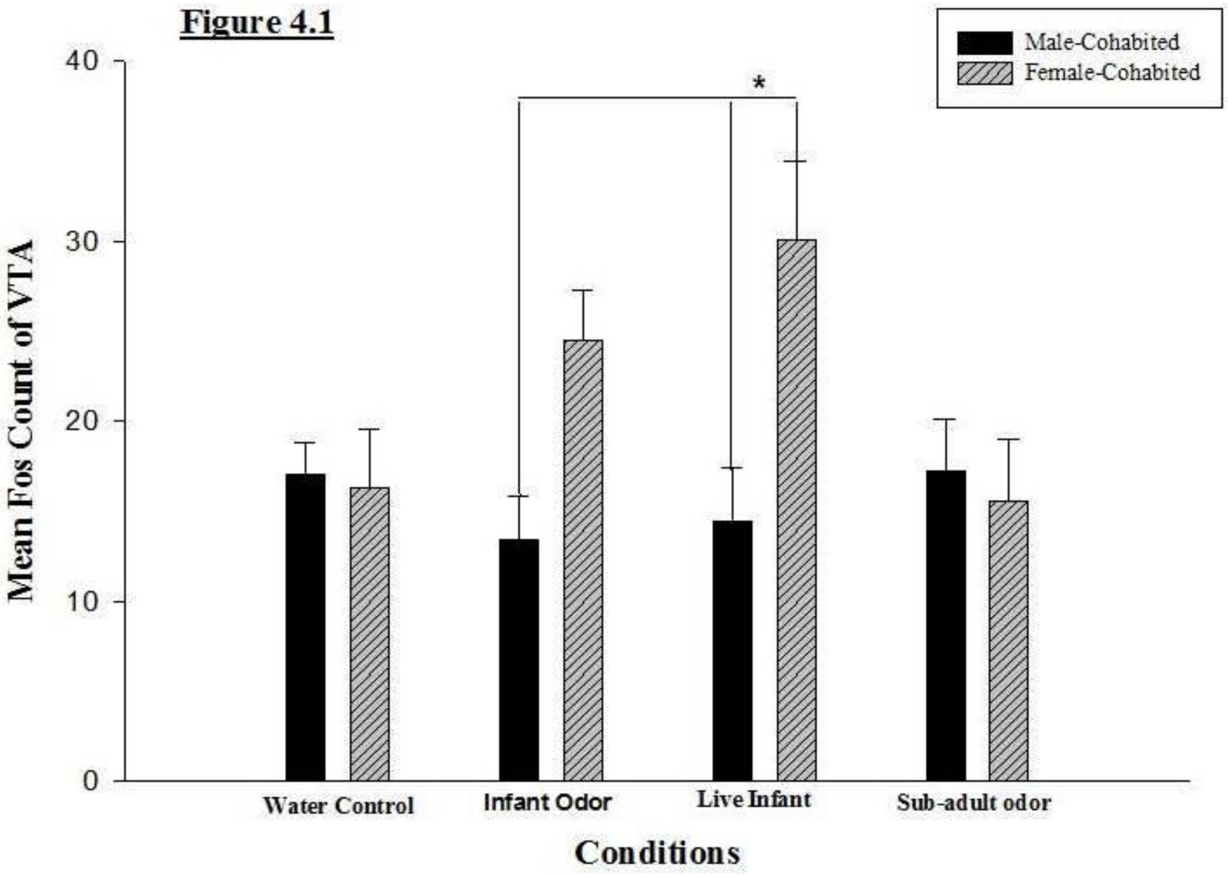
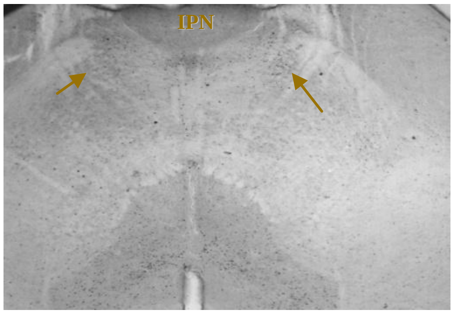
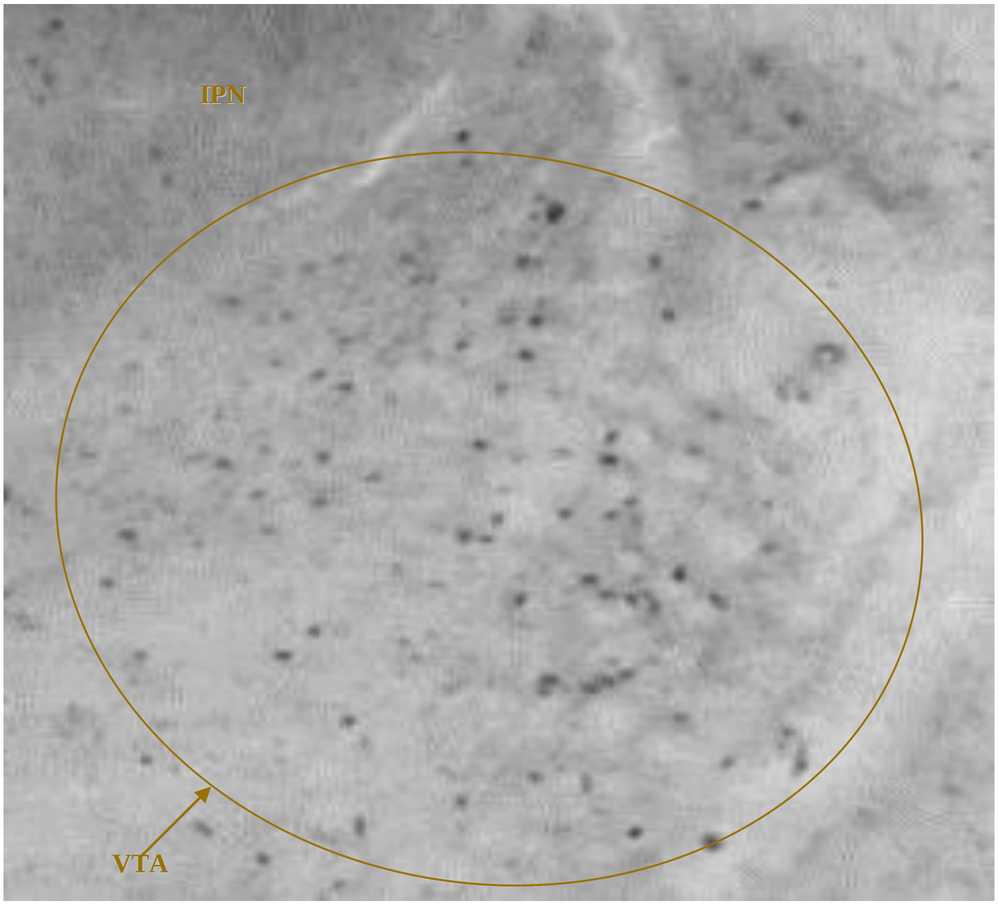


Figure 4.1: Mean of Fos-positive cells in the Ventral Tegmental Area (VTA) for male prairie voles that cohabited with a same-sex sibling (Male-Cohabited) and male prairie voles that cohabited with an unrelated female (Female-Cohabited) for 13 days. Male-Cohabited and Female-Cohabited subjects were exposed to an object cup containing live infants or doll pups infused with infant odor, sub-adult odor or water. Bars represent means (± SEM). The symbol above the bars (*) indicates significant differences at $P \leq 0.05$. Female-Cohabited voles had more Fos-positive nuclei compared to Male-Cohabited male voles when they were exposed to live infants.

Brain Areas and FOS

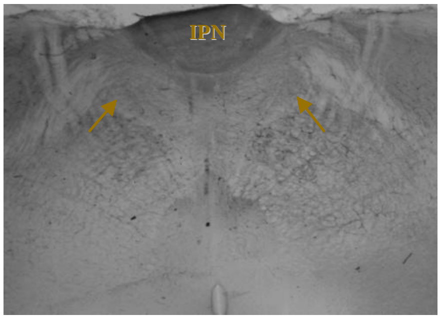
Slide 4.1: Ventral Tegmental Area (VTA) → Fos

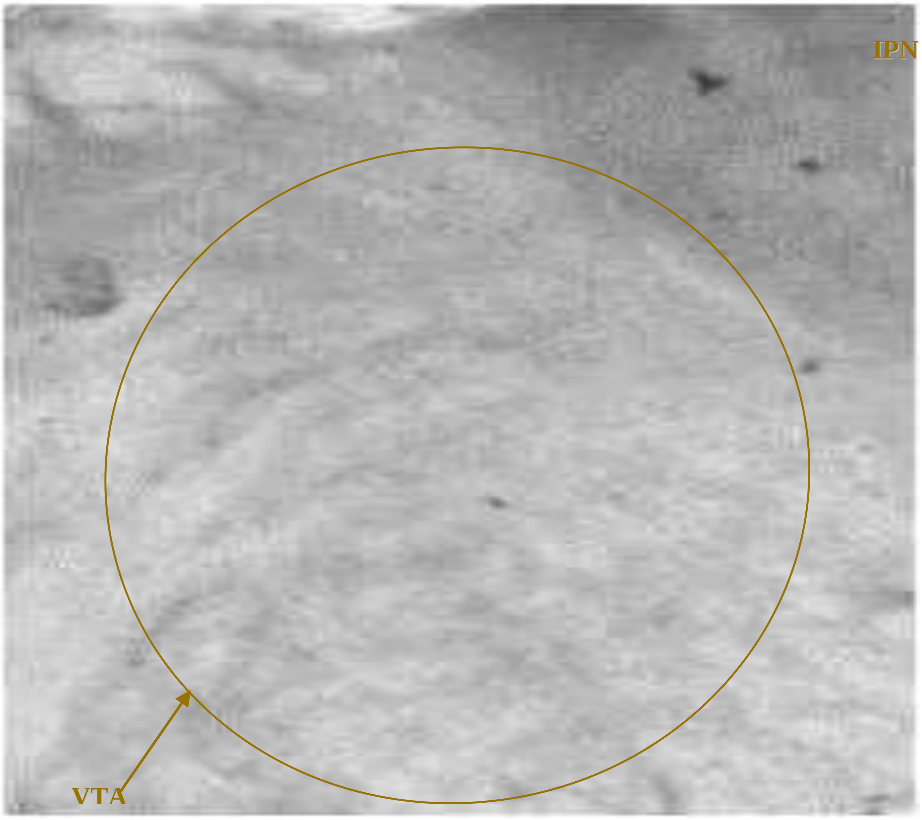




Slide 4.1: Arrows point to ventral tegmental area (VTA). Slide shows more Fos expressing cells in the VTA of Female-Cohabited male prairie voles. IPN is the Inter Penduncular Nucleus

Slide 4.2: Ventral Tegmental Area (VTA)→ Fos





Slide 4.2: Arrows point to ventral tegmental area (VTA). Slide shows less *Fos* expressing cells in the VTA of Male-Cohabited male prairie voles. IPN is the Inter Penduncular Nucleus

Discussion:

This experiment was designed to identify brain areas activated by infant-related sensory cues in ‘expectant’ fathers. I predicted that since ‘expectant’ fathers were more attentive in the presence of infant-related sensory cues compared to ‘non-expectant’ males, specific brain area(s) may be more activated. Results showed that *Fos* expression in the VTA was high in ‘expectant’ fathers that were exposed to live infants compared to the VTA of ‘non-expectant’ males. I had also predicted that in the BNST which is the brain area involved in the onset of parental care (Katz et al. 1999), *Fos* will be expressed in both the ‘expectant’ fathers and ‘the non-expectant’ males equally, as male prairie voles show paternal care throughout their adult life (Roberts et al. 1998). Finally as the LS is vital for the performance of paternal behavior in male prairie voles (Wang et al. 1994), I had predicted that this region will be associated with enhanced behavioral output associated with manipulating and retrieving of objects.

Research in rats has shown that the VTA influences behavioral enhancement associated with approach responses, exploratory tendencies, spontaneous behavior and attention processing activity (Kelley et al. 1985). Therefore higher *Fos* expression in the VTA of ‘expectant’ fathers suggests that these males had higher approach responses tendencies which could lead to their enhanced exploratory tendency and attention processing activity. In addition, VTA which is rich in dopamine, a neurotransmitter involved in reward and appetitive behavior (Numan et al. 2003) could have produced a rewarding effect in the ‘expectant’ fathers leading to the alert behavioral output observed. Furthermore VTA is involved in social bonding in the monogamous prairie voles (Curtis et al. 2005) and could possibly influence a father-infant bond formation in the ‘expectant’ fathers.

In the BNST which is a brain area involved in the onset of parental care in female rats and female prairie voles (Katz et al. 1999), data confirmed that the onset of parental behavior occurs in male prairie voles irrespective of their reproductive state (Getz et al. 1981; Roberts et al. 1998; Lonstein et al. 1999a) and cohabitation state, since the level of *Fos* expressed in the BNST was the same for both the ‘expectant’ and ‘non-expectant’ male prairie voles. Thus irrespective of the quality of the behavioral output, BNST is necessary for the onset of parental care in male prairie voles and hence BNST activation induced the onset of paternal care in both the ‘expectant’ and ‘non-expectant’ males.

Although male prairie voles manipulated the objects which confirm the involvement of LS in parental responsiveness (Wang et al. 1994), the data for *Fos* expression in the LS, was not clear. A confounding variable that could have affected the outcome of this result was the variability of the sample size in the sub-groups. Further studies are required to clarify the role of LS in infant-odor induced behaviors of male prairie voles.

In summary, ‘expectant’ male prairie voles showed a difference in neuronal activation in the VTA compared to ‘non-expectant’ males consistent with the increased arousal seen in the ‘expectant’ fathers in the presence of infant-related sensory cues.

CHAPTER 5

GENERAL DISCUSSION

GENERAL DISCUSSION

Together, the results of my studies suggest that a higher number of male prairie voles that cohabit with unrelated females show attentiveness and sensitivity towards odors on the 13th day of cohabitation. In the presence of infant related sensory cues, these males showed enhanced paternal behavior. Infant odors alone induced indirect paternal care in these males however infant odors alone were not sufficient to elicit direct paternal behavioral responses. The increased activation of the ventral tegmental area (VTA) in the brain of males that cohabited with unrelated females for 13 days suggests that this area is involved in enhanced exploratory behavioral tendencies associated with indirect paternal care.

Parental behavior is a social behavior directed towards a reproductively immature conspecific that could enhance it’s survival to maturity (Kleiman et al. 1981; Numan et al. 2003). Parental care occurs in other vertebrate and invertebrate species but the behavior is highly developed in birds and mammals (Clutton-Brock, 1991). In most mammals, the female cares for young as she is the one which becomes pregnant, gives birth and lactates (Gubernick et al. 1981; Rosenblatt, 1990; Numan et al. 1994; Hayes et al. 2007). Although paternal care is rare among mammals, it is displayed by those species that have a monogamous mating system such as humans (Kleiman et al. 1981).

In certain species, the male’s presence and involvement in parental care is necessary for the survival of the offspring (Trivers et al. 1972; Kleiman et al.

1981).Several factors could stimulate the onset and maintenance of paternal care in mammals. Among these are external sensory input from both the female partner (Gubernick et al. 1989; Bamshad et al., 1993) and the infant. The studies showed that in a monogamous and biparental mammal such as the prairie vole, female sensory stimuli play an important role in enhancing paternal responsiveness by increasing male attentiveness to sensory stimuli in his immediate environment and by activating brain regions involved in parent-infant bonding and exploratory behaviors.

The results have implications for human parenting. Male humans, like prairie voles, remain with their mate throughout the gestation period. Therefore, they are also exposed to the sensory cues of their spouse. A sensory modality that is efficiently used by rodents including the prairie vole to impact social behavior is olfaction (Carter et al. 1987; Kirkpatrick et al. 1991). In humans however, olfaction may subconsciously impact the brain thus subtly influencing the modalities of social behavior. However, other senses such as visual, auditory and tactile may reach the conscious regions of the brain thus influencing behavior in a more controlled cognitive manner.

APPENDICES

Appendix A

Summary of Data Analysis

Chapter 2

Table2.1

			Cohabitation Interval					
Smell	**Measure**	**Sex**	0-Days	13-Days	18-Days	26-Days	P(Cohabit)	P(Sex)
			$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	df (3,59)	df (1,59)
Saline	**Duration**	**Female**	10.0±2.7	10.3±3.0	19.4±8.7	30.3±7.8	0.10	0.09
Saline	**Duration**	**Male**	6.2±1.7	10.6±2.2	10.5±3.8	10.0±2.6	0.10	0.09
Saline	**Frequency**	**Female**	6.0±1.1	4.4±0.5	5.9±0.8	7.9±1.1	0.04	--
Saline	**Frequency**	**Male**	5.2±1.9	6.2±0.5	7.0±1.2	7.6±0.7	0.04	--
Citrus	**Duration**	**Female**	6.7±0.9	4.3±1.0	7.5±1.5	5.3±0.5	--	--
Citrus	**Duration**	**Male**	6.0±0.9	5.5±1.3	5.5±1.0	8.9±2.7	--	--
Citrus	**Frequency**	**Female**	6.9±0.9	4.5±0.8	6.0±0.9	6.5±0.7	--	--
Citrus	**Frequency**	**Male**	6.0±0.9	6.3±0.9	7.1±1.0	7.6±1.6	--	--
Pup	**Duration**	**Female**	8.7±1.8	8.6±2.7	21.3±4.3	16.2±3.8	--	--
Pup	**Duration**	**Male**	12.6±2.8	18.8±4.7	13.7±2.4	15.2±3.8	--	--
Pup	**Frequency**	**Female**	7.3±1.0	4.9±1.3	7.5±0.9	8.0±1.5	--	0.04
Pup	**Frequency**	**Male**	6.2±1.2	9.3±0.8	9.9±1.6	9.6±1.4	--	0.04

***Table 2.1* -** Duration and frequency of sniffing each odor-covered filter paper by female and male prairie voles were analyzed by a two-way ANOVA. Significant differences of ≤0.05 and values that were close to significance are shown in the table.

Table 2.2

			Cohabitation Interval						
Manipulate	**Measure**	**Sex**	0-Days	13-Days	18-Days	26-Days	P (Cohabit)	P (Sex)	P (Cohabit x Sex)
			$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	df (3,59)	df (1,59)	df (3,59)
Saline	**Duration**	**Female**	0.2±0.2	3.3±2.4	13.1±7.9	23.3±7.8	0.002	0.08	0.06
Saline	**Duration**	**Male**	0.3±0.4	4.2±2.0	4.3±2.7	3.3±1.5	0.002	0.08	0.06
Saline	**Frequency**	**Female**	0.1±0.1	0.5±0.3	1.4±0.6	2.3±0.5	0.001	0.09	0.09
Saline	**Frequency**	**Male**	0.1±0.1	0.7±0.3	0.8±0.4	0.8±0.3	0.001	0.09	0.09
Citrus	**Duration**	**Female**	0.0±0.0	0.0±0.0	0.6±0.4	0.3±0.2	--	--	--
Citrus	**Duration**	**Male**	0.0±0.0	1.3±1.2	0.0±0.0	1.5±1.0	--	--	--
Citrus	**Frequency**	**Female**	0.0±0.0	0.0±0.0	0.0±0.0	0.2±0.2	--	--	--
Citrus	**Frequency**	**Male**	0.0±0.0	0.2±0.2	0.0±0.0	0.2±0.2	--	--	--
Pup	**Duration**	**Female**	4.3±4.3	2.3±2.0	11.3±3.1	6.5±2.7	--	--	--
Pup	**Duration**	**Male**	1.7±1.7	5.8±2.5	1.8±0.9	4.9±2.9	--	--	--
Pup	**Frequency**	**Female**	0.3±0.3	0.4±0.3	2.1±0.5	1.6±0.4	0.01	--	--
Pup	**Frequency**	**Male**	0.2±0.2	1.2±0.6	0.8±0.4	1±0.5	--	--	--

Table 2.2 **-** Duration and frequency of manipulating each odor-covered filter paper by female and male prairie voles were analyzed by a two-way ANOVA. Significant differences of ≤ 0.05 and values that were close to significance are shown in the table.

Appendix B

Summary of Data Analysis

Chapter 3

Table 3.1

			Conditions Interval						
Behavior	**Measure**	**Group**	Water Odor	Infant Odor	Live Infant	Sub-Adult Odor	P (Group) A	P (Condition) B	P (AB)
			$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	df (1,53)	df (3,53)	df (3,53)
Latency	**Duration**	**Male Cohabited**	230.2±86.2	309.8±95.5	59.7±23.5	139.3±24.8	--	--	0.02
Latency	**Duration**	**Female Cohabited**	259.9±82.5	56.5±19.5	215.5±95.8	141.7±45.3			
Self-Groom	**Duration**	**Male Cohabited**	13.7±12.2	28.3± 20.2	18.1±12.7	7.2±3.2	--	--	0.06
Self-Groom	**Duration**	**Female Cohabited**	38.4±20.7	5.1±2.2	23.1±5.4	3.0±1.9			
Self-Groom	**Frequency**	**Male Cohabited**	1.1±0.5	3.9±2.1	3.1±1.18	2.3±0.8	--	--	0.02
Self-Groom	**Frequency**	**Female Cohabited**	3.5±0.6	1.6±0.5	3.9±0.4	0.8±0.3			
Visit/ Manipulation	**Duration**	**Male Cohabited**	53.7±20.8	65.7±36.4	171.0±43.2	75.5±9.7	--	0.00006	--
Visit/ Manipulation	**Duration**	**Female Cohabited**	48.5±18.6	94.9±30.6	205.1±45.7	60.4±13.4			
Visit/ Manipulation	**Frequency**	**Male Cohabited**	7.7±2.6	4.9±1.6	17.6±1.8	11.4±1.2	--	0.002	--
Visit/ Manipulation	**Frequency**	**Female Cohabited**	5.6±1.6	8.9±2.2	12.1±3.4	8.0±1.5			

***Table 3.1* -** Duration and frequency of self-grooming, object visit and object manipulation, latency duration by sexually inactive and Female-Cohabited male prairie voles were analyzed by a two-way ANOVA comparing means between conditions by a two-way ANOVA. Significant differences of ≤0.05 and values that were close to significance are shown in the table.

Table 3.2

			Conditions Interval						
Behavior	**Measure**	**Group**	Water Odor	Infant Odor	Live Infant	Sub-Adult Odor	P (Group) A	P (Condition) B	P (AB)
			$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	df (1,53)	df (3,53)	df (3,53)
Runway Build	**Duration**	**Male Cohabited**	12.8±6.7	11.7±6.3	17.1±4.6	5.3±2.7	0.08	--	--
Runway Build	**Duration**	**Female Cohabited**	18.7±7.8	17.6±8.3	20.1±6.9	24.7±7.9			
Runway Build	**Frequency**	**Male Cohabited**	2.4±1.1	4.3±2.5	4.4±1.5	2.1±1.19	--	--	--
Runway Build	**Frequency**	**Female Cohabited**	3.3±1.3	5.6±2.3	3.9±1.4	4.9±1.5			
Locomotion	**Duration**	**Male Cohabited**	449.7±46.6	350.0±69.5	411.7±62.6	487.8±10.6	--	--	--
Locomotion	**Duration**	**Female Cohabited**	414.6±59.6	479.9±32.4	334.5±57.9	490.6±23.1			
Locomotion	**Frequency**	**Male Cohabited**	13.1±2.6	13.4±4.0	16.6±2.9	16.9±1.0	--	--	--
Locomotion	**Frequency**	**Female Cohabited**	13.5±2.5	15.6±3.9	14.3±3.4	13.8±2.5			
Odor Container	**Duration**	**Male Cohabited**	334.8±58.3	287.3±76.4	282.0±68.6	289.1±40.5	--	--	--
Odor Container	**Duration**	**Female Cohabited**	304.9±57.5	370.2±27.5	282.9±63.9	314.1±62.7			
Odor Container	**Frequency**	**Male Cohabited**	7.4±2.3	6.9±2.1	11.0±2.7	10.4±1.7	--	--	--
Odor Container	**Frequency**	**Female Cohabited**	6.8±1.7	11.5±2.6	7.1±2.5	7.9±1.4			

Table 3.2 - Duration and frequency of runway building, locomotion and presence in odor container by sexually inactive and Female-Cohabited male prairie voles were analyzed by a two-way ANOVA. Significant differences of $\leq$0.05 and values that were close to significance are shown in the table.

Appendix C

Summary of Data Analysis

Chapter 4

Table 4.1

		***Fos* Count**						
Brain Area	**Group**	Water Odor	Infant Odor	Live Infant	Sub-Adult Odor	P (Group) A	P (Condition) B	P (AB)
		$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE	$\bar{x}$ ± SE			
Lateral Septum (LS)	**Male Cohabited**	246.8±77.8	119.0±46.7	247.9±44.9	50.6±5.4	df (1,56) 0.06	df (3,56) 0.09	df 0.04
Lateral Septum (LS)	**Female Cohabited**	123.4±56.7	205.3±53.5	45.5±15.6	58.2±24.7			
Bed Nucleus of Stria Terminalis (BNST)	**Male Cohabited**	79.1±12.4	26.9±9.5	45.3±10.5	25.3±7.2	df (1,57) --	df (3,57) --	df 0.08
Bed Nucleus of Stria Terminalis (BNST)	**Female Cohabited**	36.1±12.3	36.3±8.0	30.3±9.5	31.7±10.9			
Ventral Tegmental Area (VTA)	**Male Cohabited**	17.0±1.8	13.4±2.4	14.4±2.9	17.3±2.8	df (1,44) 0.02	df (3,44) --	df 0.01
Ventral Tegmental Area (VTA)	**Female Cohabited**	16.3±3.3	24.5±2.8	30.1±4.4	15.5±3.5			

Table 4.1 - *Fos* immune reactivity in the brain regions, LS, BNST and VTA of male prairie voles exposed to water, infant odors, live infants and sub adult odors. The data were analyzed by a two-way ANOVA. Significant differences of ≤ 0.05 and values that were close to significance are shown in the table.

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