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CONDITION-DEPENDENT EFFECTS ON PROCEPTIVE BEHAVIORS IN
MEADOW VOLES

By

Nicholas James Hobbs

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

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ABSTRACT

Hobbs, Nicholas James. Ph.D. The University of Memphis. May 2012. Condition-dependent Effects on Proceptive Behaviors in Meadow Voles. Major Professor: Michael H. Ferkin.

Meadow voles, *Microtus pennsylvanicus*, live in transitional grasslands where food is available in patches of uneven quality. As such, voles may differ in their access to quality and sufficient forage. It is hypothesized that voles that have access to better quality and more abundant forage will be more likely to signal their interest in opposite-sex conspecifics and be more likely to secure mates compared to voles that do not have such access. This and related hypotheses were tested. The expression of the behaviors used to indicate interest in a potential mate varied with the nutritional status of the actor and that of the potential mate. The results of the first study showed that the protein content of a vole's diet did not affect the rate at which it self-groomed. However, the amount of time female, but not male, voles self-groomed was affected by the protein content of the vole's diet to whose odors they were exposed. Neither the protein content of a vole's diet nor that of a nearby conspecific affected the number of scent marks or proportion of over-marks a vole deposits. However, the protein content of a vole's diet did affect how it responds to the scent donors of a same-sex over-mark. Similarly, the protein content of the top- and bottom-scent donors' diets also affected how voles respond to these scent donors. Finally, food availability affected the self-grooming behavior of meadow voles, but not their scent marking and over-marking behavior. Overall, the behaviors that convey interest in opposite-sex conspecifics, such as scent marking, odor preferences, and self-grooming, depend on the nutritional status of both

the actor and the opposite-sex conspecifics in which the actor may be interested in as a potential mate.

PREFACE

The goal of my research was to examine how the condition of meadow voles, specifically the quality and availability of food, affects proceptive behaviors, those behaviors that indicate an individual's interest in the opposite sex. Meadow voles, *Microtus pennsylvanicus*, are an ideal species to address this research because of the habitat in which they are found in the wild as well as their natural history. Meadow voles live in transitional grasslands where the availability and quality of forage can vary. Additionally, the reproductive state of female voles can vary during the breeding season. The proceptive behaviors voles use include scent marking, over-marking, and self-grooming. The rate at which voles perform such behaviors or respond to over-marks may be affected by their diet or that of nearby opposite-sex conspecifics. My dissertation examined how food availability, dietary protein content, and female reproductive state affected self-grooming, over-marking, and the response to over-marks. I am the primary author on all of these co-authored manuscripts. Chapter 1 (Introduction) has been formatted according to the journal *Animal Behaviour*. Chapter 2 (Self-grooming response of meadow voles to the odor of opposite-sex conspecifics in relation to the dietary protein content of both sexes) has been published in the journal *Ethology* (Hobbs et al. 2008). Chapter 3 (Effect of protein content of the diet on scent marking and over-marking behavior in meadow voles, *Microtus pennsylvanicus*) has been published in the journal *Behaviour* (Hobbs & Ferkin 2011a). Chapter 4 (Dietary protein content affects the response of meadow voles, *Microtus pennsylvanicus*, to over-marks) has been published in the journal *Acta Ethologica* (Hobbs & Ferkin 2011b). Chapter 5 (Effects of food availability on proceptivity: a test of the reproduction at all costs and metabolic fuels

hypotheses) has been submitted and is currently under review at *Animal Behaviour*.

Chapter 6 (The response of male meadow voles, *Microtus pennsylvanicus*, to same- and mixed-sex over-marks depends on the reproductive state of the top- and bottom-female scent donors) has been submitted and is currently under review at *Behavioral Processes*.

Chapter 7 (Conclusion) has been formatted according to the journal *Animal Behaviour*.

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Hobbs, N. J. & Ferkin, M. H. The response of male meadow voles, *Microtus pennsylvanicus*, to same- and mixed-sex over-marks depends on the reproductive state of the top- and bottom-female scent donors. In review.

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Chapter 1: Introduction

Proceptivity is one of three components that make up sexual behavior (Beach 1976). It is defined as the set of behaviors animals use to attract or indicate interest in opposite-sex conspecifics as potential mates. Two such behaviors used by terrestrial mammals are self-grooming and over-marking (Thiessen 1977; Brown and Macdonald 1985). As the sources of scent are often digestive exudates, such as urine or feces (Albone 1984), the rate at which an individual self-grooms or deposits over-marks may be affected by its or its potential mate's nutritional state. An animal's nutritional state is determined by the quality and amount of food it consumes. As such, diet may play an important role in the expression of proceptive behaviors, particularly for animals that live in transitional grasslands where the availability and quality of food can vary, such as the meadow vole, *Microtus pennsylvanicus* (Lindroth & Batzli 1984; Bergeron & Jodoin 1987, 1989).

Many animals self-groom when they encounter the scent marks and odors of opposite-sex conspecifics (Thiessen 1977). Self-grooming transmits odiferous substances that contain information about the groomer's condition, which can affect interactions with opposite-sex conspecifics (Ferkin 2006). In chapter 2, we tested the hypothesis that the amount of time that individuals self-groom to opposite-sex conspecifics is affected by the amount of protein in their diet and that of the scent donor. Previous work has shown that dietary protein content affects the attractiveness of a vole's scent (Ferkin et al. 1997). As such, we predicted that voles spend more time grooming when exposed to the odor of an opposite-sex conspecific that has been fed a diet high in protein content compared to if it was exposed to the odors of an opposite-sex conspecifics

fed a low-protein diet. Similarly, we predicted that voles fed a high-protein diet would spend more time self-grooming compared to voles fed a low-protein diet.

Differences in the protein content of individuals may affect their response to signals produced by opposite-sex conspecifics and how opposite-sex conspecifics respond to the individual's own signals. In terrestrial mammals, such signals include over-marks, which can be used to communicate with potential mates (Brown & Macdonald 1985). In chapter 3, we tested the hypothesis that the protein content of the top- and bottom-scent donors of an over-mark's diets affects the scent marking and over-marking behavior of the top-scent vole. We tested this hypothesis by performing two experiments on meadow voles in which the top- or bottom-scent donors of an over-mark were fed a diet containing either 9%, 13%, or 22% protein; these protein concentrations are similar to those that voles may consume in free-living populations (Lindroth & Batzli 1984). In experiment 1, we varied the protein content of the top-scent voles but not that of the bottom-scent donor. We predicted that top-scent donor voles fed a diet high in protein content deposit more scent marks and more over-marks than do top-scent donor voles fed diets lower in protein content. In experiment 2, the top-scent voles were fed a 22% protein diet but the protein content of the diet of the bottom-scent donor varied. We predicted that top-scent donor will deposit more scent marks if the bottom-scent vole was fed a diet high in protein content than if it was fed a diet lower in protein content.

How voles respond to the scent donors of an over-mark is affected by their condition as well as the condition of the top- and bottom-scent donors of the over-mark (Leonard et al. 2001). Dietary protein affects the behavior of animals and is an important contributor to their condition. The aim of Chapter 4 was to examine how the protein

content affects a meadow vole's response to the top- and bottom-scent donor of a same-sex over-mark. We did so by conducting two experiments. In experiment 1, meadow voles were fed a 9%, 13%, or 22% protein diet to assess their response to an over-mark in which the top- and bottom-scent donors were fed the 22% protein diet. In experiment 2, voles fed a 22% protein diet were exposed to an over-mark in which the top- and bottom-scent donors were fed a different protein diet.

Proceptive behaviors are used by animals to indicate interest in opposite-sex conspecifics. These behaviors can be affected by an individual's nutritional status (Pierce et al. 2005). Two mutually exclusive hypotheses have been proposed to account for the effects of food availability on reproduction. These are the metabolic fuels hypothesis and the reproduction at all costs hypothesis (Wade & Schneider 1992; Boonstra et al. 2001). It is not known if food availability affects proceptive behaviors such as scent marking, over-marking, and self-grooming. In Chapter 5, we tested the hypothesis that food-deprived and nonfood-deprived meadow voles differ in the number of scent marks they deposit, the proportion of over-marks they deposit, and the amount of time they spend self-grooming when they encounter the scent marks of opposite-sex conspecifics.

A vole's condition is also influenced by its reproductive state. During the breeding season, a female vole's reproductive state may vary. Females may be pregnant, pregnant and lactating, neither pregnant nor lactating, or in a heightened state of sexual receptivity known as postpartum estrus (PPE) (Keller 1985). Our goal in Chapter 6 was to determine if male meadow voles respond differently to the top- and bottom-scent marks of females in different reproductive states after being exposed to same-sex and mixed-sex over-marks. As males benefit by mating with a PPE female (Gilbert 1984;

Keller 1985), they may spend more time investigating the mark of a PPE female to that of a female that was not in PPE, independent of the former's position in an over-mark. However, how male voles respond to the scent mark of a female whose mark the male first encountered as part of a mixed-sex over-mark may be affected by the female's reproductive state.

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Chapter 2: Self-grooming response of meadow voles to the odor of opposite-sex conspecifics in relation to the dietary protein content of both sexes

Abstract

Many animals self-groom when they encounter the scent marks of opposite-sex conspecifics. Self-grooming transmits odiferous substances that contain information about the groomer's condition, which is affected by its nutritional state. We tested the hypothesis that the amount of time that individuals self-groom to opposite-sex conspecifics is affected by the amount of protein in their diet and that of the scent donor. We did so by feeding meadow voles (*Microtus pennsylvanicus*) a diet containing 9%, 13%, or 22% dietary protein for 30 days and observing their self-grooming behavior when they were exposed to bedding scented by an opposite-sex conspecific (odor donor) fed one of the three diets, or fresh cotton bedding (control). The hypothesis was partially supported. We found that the protein content of the diet of male and female groomers did not affect the amount of time they self-groomed. However, the protein content of the diet of male odor donors affected the amount of time that female voles spent self-grooming. Female voles self-groomed more in response to male odor donors fed a 22% protein-content diet than to those produced by male odor donors fed either a 9% or a 13% protein-content diet. Interestingly, the amount of time males self-groomed was not affected by the protein content of the diet of the female odor donor. These results may, in part, be explained by the natural history of free-living meadow voles, sex differences in costs associated with mate attraction and reproduction, and the direct or indirect benefits that females receive from males fed a diet high in protein content.

Introduction

Scent-marking (Johnston 1983, 2003; Gosling & Roberts 2001; Roberts 2007) and self-grooming (Thiessen 1977; Thiessen & Harriman 1986; Ferkin et al. 1996) can signal interest in the opposite sex for many mammals. Self-grooming spreads odiferous substances that increase the active space of the odor field that surrounds groomers (Bossert & Wilson 1963), making them more easily detected by nearby opposite-sex conspecifics (Ferkin & Leonard 2005). Self-grooming may transmit information about the groomer that affects interactions with opposite-sex conspecifics (Steiner 1973, 1974; Brockie 1976; Thiessen 1977; Gosling 1985; Thiessen and Harriman 1986; Witt et al. 1988; Ferkin et al. 2001; Ferkin 2006; Ferkin & Leonard in press). For example, meadow voles spend more time self-grooming in response to odors of opposite-sex conspecifics in heightened states of sexual receptivity than those not in such states (Ferkin et al. 1996). Voles also spend more time self-grooming when exposed to odors of unfamiliar, opposite-sex conspecifics than to those of their opposite-sex siblings (Paz-y-Miño et al. 2002). Together, these observations suggest that the amount of time that animals (groomers) spend self-grooming depends on features of their identity as well as that of the individuals that produced the odor (the odor donor) (Ferkin 2005, 2006; Ferkin & Leonard 2005, in press).

The odiferous substances produced by voles and other mammals during bouts of self-grooming are composed of anogenital secretions, and digestive exudates such as saliva, urine, and feces (Albone 1984) and depend on an individual's condition (Zeh & Zeh 1988; Grafen 1990; Andersson 1994; Johnstone 1995) and its nutritional state (Wade et al. 1992, 1996; Gill & Rissman 1997; Jones & Wade 2002). Thus, nutritional state

provides reliable signals and information about the current condition and potential quality of conspecifics (Kodric-Brown & Brown 1984; Rowe & Houle 1996; Pierce & Ferkin 2005a, b; Pierce et al. 2005a, 2007a, b). For example, animals that are food deprived or restricted are treated as if they are lower quality relative to their counterparts who were fed ad libitum (Dickerman et al. 1993; Gill & Rissman 1997; Panicker et al. 1998; Jones & Wade 2002; Pierce et al. 2005b; Punzo & Parker 2006).

The quality of an animal's diet also affects its ecology, development, and behavior (Kodric-Brown 1984; Hill 1991, 1992; Martin & Lopez 2006; McGraw 2007). For example, reproductive maturation, maternal behavior, growth of young, and space use are affected by the protein content of the diet of many animals, including voles (Batzli 1985; Bergeron & Jodoin 1987, 1989; Bergeron et al. 1990; Jones 1990). Dietary protein also influences the attractiveness of conspecific signals to opposite-sex individuals, interest in the opposite sex, and reproduction (Louis et al. 1994; Ferkin et al. 1997; Droney 1998; Schoech et al. 2004; McGlothlin et al. 2007; Engels & Sauer 2008). Voles fed a diet high in protein produce odors that are more attractive to the opposite-sex than do voles fed a diet low in protein content (Ferkin et al. 1997). Interestingly protein content of the diet does not affect the preferences voles have for the odors of opposite-sex conspecifics (Pierce et al. 2005b). In that odor attractiveness and responses to odors are components of self-grooming (Ferkin & Leonard in press), it is not clear if self-grooming behavior is affected by the protein content of the diet of voles.

If the ability of males and females to find patches of high and low quality forage affects self-grooming, then it provide a condition-dependent, honest and relatively cheat-proof signal of that individual's condition (Gosling & Roberts 2001; Ferkin 2006;

Roberts 2007; Ferkin and Leonard in press). The present study tested the hypothesis that the amount of time that individuals self-groom to opposite-sex conspecifics is affected by the amount of protein in their diet and that of the odor donor. If self-grooming is affected by the diet of the odor donor, then voles will spend more time self-grooming in response to odors of opposite-sex conspecifics that are fed a diet higher in protein content than they would to those of opposite-sex conspecifics fed a diet lower in protein content. If self-grooming is affected by the amount of protein in the diet of the groomer (subject), then voles fed a high protein diet will spend more time self-grooming than voles fed a lower protein diet when they are exposed to bedding scented by an opposite-sex conspecific.

Methods and Materials

Animals

Meadow voles used were third or fourth generation offspring of animals caught in the field during the summer of 2005. Experiments were conducted in 2007 between February and July. Forty-six male meadow voles and 43 female meadow voles were raised under a long photoperiod (14:10 h light:dark, lights on at 0700 Central Standard Time), simulating the amount of light during the breeding season in meadow voles. Voles were weaned at 19 days of age and housed with their littermates in a clear plastic cage (26 x 32 x 31 cm) with clean woodchip bedding and cotton nesting material. Fourteen days after the animals were weaned they were separated from their littermates and housed individually in plastic cages (27 x 16.5 x 12.5 cm) with clean woodchip bedding and cotton nesting material. Cages were changed weekly unless otherwise

stated. Voles were given water and the appropriate diet depending on the treatment group they were assigned (please see below). We followed Animal Care Protocol 505, which was approved by the IACUC at The University of Memphis. We adhered to the ‘Guidelines for the use of animals in research’ as published in *Animal Behaviour* (1991, 41, 183–186) and the laws of the country where the research was conducted.

Protein Diets

We chose diets containing 9%, 13%, or 22% protein because they roughly corresponded to the range of protein content available to free-living meadow voles during the breeding season (Batzli 1985). The 9% protein diet represents the quality of food (e.g. hay) that a vole may encounter in a poor habitat in the wild (Lindroth & Batzli 1984). The 13% protein diet represents the quality of food (e.g. alfalfa) voles may encounter when living in a good habitat (Lindroth & Batzli 1984). The 22% protein diet simulates the quality of food (e.g. legumes) a vole may encounter when living near a cultivated field (Lindroth & Batzli 1984). The 9% and 13% protein diets were specially prepared by Harlan Teklad (Madison, WI, USA); the 22% diet was Harlan Teklad’s standard rodent chow. The 9% diet contained 53.2% carbohydrate and 5.0% fat. The 13% diet contained 49.7% carbohydrate and 5.1% fat. The 22% diet contained 51.2% carbohydrate and 5.3% fat. The three diets were isocaloric and calculated to provide 3 Kcal/g of digestible energy.

Experimental procedure

We randomly assigned voles ($n = 46$ males and $n = 43$ females) into 1 of 3 groups corresponding to the specific protein diets that they were to be fed during this study. Sixteen male and 14 female meadow voles were fed a 9% protein diet. Sixteen male and 13 females were placed on the 13% protein diet. Fourteen males and 16 females were fed a 22% protein diet. Subjects were weighed before being placed on the diet and at the conclusion of the experiment, 35 d later. We use the terms subject for the individuals scored for self-grooming and odor donor for the individuals that contribute bedding as a stimulant to the subject. Subjects also provided scented bedding in tests involving opposite-sex subjects. For any given test, the subject and odor donor were unfamiliar and unrelated to each other. Subjects were not exposed to their own odors in any of the tests. All self-grooming tests began 30 d after the voles were placed on their specific diets. Previous studies have demonstrated that 30 d are sufficient to induce changes in the attractiveness of voles fed similar protein-content diets (Ferkin et al. 1997). Cotton bedding, which served as the stimulus odors, had remained in the cage of the odor donors for 10 d prior to being used in an odor test. Cotton bedding from an odor donor was used in only one self-grooming test before it was discarded. All testing took place between 0700 and 1100 (CST). The self-grooming tests took place over the course of four days. We used a Latin Squares assignment to ensure that each subject was randomly exposed to all of the 4 treatment combinations over the four day experiment.

We followed the procedure for measuring self-grooming in voles detailed elsewhere (Ferkin et al. 1996; Leonard et al. 2005; Ferkin 2006). Briefly, self-grooming tests were performed by replacing the nesting material of the subject vole with the

stimulus nesting material. The stimulus nesting material was 8 g of either fresh, clean nesting material, or nesting material scented by an opposite-sex conspecific fed a 9% protein-content diet, or nesting material scented by an opposite-sex conspecific fed a 13% protein-content diet, or nesting material scented by an opposite-sex conspecific fed a 22% protein-content diet. Ten seconds after placing the odor donor's nesting material in the subject's (groomer's) cage, we recorded continuously for 5 min the amount of time the subject vole self-groomed. The experimenter was blind to the diet of the odor donors and subjects. An animal was considered to be self-grooming when it licked, scratched, or rubbed its face, over the ears, flank, ventrum, anogenital area, or tail; these areas contain sexually discriminable odors (Ferkin et al. 1996, 2001; Ferkin 2006). At the end of the self-grooming test the odor donor's nesting material was removed from the subject's cage and discarded, and the subject's own nesting material was returned to its home cage. For each self-grooming test, the experimenters wore clean floor exam gloves to minimize contact with human odors.

Statistical Analysis

We analyzed the data using a two-way ANOVA (diet of subject x diet of donor) with repeated measures for each sex to determine if significant differences existed in the amount of time that subjects spent self-grooming in response to the different donors. We also measured the effects of the protein content of the diet on weight gain or loss of a subset of the voles used in this experiment with a two-way ANOVA (diet x sex). If any significant effect was found, we used the Holm-Sidak method to determine significant differences between the pairwise comparisons. We also used separate paired t-tests (one

t-test for male animals, one t-test for female animals) to determine if there was a significant change in weight regardless of the protein diet the animals were fed. For all analyses, statistical significance was accepted at α level ≤ 0.05 .

Results

Female subjects and male odor donors

We tested whether the amount time female meadow voles spent self-grooming was affected by the protein content of their diet and/or that of the male odor donors. We found no significant interaction between the subject's diet and the diet of the odor donor ($F_{6, 120} = 1.152$, $p = 0.34$). Similarly, we found no significant main effect for the amount of time female meadow voles self-groomed based on their diet ($F_{2, 120} = 1.348$, $p = 0.27$). In contrast, we found a significant main effect for the amount of time female meadow voles spent self-grooming based on the diet of the odor donor ($F_{3, 120} = 6.890$, $p < 0.001$). Our post hoc comparisons indicated that female voles self-groomed significantly longer to odors provided by male meadow voles fed a 22% protein diet than to males fed a 9% or 13% protein diet or the unscented cotton bedding (Holm-Sidak, $p < 0.05$, Figure 2.1A). There were no significant differences in the amount of time spent by female voles spent self-grooming in response to odors of males fed a 9% or 13% diet (Holm-Sidak, $p > 0.05$, Figure 2.1A). Female voles, however, spent significantly more time self-grooming when they were exposed to odors provided by male donors fed a 9% or 13% protein diet than to the unscented cotton bedding (Holm-Sidak, $p < 0.05$, Figure 2.1A).

Male subjects and female odor donors

We tested whether the amount of time male voles spent self-grooming was affected by the protein content of their diet and/or that of the female odor donors. We found no significant interaction between the diet of the male groomers and diet of the female scent donors ($F_{6, 120} = 0.383$, $p = 0.89$). Similarly, we found no significant differences in the amount of time male meadow voles spent self-grooming based on the protein content of their diet ($F_{2, 129} = 1.046$, $p = 0.36$). Likewise, there were no significant differences in the amount of time spent self-grooming by males due to the protein content of the diet of the female odor donors ($F_{3, 129} = 0.180$, $p = 0.91$). Male meadow voles, independent of the protein content of their diet, spent similar amounts of time self-grooming when they were exposed to unscented cotton bedding or odors provided by female scent donors fed a 9%, 13%, or 22% protein diet (Figure 2.1B).

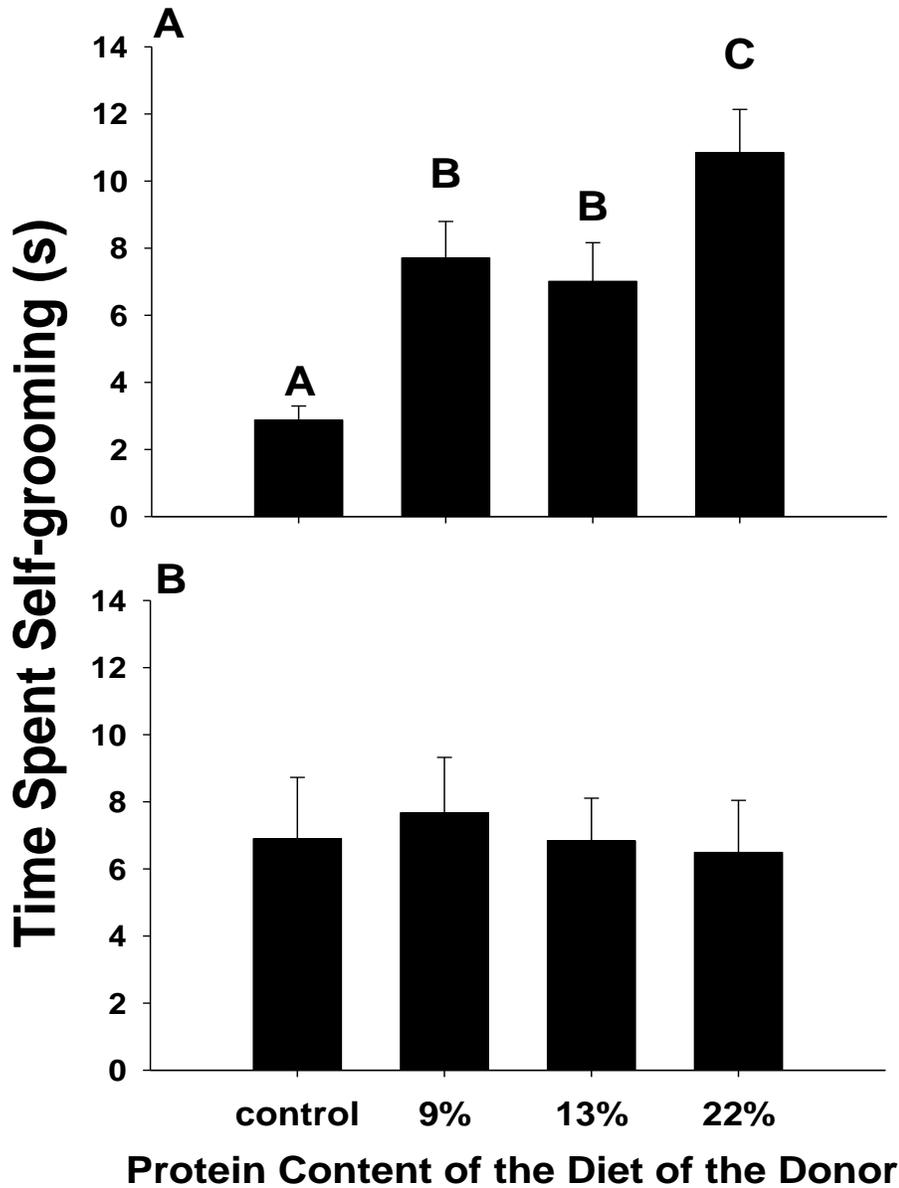


Figure 2.1: The amount of time spent self-grooming (mean + standard error) by (A) female and (B) male meadow voles when exposed to fresh cotton bedding (control) or bedding scented by an opposite-sex conspecific either fed a 9% protein diet, 13% protein diet, or 22% protein diet. Histograms capped with different letters represent statistically significant differences among the groups ($P < 0.05$; Holm-Sidak method multiple pairwise comparisons).

Effect of protein content of the diet on body weight and weight loss

The differences in the protein content of the diet did not significantly alter the weight gain/loss among male and female subjects and odor donors. We found no interaction between diet of the vole and its sex ($F_{2, 48} = 2.499$, $p = 0.09$). Similarly, we found no significant difference in the change in weight due to the protein content of the diet ($F_{2, 48} = 2.901$, $p = 0.07$) or the sex of the vole ($F_{1, 48} = 0.000$, $p = 0.99$). At the beginning of the experiment the mean weight of the female odor donors and subjects was 36.39 ± 2.1 g. At the completion of the experiment 34 days later, they weighed 36.46 ± 2.1 g, having gained an average of 0.07 g, which was not a statistically significant change in body weight ($t_{26} = 0.074$, $p = 0.94$). At the start of the experiment the mean weight of the male odor donors and subjects was 54.75 ± 2.4 g. At the end of the experiment 34 days later, subjects and odor donors weighed 54.85 ± 2.2 g, having gained an average of 0.10 g, which was not a statistically significant change in body weight ($t_{26} = 0.083$, $p = 0.94$). At the completion of the experiment, the weight for females fed a 9% diet was 40.51 ± 4.8 g, the weight for females fed a 13% protein diet was 33.40 ± 2.8 g, and the weight for females fed a 22% diet was 35.46 ± 2.7 g. At the end of the experiment, the weight of males fed a 9% diet was 49.39 ± 3.9 g, the weight of males fed a 13% protein diet was 57.82 ± 4.0 g, and the weight of males fed a 22% diet was 57.34 ± 3.5 g. These data suggest that changes in body weight were not responsible for changes in self-grooming behavior.

Discussion

Our results support the prediction that the amount of time female voles self-groom depends on the protein content of the diet of the male odor donors. Female meadow voles self-groomed more to males fed a 22% protein diet than to males fed a 9% or 13% protein diet. Similar results were obtained in an earlier study, showing that male voles fed diets higher in protein produced odors that were more attractive to female voles than males fed a diet lower in protein content (Ferkin et al. 1997). Dietary protein also influences the growth of a white patch used by males to attract mates in dark-eyed juncos (*Junco hyemalis*) (McGlothlin et al. 2007). Diet quality has also been shown to affect reproduction in a variety of male animals. Boars fed a low protein diet take longer to ejaculate and have reduced semen volumes (Louis et al. 1994). Male fruit flies (*Drosophila grimshawi*) are in poorer reproductive condition when fed a diet low in protein (Droney 1998). Similarly, manipulations of micronutrients such as calcium, vitamin D and carotenoids affect the attractiveness of males to females in zebra finches (*Taeniopygia guttata*) and Iberian rock lizards (*Lacerta monticola*), guppies and house finches, with females responding preferentially to males fed diets richer in these substances (Kodric-Brown 1985; Hill 1991, 1992; Martin & Lopez 2006; McGraw 2007). Similarly, the amount of food available to male rodents is positively correlated with the attractiveness to female conspecifics (Wade et al. 1992, 1996; Dickerman et al. 1993; Panicker et al. 1998; Jones & Wade 2002) and their reproductive success (Pierce et al., 2005b; Engels & Sauer 2008).

Female voles, however, spent a similar amount of time self-grooming regardless of the amount of protein in their diet. Thus, the protein content of a female groomer's

diet did not affect the amount of time that it self-grooms when exposed to the scents of a male donor. This result was somewhat surprising in that previous work has shown that food availability for female voles affects their response to the odors of male voles. Female voles that were not food deprived prefer the odors of male voles to those of female voles, whereas as those that were food deprived no longer show such a preference for the odors of male voles (Pierce et al. 2005b). Therefore, it appears that food availability and the protein content of the diet have different effects on the response of female voles to male conspecifics and are possibly mediated by different factors (Leonard et al. 2005; Pierce et al. 2007b). Likewise, protein content of the diet of both male subjects and female odor donors did not affect male self-grooming, which is consistent with Pierce et al. (2005b), but not those of Ferkin et al. (1997), who reported that males fed diets higher in protein content spend more time self-grooming than males fed diets lower in protein content.

These sex differences in self-grooming behavior in meadow voles with respect to the protein content of the diets may be explained, in part, by considering their natural history during the breeding season and the differences in investment that each sex puts into signaling to opposite-sex conspecifics. During the breeding season, male meadow voles occupy large overlapping home ranges that encompass the territories of one of more females in transitional grasslands, where patches of food can vary greatly in abundance and quality (Batzli 1985; Bergeron & Jodoin 1989). Thus, males are likely to encounter areas that contain forage (grasses and sedges) of different quality and have an opportunity to eat diets that vary in protein content (Bergeron & Jodoin 1987; Bergeron et al. 1990). Within these areas, males will also encounter the scent marks of females and

begin to self-groom to indicate their presence in an area to that female (Ferkin et al. 1996). Upon encountering sexually receptive non-sibling females, males will self-groom at high rates to increase both their attractiveness and the likelihood that the female will respond favorably and mate with him (Ferkin et al. 1996, 2001; Paz-y-Miño et al. 2002; Ferkin & Leonard 2005, in press; Ferkin 2006). Since male fitness depends on the number of females that he mates with (Boonstra et al. 1993; Berteaux et al. 1999; delBarco-Trillo & Ferkin 2004), male voles should attempt to self-groom and indicate their presence in an area to nearby females. By producing odors that are attractive to females, which are dispersed to female voles by self-grooming, male voles may be able to convey their nutritional state and other features of their condition-dependent quality in odors to nearby female voles in an attempt to mate with them (Ferkin & Leonard in press).

The fact that female meadow voles self-groom at different rates when they encounter the scents of male conspecifics fed diets that differed in protein content is consistent with their natural history. Female meadow voles occupy territories that are visited by male conspecifics (Madison 1980; Madison & McShea 1987; Boonstra et al. 1993). Consequently, females should exert some degree of mate choice when they encounter the odors of preferred males that visit their territories (Ferkin & Leonard in press). Female voles may choose males based on differences in features of their relative quality (Trivers 1972; Grafen 1990; Andersson 1994), which depends in part on his diet (Ferkin et al. 1997). In that diet is an accurate reflection of his condition (Kodric-Brown & Brown 1984), females should be attempting to attract higher quality mates. This may explain why we found that female voles spend more time self-grooming when they

encounter the odors produced by males fed a diet that is high in protein content than by males fed a diet low in protein. By doing so, female voles may be able to signal to the former males their location and willingness to mate with them. By selecting a male fed a diet higher in protein a female may receive additional direct or indirect benefits (Zeh & Zeh 1988; Andersson 1994). Female meadow voles do not receive nuptial gifts from males, males do not provide nests to females, and males do not generally offer paternal care (Madison 1980; Boonstra et al. 1993). The type of direct benefits a female meadow vole gains by mating with a male fed a protein-rich diet may be associated with features of his ejaculate. As in other species with similar promiscuous/polygynous mating systems (Trivers 1972; Boonstra et al. 1993), females may gain direct benefits if males fed a diet rich in protein produce more or better quality sperm that will increase the female's chance to become pregnant (Louis et al. 1994; Droney 1998; Birkhead 2000; Schulte-Holstedde et al. 2005). Moreover, because female voles can multiply mate and deliver litters of mixed paternity (Boonstra et al. 1993; Berteaux et al. 1999), sperm competition may favor males fed a diet high in protein content (delBarco-Trillo & Ferkin 2004; Snook 2005). Female voles may gain indirect benefits from mating with male voles that have consumed a diet rich in protein if the ability to find high quality forage and the ability to identify these males is heritable (i.e., Grafen 1990; Andersson 1994; Johnstone 1995).

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Chapter 3: Effect of protein content of the diet on scent marking and over-marking
behavior in meadow voles, *Microtus pennsylvanicus*

Summary

Differences in the protein content of individuals may affect their response to signals produced by opposite-sex conspecifics and how opposite-sex conspecifics respond to the individual's own signals. Many terrestrial mammals use over-marks to communicate with potential mates. In this study, we determined whether over-marking behavior is affected by the protein content of the top- and bottom-scent donors of an over-mark. We tested two hypotheses by performing two experiments on meadow voles, *Microtus pennsylvanicus*, in which the top- or bottom-scent donors of an over-mark were fed a diet containing either 9%, 13%, or 22% protein; these protein concentrations are similar to those that voles may consume in free-living populations. In experiment 1, we varied the protein content of the top-scent voles but not that of the bottom-scent donor. We tested the hypothesis that top-scent donor voles fed a diet high in protein content deposit more scent marks and more over-marks than do top-scent donor voles fed diets lower in protein content. In experiment 2, the top-scent voles were fed a 22% protein diet but the protein content of the diet of the bottom-scent donor varied. We tested the hypothesis that top-scent donor will deposit more scent marks if the bottom-scent vole was fed a diet high in protein content than if it was fed a diet lower in protein content. Protein content of the top-scent vole's diet did not affect the number of scent marks and over-marks it deposited. Likewise, the protein content of the bottom-scent vole did not affect the number of scent marks and over-marks deposited by the top-scent vole.

Introduction

The nutritional condition of an individual may affect both how it responds to the signals of opposite-sex conspecifics and the response of opposite-sex conspecifics to its signals they use to communicate their interest in potential mates (Bronson, 1989). For example, swordtail fish, *Xiphophorus birchmanni*, (Fisher & Rosenthal, 2006), Atlantic mollies, *Poecilia Mexicana*, (Plath et al., 2005), guppies, *Poecilia reticulata*, (Kodric-Brown, 1985, 1989) and meadow voles, *Microtus pennsylvanicus*, (Pierce et al., 2007), are less interested in opposite-sex conspecifics that have been nutritionally challenged as compared to opposite-sex conspecifics that have not been challenged. Male meadow voles that had been food deprived for 6 hours or longer spent less time investigating the scent marks of sexually receptive female conspecifics than did males that were not food deprived (Pierce et al., 2005). In addition, the former male voles were less likely than the latter male voles to mate with a sexually receptive female vole (Pierce et al., 2005). Male water mites, *Neumania papillator*, (Proctor, 1992), lizards, *Sceloporus jarrovii*, (Punzo & Parker, 2006), and freshwater prawns, *Macrobrachium malcolmsonii*, (Samuel et al., 2006), were less likely to display reproductive behaviors if they were nutritionally stressed relative to those that were not nutritionally stressed.

The nutritional status of individuals would likely be highly variable among temperate-zone animals. Specifically, individuals that inhabit transitional grasslands where the quality of forage, particularly its protein content, is distributed in patches, may vary in their nutritional states (Lindroth & Batzli, 1984; Batzli, 1985). Many of these terrestrial animals are faced with the challenge of locating or gaining access to forage high in protein (Bergeron & Jodoin, 1987, 1989). Access to such forage can affect an

individual's nutritional state, which may be reflected in the attractiveness of its signals to opposite-sex conspecifics. Studies on a variety of animals, including boars, *Sus scrofa*, (Louis et al., 1994), Florida scrub jays, *Aphelocoma coerulescens* (Schoech et al., 2004), dark-eyed juncos, *Junco hyemalis* (McGlothlin et al., 2007), scorpionflies, *Panorpa vulgaris* (Engels & Sauer, 2008), fruit flies, *Drosophila grimshawi* (Droney, 1998), and meadow voles (Ferkin et al., 1997; Hobbs et al., 2008; Ferkin & Leonard, 2010), have shown that opposite-sex conspecifics fed a high-protein diet are more attractive than those fed a low-protein diet to potential mates. Studies have also reported that the protein content of an animal's own diet also affects how it responds to signals from opposite-sex conspecifics. Male boars that are fed a low-protein diet exhibit a reduced libido, and are less likely to respond to signals from females (Louis et al., 1994). However, male and female meadow voles fed different protein diets spent similar amounts of time self-grooming when they were exposed to the odors of opposite-sex conspecifics (Hobbs et al., 2008). It appears that opposite-sex conspecifics that have consumed diets lower in protein may be less attractive and not as interested in mating relative to those that have consumed diets higher in protein (Ferkin et al., 1997). If so, the former opposite-sex conspecific may not be viewed as a preferred mate or consider itself a potential mate (Hobbs & Ferkin, in press).

For many terrestrial rodents, communication between conspecifics is conducted through scent marks. The sources of much of these scent marks are digestive exudates, such as urine or feces (Albone, 1984), which convey accurate information about an individual's diet and nutritional status to opposite-sex conspecifics (Roberts, 2007). These scent marks also convey information about a donor's protein content to opposite-

sex conspecifics (Ferkin et al., 1997; Hobbs et al., 2008). Ferkin et al. (1997) found that male and female voles spent more time investigating the scent marks of opposite-sex conspecifics fed a diet with 22% protein content to those of opposite-sex conspecifics fed either a 9% or 15% protein diet. Voles may also encounter the overlapping scent marks of two conspecifics, which is referred to as an over-mark (Johnston et al., 1994; Ferkin & Pierce, 2007). Recently, Hobbs and Ferkin (in press) discovered that male and female voles fed a 22% protein diet spent more time investigating the scent mark of the top-scent donor relative to that of the bottom-scent donor, but voles fed a 9% or 13% protein diet spent similar amounts of time investigating the scent marks of the top- and bottom-scent donors. Male voles spent more time investigating the top-scent female's mark when she was fed a diet higher in protein relative to the bottom-scent female (Hobbs & Ferkin, in press). This finding suggests that for male voles a preference for a potential mate was determined by the condition of the top-scent female relative to that of the bottom-scent female. In contrast, female voles spent more time investigating the mark of the top-scent male, independent of the protein content of his or the bottom-scent male's diets (Hobbs & Ferkin, in press). For female voles a preference for a potential mate was determined by the position of the scent marks of the two male donors relative to one another in the over-mark.

Voles also signal their interest in these opposite-sex conspecifics as potential mates by over-marking their scent marks (Ferkin et al., 2004a, b; Ferkin & Pierce, 2007). The protein content of the top-scent donor's diet may affect the proportion of scent marks it over-marks when it encounters the scent marks of an opposite-sex conspecific. Likewise, the protein content of the bottom-scent donor's diet may affect the proportion

of their scent marks that are over-marked by an opposite-sex conspecific. Thus, the objective of the current study was to determine whether the over-marking behavior of meadow voles is affected by the protein content of the top- and bottom-scent donors of an over-mark. We did so by allowing voles fed diets that differed in protein content, 9%, 13%, or 22%, to over-mark the scent marks of opposite-sex conspecifics whose diets also differed in protein content. In experiment 1, we varied the protein content of the top-scent voles but the bottom-scent donor was fed a 22% protein diet. We tested the hypothesis that top-scent donor voles fed a diet high in protein content deposit more scent marks and more over-marks than do top-scent donor voles fed diets lower in protein content in response to the scent marks of the bottom-scent donor. In experiment 2, the top-scent voles were fed a 22% protein diet but the protein content of the diet of the bottom-scent donor was varied. We tested the hypothesis that top-scent donor will deposit more scent marks if the bottom-scent vole was fed a diet high in protein content than if it was fed a diet lower in protein content.

Material and methods

Animals

Meadow voles used in this study were 6th-8th generation offspring of field-caught animals captured in Ohio and Pennsylvania, USA. All voles used in this study were born and raised under a long photoperiod (14 L: 10 D, lights on at 0700 hours CST and off at 2100 hours CST). This long photoperiod simulates the typical amount of light present during the breeding season. All voles were weaned at 19 days of age, housed with littermates until 34 days of age, and then housed singly in clear plastic cages (30.5 x 35.5

x 22.8 cm). Top- and bottom-scent donors were between 60-100 days of age when placed on their designated experimental diet (see below). Cages contained woodchip bedding and cotton nesting material. Voles were provided with food and water *ad libitum*. We followed Animal Care Protocol 505, which was approved by the IACUC at The University of Memphis. We adhered to the ‘Guidelines for the use of animals in research’ as published in *Animal Behaviour* (1991, 41, 183–186) and the laws of the country where the research was conducted.

Diet

Scent donors were fed one of three diets that varied in protein content. The diets we selected were similar in protein content to those used in vole studies that examined the attractiveness of scent marks and over-marks to opposite-sex conspecifics (Ferkin et al., 1997; Hobbs et al., 2008; Hobbs & Ferkin, in press). These diets are representative of the amount of protein available to meadow voles in the wild. The 9% protein diet represents the quality of food (e.g. hay) that a vole may encounter in a poor habitat in the wild (Lindroth & Batzli, 1984). The 13% protein diet represents the quality of food (e.g. alfalfa) voles may encounter when living in a good habitat (Lindroth & Batzli, 1984). The 22% protein diet simulates the quality of food (e.g. legumes) a vole may encounter when living near a cultivated field (Lindroth & Batzli, 1984). The 9% and 13% protein diets were specially prepared by Harlan Teklad (Madison, WI, USA); the 22% diet was Harlan Teklad’s standard rodent chow. The three protein diets were isocaloric and calculated to provide 3 Kcal/g of digestible energy. The 9% diet contained 53.2% carbohydrate and 5.0% fat. The 13% diet contained 49.7% carbohydrate and 5.1% fat. The 22% diet

contained 51.2% carbohydrate and 5.3% fat. Table 1 provides a summary of the sources of protein for each diet.

Table 1: Protein Sources (% of total protein coming from various ingredients)

	9% Protein Diet	13% Protein Diet	22% Protein Diet
Corn	18	13	10
Wheat	37	27	16
Soybean meal	10	35	54
Fish meal	25	18	11
Other	10	7	9

Scent donors

In experiment 1, top-scent donors were fed 1 of the 3 experimental diets and all bottom-scent donors were fed the 22% protein diet. Thus we created 6 types of over-marking dyads with an opposite-sex conspecific fed the 22% protein diet as the bottom-scent donor and as the top-scent donor 1) a male vole fed the 9% protein diet, 2) a female vole fed the 9% protein diet, 3) a male vole fed the 13% protein diet , 4) a female vole fed the 13% protein diet, 5) a male vole fed the 22% diet , and 6) a female vole fed the 22%

diet. There were 12 subjects for each dyad, resulting in a total of 36 male and 36 female subjects. Bottom-scent donors were selected from a pool of scent donors consisting of 25 males and 25 female voles. The top- and bottom-scent donors were unfamiliar and unrelated to one another. We used unique combinations of top- and bottom-scent donors.

In experiment 2, top-scent donors were all fed the 22% protein diet and bottom-scent donors were fed 1 of the 3 experimental diets. Again, this created 6 types of over-marking dyads with an opposite-sex conspecific fed the 22% protein diet acting as the top-scent donor and as the bottom-scent donor 1) a male vole fed the 9% protein diet, 2) a female vole fed the 9% protein diet, 3) a male vole fed the 13% protein diet, 4) a female vole fed the 13% protein diet, 5) a male vole fed the 22% diet, and 6) a female vole fed the 22% diet. Once again, there were 12 subjects for each dyad, resulting in a total of 36 male and 36 female subjects. Bottom-scent donors were selected from a pool consisting of 30 males and 30 females. The top- and bottom-scent donors were unfamiliar and unrelated to one another. We used unique combinations of top- and bottom-scent donors.

Arena

The top-scent donors were allowed to scent mark in a T-shaped arena. This arena was constructed of green acrylic sides. Each arm and the stem of the arena was 25 cm (length) x 13 cm (width) x 16 cm (height). The color, shape and size of the arena simulate an intersection of two natural vole runways (Ferkin et al., 2004a, b). White photocopy paper served as the substrate and was placed on the floor of the arena. The experimenter wore disposable floor-exam gloves when handling the paper to prevent the introduction of human scents.

Procedure

Feces from bottom-scent donors were collected fresh for each trial. Voles typically deposit feces when scent marking (Ferkin et al., 2004a, b) and can detect differences in the protein content of two individual's diets by investigating their feces (Ferkin et al., 1997). Briefly, the bottom-scent donors provided a fixed number (16 marks) of scent marks that were placed in one arm of the arena (Ferkin, 2010). The paper contained 16 fecal scent marks (two equidistant rows of 8 marks) from a male or a female vole (the bottom-scent donor); each fecal scent mark was approximately 0.4-0.5 cm in length and 0.1-0.2 cm in width. As in other studies (Ferkin et al., 2004a, b; Ferkin 2010), this method of scent deposition allowed the investigator to control for the size and the number of marks deposited by the bottom-scent donor and to prevent a bias in the scent marking behavior of the bottom-scent donor. Under UV light, each of these 16 marks was visualized and circled by a pen with blue ink to distinguish them from marks that would be deposited by the top-scent donor. Sixteen marks were selected as it was approximately the mean number of marks deposited by meadow voles while exploring an empty arena for 10 min (Ferkin, 2010). Previous tests indicated that the presence or color of the ink used in tracing has no effect on the marking behavior of voles (Ferkin et al., 2004a, b). The resulting configuration of the T-arena was such that it contained an arm that contained the scent marks of the bottom-scent donor (the scented arm) and an arm that was not marked by that donor (the unscented arm).

The top-scent donor was placed into the stem of the arena 15 min after marks of the bottom-scent donor were placed in the arena. The top-scent donor was allowed to explore and scent mark within the T-maze for 10 min and was then returned to its home

cage. The paper substrate was allowed to dry for 5 min. Under UV illumination (Blak-Ray Longwave UV lamp, UVP Model B100 AP, Upland CA, USA) in an otherwise dark room, we recorded the number and location of scent marks that the top-scent donor deposited in the T-shaped arena (Ferkin et al., 2004a, b; Ferkin, 2010). Scent marks deposited included feces, urine, and anogenital area secretions. Under UV light, feces appears as brown streaks or boli, urine appears as blue spots, and anogenital area secretions appear as silver-white slivers. We assumed their signal values to be similar (Ferkin et al., 2004a, b; Ferkin, 2007). Thus, one urine mark, two anogenital area marks, and two fecal boli were considered to represent five marks. The three types of scent marks convey sexually discriminable information to conspecifics (Ferkin & Johnston, 1995). We used red ink to trace the outline of the scent marks deposited by the top-scent donor. This allowed the observer to distinguish between the marks of the top-scent donor from those of the bottom-scent donor, whose scent marks were previously traced in blue ink.

We considered over-marks to be marks by the top-scent donor that overlapped or touched those deposited by the bottom-scent donor (Ferkin et al., 1999, 2004a, b, Ferkin, 2010). We counted the total number of scent marks deposited in each arm by the top-scent donor. We also counted the number of marks of the bottom-scent donor that were over-marked by the top-scent donor. Next, we calculated the proportion of the bottom-scent donor's marks that were over-marked by those of the top-scent donor's marks. We also calculated the proportion of the top-scent donor's scent marks that were used to over-mark the scent marks of the bottom-scent donor.

Statistical methods

For experiment 1, we used a 3-way ANOVA with sex of the top-scent donor (male or female), arm (scented or unscented), and amount of protein in the top-scent donor's diet (9%, 13%, or 22%) as main effects to determine whether differences existed in the total number of scent marks that were deposited by the top-scent donor. We used *a posteriori* multiple pairwise comparisons (Holm-Sidák method) to assess statistical differences across groups. We used separate 2-way ANOVA's with sex of the top-scent donor (male or female) and the amount of protein in the top-scent donor's diet (9%, 13%, or 22%) as main effects to determine whether differences existed in the proportion of bottom-scent donor's marks that were over-marked by the top-scent donor and the proportion of marks used by the top-scent donor to over-mark the bottom-scent vole's marks. In both cases, we arcsine-square root transformed the proportions prior to analysis. We used *a posteriori* multiple pairwise comparisons (Holm-Sidák method) to assess statistical differences across groups. Statistical procedures were identical for experiment 2 except that the independent variable dealing with protein content of the diet was now in terms of the bottom-scent donor rather than the top-scent donor. Statistically different significance was accepted at $\alpha=0.05$. The non-transformed data are presented in the figures.

Results

Experiment 1

The number of scent marks deposited in the arena by the top-scent voles was not affected by the protein content of their diet ($F_{2, 132} = 1.681, p = 0.190$). However, the

number of scent marks deposited by the top-scent voles was affected by their sex ($F_{1, 132} = 30.500, p = 0.0001$) and by whether those marks were placed in the previously scented or unscented arm of the arena ($F_{1, 132} = 15.457, p = 0.0001$). There was no interaction between the subject's sex and protein content of the diet ($F_{2, 132} = 1.101, p = 0.336$), sex and arm of the arena ($F_{1, 132} = 0.246, p = 0.621$), the protein content of the diet and the arm of the arena ($F_{2, 132} = 0.245, p = 0.783$), and protein content of the diet, sex, and arm of the arena ($F_{2, 132} = 0.684, p = 0.507$). Male and female voles placed more scent marks in the arm with the scent marks of an opposite-sex conspecific than they did in the clean, unscented arm (Holm-Sidák, $p < 0.05$, Figure 3.1a). Male voles deposited more scent marks than did female voles when they encountered areas containing the scent marks of conspecifics (Holm-Sidák, $p < 0.05$, Figure 3.1a).

The proportion of the bottom-scent donor's marks that were over-marked by the top-scent donor was affected by the sex of the top-scent donor ($F_{1, 66} = 24.328, p = 0.0001$), but not by the amount of protein in its diet ($F_{2, 66} = 1.372, p = 0.261$). There was no significant interaction between the sex of the top-scent donor and the amount of protein in its diet ($F_{2, 66} = 1.193, p = 0.310$). Thus, males fed the 9%, 13%, and 22% protein diets over-marked a similar proportion of the bottom-scent female's marks (Holm-Sidák, $p > 0.05$, Figure 3.1b). Likewise, females fed the 9%, 13%, and 22% protein diets over-marked a similar proportion of the bottom-scent male's marks (Holm-Sidák, $p > 0.05$, Figure 3.1b). Male voles, however, over-marked a greater percentage of the bottom-scent donor's marks than did female voles (Holm-Sidák, $p < 0.05$, Figure 3.1b).

The proportion of the top-scent vole's marks used to over-mark the bottom-scent donor's marks was affected by the top-scent donor's sex ($F_{1, 66} = 5.817, p = 0.019$), but not by the amount of protein in its diet ($F_{2, 66} = 0.165, p = 0.848$). There was no significant interaction between the subject's sex and the amount of protein in its diet ($F_{2, 66} = 0.533, p = 0.590$). Males fed the 9%, 13%, and 22% protein diets used a similar proportion of their scent marks as over-marks (Holm-Sidák, $p > 0.05$, Figure 3.1c). Likewise, females fed the 9%, 13%, and 22% protein diets used a similar proportion of their scent marks as over-marks (Holm-Sidák, $p > 0.05$, Figure 3.1c). Male voles, however, used a greater proportion of their scent marks as over-marks than did females (Holm-Sidák, $p < 0.05$, Figure 3.1c).

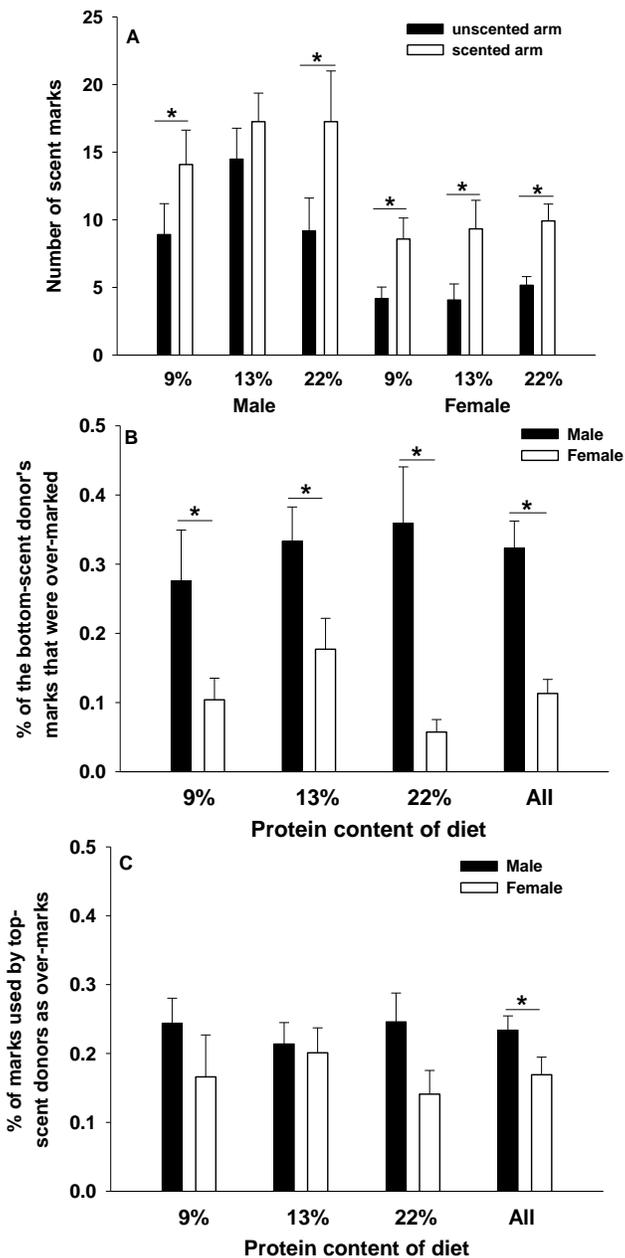


Figure 3.1: Upon altering the protein content of the top-scent vole's diet a) the number (mean \pm SEM) of scent marks deposited by male and female meadow voles in the arm of the arena containing the scent marks of an opposite-sex conspecific (scented arm) and the arm of the arena that did not contain any scent marks (unscented arm), b) the proportion (mean \pm SEM) of the scent marks of the bottom-scent donor over-marked by the top-scent donor within and across each protein diet, and c) the proportion (mean \pm SEM) of the top-scent donor's scent marks used to over-mark the marks of the bottom-scent donor within and across each protein diet. * indicates significant differences across groups at $p < 0.05$.

Experiment 2

The number of scent marks deposited in the arena by the top-scent voles was not affected by the protein content of the bottom-scent donor's diet ($F_{2, 132} = 0.0813$, $p = 0.922$) or the arm of the arena in which these marks were placed ($F_{1, 132} = 0.324$, $p = 0.570$). However, the number of scent marks deposited by the top-scent voles was affected by their sex ($F_{1, 132} = 23.600$, $p < 0.001$). There was no interaction between the top-scent vole's sex and protein content of the diet of the bottom-scent donor ($F_{2, 132} = 0.441$, $p = 0.644$), sex and arm of the arena ($F_{1, 132} = 0.441$, $p = 0.508$), the protein content of the bottom-scent donor's diet and the arm of the arena ($F_{2, 132} = 3.019$, $p = 0.052$), and protein content of the bottom-scent donor's diet, top-scent vole's sex, and arm of the arena ($F_{2, 132} = 1.567$, $p = 0.213$). Male voles deposited more scent marks than did female voles when they encountered areas containing the scent marks of conspecifics (Holm-Sidák, $p < 0.05$, Figure 3.2a). Male and female voles placed more scent marks in the scented arm relative to the unscented arm when the bottom-scent donor was fed a 22% protein diet (Holm-Sidák, $p < 0.05$, Figure 3.2a). Top-scent male voles placed a similar number of scent marks in the scented and unscented arms when the bottom-scent female was fed a 13% protein diet (Holm-Sidák, $p > 0.05$, Figure 3.2a), whereas top-scent females placed more scent marks in the unscented arm relative to the scented arm when the bottom-scent male was fed a 13% protein diet (Holm-Sidák, $p < 0.05$, Figure 3.2a). Male and female voles placed a similar number of scent marks in the scented and unscented arms when the bottom-scent donor was fed a 9% protein diet (Holm-Sidák, $p > 0.05$, Figure 3.2a).

The proportion of the bottom-scent donor's marks that were over-marked by the top-scent donor was affected by the sex of the top-scent donor ($F_{1,66}=19.818, p < 0.001$), but not by the amount of protein in the bottom-scent donor's diet ($F_{2,66} = 0.666, p = 0.517$). There was no significant interaction between the sex of the top-scent donor and the amount of protein in the bottom-scent donor's diet ($F_{2,66} = 1.762, p = 0.180$). Thus, males over-marked a similar proportion of scent marks when the bottom-scent female was fed a 9%, 13%, or 22% protein diet (Holm-Sidák, $p > 0.05$, Figure 3.2b). Likewise, females over-marked a similar proportion scent marks when the bottom-scent male was fed a 9%, 13%, or 22% protein diet (Holm-Sidák, $p > 0.05$, Figure 3.2b). Male voles, however, over-marked a greater percentage of the bottom-scent donor's marks than did female voles (Holm-Sidák, $p < 0.05$, Figure 3.2b).

The proportion of the top-scent vole's marks used to over-mark the bottom-scent donor's marks was affected neither by the top-scent donor's sex ($F_{1,66} = 1.726, p = 0.193$), nor the protein content of the bottom-scent donor's diet ($F_{2,66} = 1.643, p = 0.201$). There was no significant interaction between the subject's sex and the amount of protein in the bottom-scent donor's diet ($F_{2,66} = 0.390, p = 0.679$). Males and females used a similar proportion of their scent marks as over-marks, independent of the protein content of the bottom-scent donor's diet (Holm-Sidák, $p > 0.05$, Figure 3.2c).

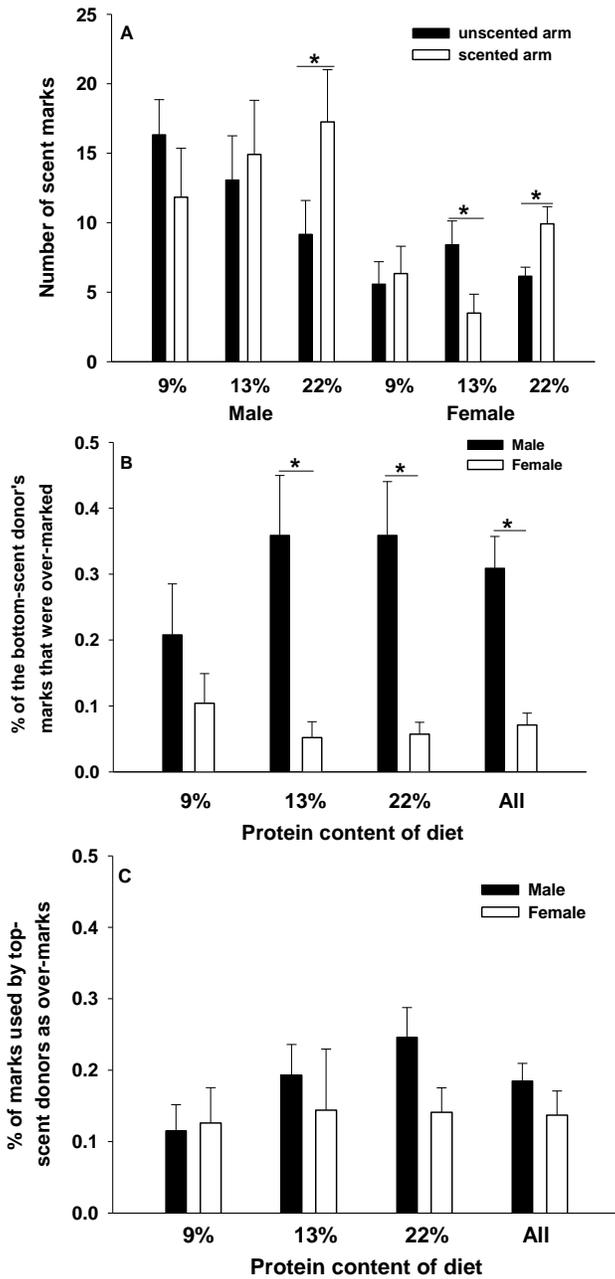


Figure 3.2: Upon altering the protein content of the bottom-scent vole's diet a) the number (mean \pm SEM) of scent marks deposited by male and female meadow voles in the arm of the arena containing the scent marks of an opposite-sex conspecific (scented arm) and the arm of the arena that did not contain any scent marks (unscented arm), b) the proportion (mean \pm SEM) of the scent marks of the bottom-scent donor over-marked by the top-scent donor within and across each protein diet, and c) the proportion (mean \pm SEM) of the top-scent donor's scent marks used to over-mark the marks of the bottom-scent donor within and across each protein diet. * indicates significant differences across groups at $p < 0.05$.

Discussion

The results of experiment 1 failed to support our hypothesis that voles fed a diet high in protein content will deposit more scent marks and over-marks compared to voles fed a diet lower in protein content upon encountering the scent marks of an opposite-sex conspecific. That is, male voles fed a 9%, 13%, or a 22% protein diet deposited a similar number of scent marks in the arm of the arena containing the scent marks of a female vole, over-marked a similar proportion of the bottom-scent female's marks, and used a similar proportion of their scent marks as over-marks. Similarly, female voles fed a 9%, 13%, or 22% protein diet deposited a similar number of scent marks in the arm of the arena containing the scent marks of a male vole, over-marked a similar proportion of the bottom-scent male's marks, and used a similar proportion of their scent marks as over-marks. These results are similar to those of a previous study which found that protein content of the diet also did not affect the rate at which meadow voles self-groom to the odors of opposite-sex conspecifics (Hobbs et al., 2008); self-grooming is a behavior that voles also employ to indicate their presence in an area to nearby opposite-sex conspecifics (Ferkin & Leonard, 2010). Taken together, these findings suggest that the protein content of a vole's diet does not affect some of the behaviors used to attract or indicate interest in opposite-sex conspecifics. Although protein content of the diet did not affect these proceptive behaviors, other factors that influence a vole's condition, such as its age and reproductive state, have been shown to affect its scent marking and over-marking behavior. Older voles and voles in heightened states of sexual receptivity deposited more scent marks and over-marks than did younger voles and voles that were not in heightened states of sexual receptivity (Ferkin et al., 2004b; Ferkin, 2010). Why

do certain features of an animal's condition (e.g. age and reproductive state) affect the animal's marking behaviors while other features (e.g. protein content of the diet) do not? One potential explanation is that although voles fed a diet low in protein content are less attractive to opposite-sex conspecifics (Ferkin et al., 1997; Hobbs et al., 2008; Hobbs and Ferkin, in press), they may still be capable of mating. In contrast, voles that are too young or are not in heightened reproductive state may not be capable of mating and may be unable to or less inclined to perform behaviors that are associated with showing interest in the opposite sex, such as self-grooming and scent marking (Ferkin et al., 2004a; Ferkin, 2006; Ferkin & Leonard, 2010).

The results of experiment 2 did not support our hypothesis that top-scent donor voles will deposit more scent marks and over-marks the scent marks of a bottom-scent donor that was fed a high-protein diet than they would the scent marks of a bottom-scent conspecific that was fed a low-protein diet. Independent of the protein content of the bottom-scent female's diet, male voles deposited a similar number of scent marks in the scented arm of the arena, over-marked a similar proportion of the female's scent marks and used a similar proportion of their scent marks as over-marks. Likewise, female voles, independent of the protein content of the bottom-scent male's diet, deposited a similar number of scent marks in the scented arm of the arena, over-marked a similar proportion of the bottom-scent male's marks, and used a similar proportion of their scent marks as over-marks. These results differ from a previous study by Hobbs et al. (2008) which showed that self-grooming, which like scent marking and over-marking can be used to attract opposite-sex conspecifics, was affected by the protein content of the scent donor's diet. Specifically, female voles spent more time self-grooming when exposed to odors of

males fed a 22% protein diet compared to when they were exposed to odors of males fed either a 13% or 9% protein diet (Hobbs et al., 2008). One potential explanation for this difference may be the context in which these behaviors are used. Self-grooming produces an ephemeral signal, and as such, is only used when opposite-sex conspecifics are nearby (Ferkin, 2006; Hobbs et al., 2008; Ferkin & Leonard, 2010). Scent marks, on the other hand, can last longer in the environment and may be deposited when there are no opposite-sex conspecifics are present (Bossert & Wilson, 1963; Brown & Macdonald, 1985). If males are known to be nearby, females may be choosier, and thus self-groom at higher rates in response to the odors of a male that has been consumed a 22% protein diet. By doing so, she can signal her presence in the area as well as her interest in that male as a potential mate (Ferkin, 2006; Hobbs et al., 2008; Ferkin & Leonard, 2010). In contrast, if no males are nearby, females may be less selective and use scent marks to try and attract males and over-mark any male scent marks she comes across, independent of the protein content of these males' diets.

It is interesting that protein content of the diet did affect the attractiveness of an individual's scent marks to conspecifics. Ferkin et al. (1997) reported that male and female voles fed a diet higher in their protein content produced scent marks that were more attractive than those of male and female voles fed a diet lower in protein content. From a mechanistic point of view, it is possible that protein content of the diet affects tissues involving the metabolism of the digestive exudates that are responsible for the production of sexually discriminable odors (Albone, 1984), but has no effect on the neural substrates that may underlie the scent marking and over-marking behavior of voles. This would suggest that protein content of the diet does not affect the act of scent

marking but does affect information about the donor's diet that is contained in the scent mark. From a functional point of view, it may be too costly for males and females, independent of the protein content of their diet, not to over-mark the marks of conspecifics and signal their presence in an area or willingness to mate (Ferkin et al., 2004a, b; Ferkin & Pierce, 2007). By not altering the frequency with which they scent mark and over-mark, voles that eat diets that differ in their protein content may still be able to communicate with multiple, potential partners (Boonstra et al., 1993; Berteaux et al., 1999).

Although scent marking and over-marking were not affected by the protein content of either the top- or bottom-scent donor's diets, these marking behaviors were affected by the sex of the top-scent donor. Male voles deposited a greater number of scent marks and over-marked a greater proportion of the bottom-scent donor's marks compared to female voles, independent of the protein content of either scent donor's diet. This is similar to other species of mammals where males scent mark and over-mark at higher rates relative to females (Ewer, 1968; Thiessen & Rice, 1976; Jordan, 2007). This sex difference in marking behavior upon encountering the scent marks of an opposite-sex conspecific may be explained by differences in a vole's natural history. Females are territorial, whereas males have large home ranges overlapping the territories of several females. As such, females may use their scent marks to maintain their territory and over-mark scent marks of other females that may be attempting to establish their own territory rather than over-marking the scent marks of male conspecifics (Ferkin et al., 2004b). Males, however, should use their scent marks to attract females as well as indicate interest in them by over-marking the female's scent marks (Ferkin & Pierce, 2007). Our

results suggest that male voles by scent marking and over-marking in response to the scent marks of females, independent of their respective protein-content diets, are attempting to seek out multiple females as potential mates. Multiple mating is a feature of the mating system of meadow voles (Boonstra et al., 1993; Berteaux et al., 1999) and over-marking and scent marking may allow males to locate and attract potential mates (Johnston et al., 1997a, b; Ferkin, 2010).

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Chapter 4: Dietary protein content affects the response of meadow voles, *Microtus pennsylvanicus*, to over-marks

Abstract

Dietary protein affects the behavior of animals and is an important contributor to their condition, and quality. In experiment 1, meadow voles (*Microtus pennsylvanicus*) were fed a 9%, 13%, or 22% protein diet to assess their response to an over-mark in which the top- and bottom-scent donors were fed the 22% protein diet. Males and females fed a 22% protein diet spent more time investigating the scent mark of the top-scent donor than that of the bottom-scent donor; voles fed 9% and 13% protein diets spent similar amounts of time investigating the top- and bottom-scent donors. In experiment 2, voles fed a 22% protein diet were exposed to an over-mark in which the top- and bottom-scent donors were fed a different protein diet. Female voles spent more time investigating the mark of the top-scent male than that of the bottom-scent male, independent of the differences in protein content of the diets of the two scent donors. Male voles, however, only showed a preference for the mark of the top-scent female when she was fed a diet higher in protein content than the bottom-scent female. Our results are discussed within the context of the natural history of voles.

Introduction

Communication between opposite-sex conspecifics can be affected by the nutritional state of those participating. For example, boars (*Sus scrofa*) and black field crickets (*Teleogryllus commodus*) fed a low protein diet are less responsive to signals from opposite-sex conspecifics than boars and crickets fed a high protein diet (Louis et

al. 1994; Hunt et al. 2005). Food-deprived meadow voles (*Microtus pennsylvanicus*) are also less responsive to signals from opposite-sex conspecifics than meadow voles that are not food-deprived (Pierce et al. 2005a, b; 2007a). In contrast, food-deprived swordtail fish (*Xiphophorus birchmanni*) are more likely to respond to the signals of opposite-sex conspecifics than those that are not food-deprived (Fisher and Rosenthal 2006). Thus, the diet and subsequent quality of the sender affect how it responds to the signals of opposite-sex conspecifics (Zeh and Zeh 1988; Grafen 1990; Andersson 1994; Johnstone 1995). In addition, the diet and subsequent quality of the sender affects how opposite-sex conspecifics respond to its signals (Wade and Schneider 1992; Ferkin et al. 1997; Gill and Rissman 1997; Jones and Wade 2002; Pierce et al. 2007b; Hobbs et al. 2008). For example, female meadow voles, dark-eyed juncos (*Junco hyemalis*), zebra finches (*Taeniopygia guttata*), house finches (*Carpodacus mexicanus*), guppies (*Poecilia reticulata*), and Iberian rock lizards (*Lacerta monticola*) are more responsive to signals of males fed diets higher in certain dietary components, including dietary protein, calcium, vitamin D, and carotenoids, than to the signals of males fed diets lower in these substances (Kodric-Brown 1985; Hill 1991, 1992; Ferkin et al. 1997; Martin and Lopez 2006; McGraw 2007; McGlothlin et al. 2007; Hobbs et al. 2008).

For many terrestrial mammals, information about their nutritional state can be conveyed via their scent marks (Brown and Macdonald 1985; Ferkin et al. 1997; Hobbs et al. 2008). Scent marks are often associated with digestive exudates, such as feces and urine. Such scent marks likely provide accurate and current information about the donor's nutritional state and subsequent quality as a mate (Albone 1984; Pierce et al. 2005a, b, 2007a, b). Thus, scent marks are frequently used by these animals to attract opposite-sex

conspecifics (Thiessen and Rice 1976; Brown and Macdonald 1985; Ferkin and Seamon 1987; Roberts 2007). However, many terrestrial mammals, including voles, place their scent mark on top of the scent mark of a same-sex conspecific, creating an over-mark (Hurst 1990; Johnston 2003; Ferkin et al. 2004a, b; Ferkin and Pierce 2007). Recent work has shown that individuals may respond differently to the same two scent donors if they encounter their scent marks first as separate and single scent marks or as if they encounter them first as part of an over-mark (Ferkin et al. in press). For example, when exposed to separate and single scent marks meadow voles respond preferentially to the scent mark of an opposite-sex conspecific that is in a better nutritional state than to those of an opposite-sex conspecific in a poorer nutritional state (Ferkin et al. 1997; Hobbs et al. 2008). Conversely, if the scent donors are in the same nutritional state, voles spend similar amounts of time investigating the scent mark of each donor (Ferkin et al. 1997; Pierce et al. 2005a, b, 2007a). Yet, if golden hamsters (*Mesocricetus auratus*), lorises (*Nycticebus pygmaeus*), prairie voles (*Microtus ochrogaster*), and meadow voles are exposed first to a same-sex over-mark of two donors in the same nutritional state, they later spend more time investigating the mark of the top-scent donor than that of the bottom-scent donor (Johnston et al. 1994, 1995; Ferkin et al. 1999; Fisher et al. 2003; Woodward et al. 2000). Thus, over-marks may provide individuals a more direct means of comparing two scent donors than single and separate scent marks (Johnston et al. 2003; Hurst and Beynon 2004; Ferkin et al. in press).

In free-living populations, individuals are likely to encounter over-marks in which the top- and bottom-scent donors differ in their respective nutritional states. However, we do not know how meadow voles or other terrestrial mammals respond to

such over-marks. Responses to over-marks of two conspecifics that differ in their nutritional may be important when we consider animals, such as meadow voles, which live in environments where quality forage is distributed in patches (Batzli 1985; Bergeron and Jodoin 1987). Studies have shown that the protein content of a meadow vole's diet affects its space use, habitat preference, and responses to the scent marks of opposite-sex conspecifics (Ferkin et al. 1997; Hobbs et al. 2008). Thus, in the present study, we carried out two experiments to examine the association between protein content of the diet and responses of meadow voles to same-sex over-marks. In experiment 1, we tested the hypothesis that protein content of the diet of a vole (subject) affects its response to same-sex over-marks. Specifically, we predicted that after exposure to a same-sex over-mark, meadow voles fed a high protein diet would spend more time investigating the mark of the top-scent donor of an over-mark, whereas voles fed a diet lower in protein content would spend similar amounts of time investigating the marks of the top- and bottom-scent donors. In experiment 2, we tested the hypothesis that protein content of the diets of the top- and bottom-scent donors affects how subjects respond to same-sex over-marks. Specifically, we determined whether relative differences in the protein content of the diet or whether their relative position in the over-mark affects the amount of time voles spend investigating the scent marks of the top-scent donor and the bottom-scent donor of a same-sex over-mark. If voles spend more time investigating the mark of the top-scent donor than that of the bottom-scent donor, independent of relative differences in the protein content of their diet, we could infer that voles use the position in the over-mark and not differences in their nutritional state to distinguish and respond preferentially to the mark of the top-scent donor to that of the bottom-scent donor. Conversely, if voles

spend more time investigating the mark of the donor fed a diet higher in protein content, independent of its position in the over-mark, we could infer that voles use differences in the nutritional states of the scent donors and not their position in the over-mark to assess and respond to the scent donor fed a diet higher in protein content.

General Methods

Animals

We used meadow voles that were descendants of those captured in Pennsylvania, Kentucky, and Ohio, USA. Voles from the different populations were bred with each other to prevent inbreeding. Every 18-24 months, the voles in the colony were mated with captured free-living voles. In this study, meadow voles were born and raised under long photoperiod (14:10 h, L: D, lights on at 0700h CST). All voles were weaned between 19-21 days of age, housed with littermates until 33-36 days of age, and thereafter housed singly in clear plastic cages (18 x 12.5 x 10 cm). Cages contained cotton nesting material, water, and food (see protein diets below). Meadow voles were housed in the animal facility at the University of Memphis. Female meadow voles are induced ovulators that do not undergo regular estrus cycles (Milligan 1982; Keller 1985). Adult female voles born and reared in long photoperiod are sexually receptive (Meek and Lee 1993). Long-photoperiod meadow voles respond preferentially to the scent marks of opposite-sex conspecifics (Ferkin and Seamon 1987; Ferkin and Johnston 1995). Subjects and scent donors used in this study were 3-7 mo-old, sexually mature, but not sexually experienced.

Protein Diets

Male and female voles were fed either a diet comprised of 9%, 13%, or 22% protein content for 30 days prior to testing. We chose diets containing 9%, 13%, or 22% protein because they roughly corresponded to the range of protein content available to free-living meadow voles during the breeding season (Batzli 1985). The 9% protein diet represents the quality of food (e.g. hay) that a vole may encounter in a poor habitat in the wild (Lindroth and Batzli 1984). The 13% protein diet represents the quality of food (e.g. alfalfa) voles may encounter when living in a good habitat (Lindroth and Batzli 1984). The 22% protein diet represents the quality of food (e.g. legumes) a vole may encounter when living near a cultivated field (Lindroth and Batzli 1984). The 9% and 13% protein diets were specially prepared by Harlan Teklad (Madison, WI, USA); the 22% diet was Harlan Teklad's standard rodent chow (Laboratory Rodent Diet # 8640). Diets with similar amounts of protein content were used in other studies of olfactory communication by voles (Ferkin et al. 1997; Hobbs et al. 2008).

The three protein diets were isocaloric and calculated to provide 3 Kcal/g of digestible energy. The 9% diet contained 53.2% carbohydrate and 5.0% fat. The 13% diet contained 49.7% carbohydrate and 5.1% fat. The 22% diet contained 51.2% carbohydrate and 5.3% fat.

Experimental Design

Our testing methods were similar to those detailed in other studies of over-marking in voles (Ferkin et al. 1999, in press; Woodward et al. 2000; Leonard et al. 2001;

Pierce et al. 2007b). Our design involved two phases, the exposure phase and testing phase, both of which took place in the subjects' home cages. All testing was carried out between 0900 and 1200 h CST. Experiment 1 was conducted between May and July 2009. Experiment 2 was conducted between August and October 2009.

Subjects and Scent Donors

In experiment 1, we used 36 male and 36 female voles that were fed either a 9%, 13%, or 22% protein diet (n=12 per group for each sex) were presented with a same-sex over-mark in which both the top- and bottom-scent donors were same-sex conspecifics but opposite in sex to the subject. Thus, males were exposed to over-marks containing the scent marks of two female donors and females were exposed to over-marks containing the scent marks of two male donors. The top- and bottom-scent donors of the over-marks were both fed a 22% protein diet. Each subject vole underwent a single exposure and a single preference test with a unique pairing of scent donors chosen from a pool of 30 male and 30 female voles. On the day of testing, subjects and scent donors had been fed their respective diets for 30 days.

In experiment 2, we chose 72 male and 72 female voles that were fed a 22% protein-content diet and exposed them to a same-sex over-mark, in which the top- and bottom-scent donors were same-sex conspecifics but opposite in sex to the subject. Thirty-six male and 36 female voles served as scent donors and each subject was exposed to a unique pair of scent donors. The top- and bottom-scent donors of the over-marks were each fed a different protein diet, resulting in 6 over-mark combinations. The over-mark combinations were: 1) scent mark of an opposite-sex conspecific fed the 22%

protein diet on top of the scent mark of an opposite-sex conspecific fed the 9% protein diet (22/9); 2) scent mark of an opposite-sex conspecific fed the 9% protein diet on top of the scent mark of an opposite-sex conspecific fed the 22% protein diet (9/22); 3) scent mark of an opposite-sex conspecific fed the 22% protein diet on top of the scent mark of an opposite-sex conspecific fed the 13% protein diet (22/13); 4) scent mark of an opposite-sex conspecific fed the 13% protein diet on top of the scent mark of an opposite-sex conspecific fed the 22% protein diet (13/22); 5) scent mark of an opposite-sex conspecific fed the 13% protein diet on top of the scent mark of an opposite-sex conspecific fed the 9% protein diet (13/9); and 6) scent mark of an opposite-sex conspecific fed the 9% protein diet on top of the scent mark of an opposite-sex conspecific fed the 13% protein diet (9/13). There were 12 different male subjects and 12 different female subjects tested for each of the six over-mark combinations.

Exposure Phase

Feces are deposited by voles in areas that are frequented by conspecifics (Brown and Macdonald 1985; Ferkin et al. 2004a, b), providing sexually distinct cues to conspecifics (Ferkin and Johnston 1995). Thus, we used feces scent marks to create the over-marks. To do so, fresh fecal boli were collected for each trial. One or two fecal boli from a scent donor were dragged across the center of a glass microscope slide (2.5 x 7.6 cm). One min later, a similar amount of the feces from another donor was dragged over the top of the previously deposited scent mark, such that the two marks overlapped, and the resulting configuration was a “+” shape. Each feces scent mark was approximately

1.2 cm in length and 0.3 cm in width. Thus, we were able to control for the size of the scent marks (Ferkin et al. 1999, in press; Woodward et al. 2000; Vlautin et al. in press).

After the second scent mark was placed on the slide, we waited 1 min before placing the slide into the subject's cage. The slide was placed against the wall opposite the subject's nest and suspended 2 cm above the substrate by a clean metal clip and hook. Subjects were exposed to this slide for 5 min. This slide was placed in the cage of only one subject and then discarded. In all observations, the observer was blind to the identity of the top- and bottom-scent mark donors. All subjects investigated the slide during the exposure phase.

Test Phase

The test phase began 1 min after completion of the 5-min exposure phase. We presented the 36 male and 36 female voles from experiment 1 and 72 male and 72 female voles from experiment 2 with a glass slide (2.5 x 7.6 cm) that contained the feces scent marks of the two opposite-sex conspecifics that provided the top- and bottom-scent marks of the over-mark during the subject's exposure phase. The scent marks were not overlapping and placed separately on different sections of the test slide. Briefly, we divided the glass test slide into three equal sections (each 2.5 cm in length); one end section of the slide contained the feces scent mark of the opposite-sex scent donor that provided the top-scent mark during the exposure phase. The other end section of the test slide contained the feces scent mark of the opposite-sex scent donor that provided the bottom-scent mark during the exposure phase. The middle section of the slide contained no scent marks. To deposit the scent marks on the slide, we dragged one or two fresh

fecal boli from one scent donor across the left-end section of a clean glass microscope slide and one or two fecal boli from the other scent donor across the right-end section of the same slide. One min separated the deposition of the scent marks of the two donors on the slide. The placement of a particular donor's scent mark on the left or right side of the slide was random. The scent marks were roughly the same size, approximately 1.2 cm x 0.3 cm (l x w). After both scent marks were placed on the slide, we waited 1 min before we suspended the slide in the home cage of the subject. We recorded the amount of time that male and female subjects licked or sniffed (the subject's nose came within 2 cm) each scent mark on the slide continuously for 3 min. The observer was blind to the position of the donors' scent marks on the slide. Each test slide was used once and then discarded (Ferkin et al. 1999, in press; Woodward et al. 2000; Vlautin et al. in press).

Statistical Methods

To test the two hypotheses, we recorded the amount of time the subject investigated the scent marks of the top- and bottom-scent donors of the over-mark. To be included in the data analysis, subjects had to have investigated the scent marks of both donors and spend more time investigating the scent marks of the two donors than they did investigating the clean portion of the slide (Ferkin et al. 1999, in press; Woodward et al. 2000; Vlautin et al. in press). We used these times to compute a ratio for the time spent investigating the scent mark of the top-scent donor vs. the scent mark of the bottom-scent donor. Since the data exhibited variance heterogeneity, we calculated the natural logarithms of these ratios prior to their use in our analysis (Lenington 1983; Drickamer 1989). Because the natural logarithm of 1 is 0, $\ln(1) = 0$, ratios of about 1 would indicate

that the subject spent a similar amount of time investigating the scent marks of the top- and bottom-scent donors. Ratios with positive values would indicate that the subject spent a greater amount of time investigating the scent mark of the top-scent donor compared to the scent mark of the bottom-scent donor. Conversely, negative ratios would indicate that subjects spent more time investigating the scent mark of the bottom-scent donor compared to the scent mark of the top-scent donor. These ratios were used in a two-way analysis of variance (ANOVA) for each experiment, with either sex of the subject and protein content of the subject's diet (experiment 1) or sex of the subject and over-mark combination (experiment 2) as main effects. Post hoc testing followed using the Holm-Sidák method for multiple pairwise comparisons. Significant differences were accepted at $\alpha = 0.05$.

Results

Experiment 1

The ratio of time spent investigating the scent mark of the top-scent donor to the amount of time investigating the bottom-scent donor's scent mark was not affected by the sex of the subject ($F = 0.900$, $df = 1, 61$, $P = 0.347$). This ratio was, however, affected by the protein content of the subject's diet ($F = 4.350$, $df = 2, 61$, $P = 0.017$). There was no interaction between a subject's sex and the protein content of its diet ($F = 0.210$, $df = 2, 61$, $P = 0.811$). Male and female subjects fed a 22% protein diet spent more time investigating the scent mark of the top-scent donor than that of the bottom-scent donor of an over-mark compared to male and female subjects fed either a 9% or 13% protein diet (Holm-Sidák, $P < 0.05$, Fig. 4.1). Male and female subjects fed either a 9% or 13%

protein diet spent similar amounts of time investigating the marks of the top- and bottom-scent donors of the over-mark (Holm-Sidák, $P > 0.05$, Fig. 4.1).

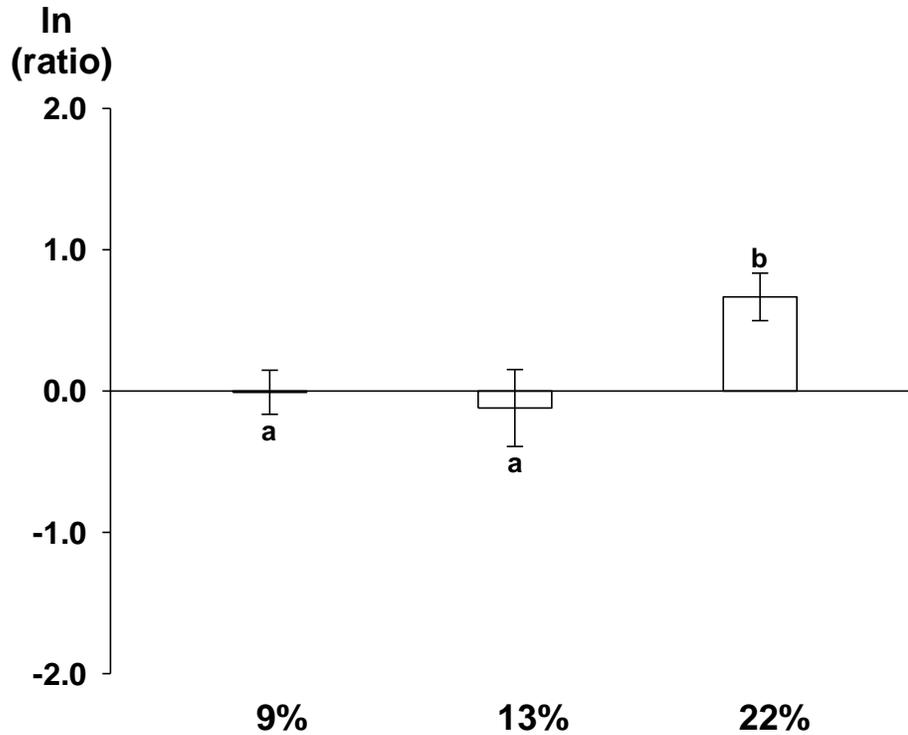


Fig. 4.1. Means (\pm SEM) of natural logarithms of the ratio of time spent by female and male meadow voles fed a 9%, 13%, or 22% protein diet investigating the scent mark of the top-scent donor over the time spent investigating the scent mark of the bottom-scent donor of a same-sex over-mark where the scent donors were both fed the 22% protein diet. Positive ratios indicate that subjects spent a greater amount of time investigating the scent mark of the top-scent donor compared to that of the bottom-scent donor. Negative ratios indicate that subjects spent a greater amount of time investigating the scent mark of the bottom-scent donor compared to that of the top-scent donor. Bars not marked with the same letter are significantly different at the 0.05 level (Holm-Sidák).

Experiment 2

The ratio of time spent investigating the scent mark of the top-scent donor to the amount of time spent investigating the scent mark of the bottom-scent donor of an over-mark in which each donor was fed a different protein diet was affected by the subject's sex ($F = 42.737$, $df = 1$, 110, $P < 0.001$). This ratio was also significantly affected by the combination of donors that comprised the over-mark that the subject was exposed to ($F = 11.829$, $df = 5$, 110, $P < 0.001$). There was a significant interaction between the subject's sex and the over-mark combination they were exposed to ($F = 4.951$, $df = 5$, 110, $P < 0.001$). Female meadow voles spent more time investigating the scent mark of the top-scent donor compared to that of the bottom-scent donor, independent of the protein content of either scent donor's diet (Holm-Sidák, $P < 0.05$, Fig. 4.2A).

Male voles spent more time investigating the scent mark of the top-scent female compared to that of the bottom-scent female when the top-scent female was fed either a 22% or 13% protein diet and the bottom-scent female was fed a 9% protein diet (Holm-Sidák, $P < 0.05$, Fig. 4.2B). Males spent more time investigating the mark of the bottom-scent female when the top-scent female was fed a 9% protein diet and the bottom-scent female was fed a 22% protein diet (Holm-Sidák, $P < 0.05$, Fig. 4.2B). Male voles spent similar amounts of time investigating the marks of the top- and bottom-scent female donors when the top-scent female was fed either a 22% or 9% protein diet and the bottom-scent female was fed a 13% protein diet (Holm-Sidák, $P < 0.05$, Fig. 4.2B). Males also spent similar amounts of time investigating the mark of the top-scent female when she was fed a 13% protein diet and the bottom-scent female was fed a 22% protein diet (Holm-Sidák, $P < 0.05$, Fig. 4.2B).

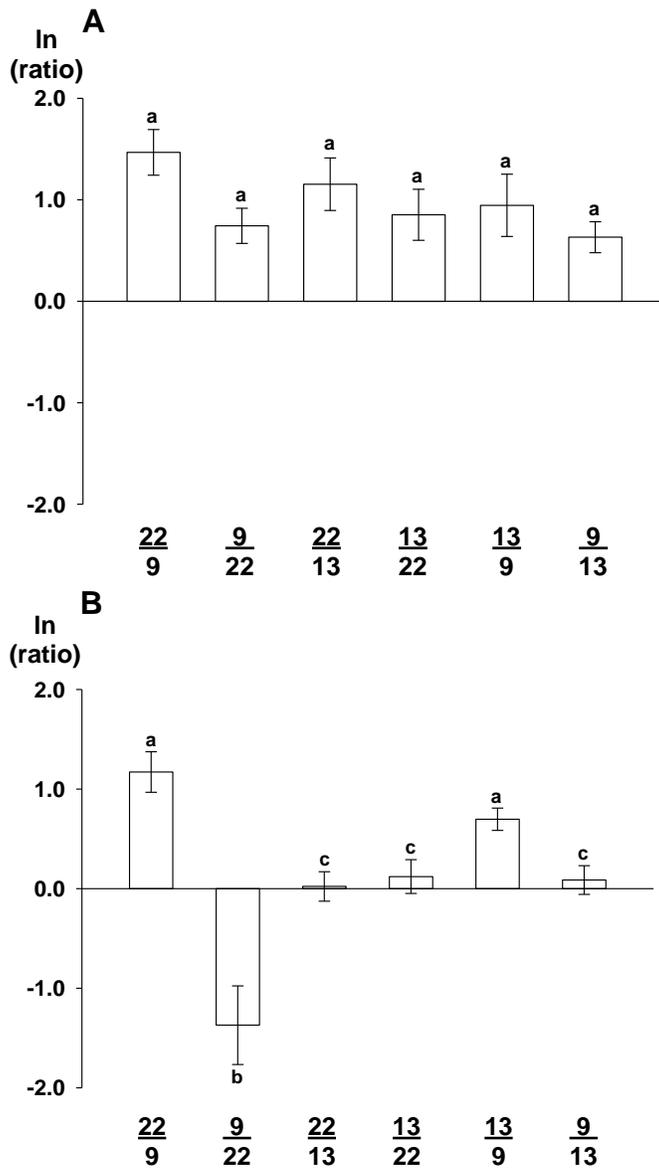


Fig. 4.2. Means (\pm SEM) of natural logarithms of the ratio of time spent by (A) female and (B) male meadow voles exposed to 6 different over-mark combinations investigating the scent mark of the top-scent donor over the time spent investigating the scent mark of the bottom-scent donor of a same-sex over-mark where the scent donors differed in the protein content of their diets. Positive ratios indicate that subjects spent a greater amount of time investigating the scent mark of the top-scent donor compared to that of the bottom-scent donor. Negative ratios indicate that subjects spent a greater amount of time investigating the scent mark of the bottom-scent donor compared to that of the top-scent donor. Bars not marked with the same letter are significantly different at the 0.05 level (Holm-Sidák).

Discussion

The results of experiment 1 support the hypothesis that the protein content of a meadow vole's diet affects its response to the top- and bottom-scent donors of a same-sex over-mark when both scent donors are fed a 22% protein diet. Male and female meadow voles fed a 22% protein diet spent more time investigating the scent mark of the top-scent donor than that of the bottom-scent donor. Preferential responses for the top-scent donor of a same-sex over-mark were also obtained in studies in which meadow voles, prairie voles and golden hamsters were fed diets comparably high in protein content (Johnston et al. 1994, 1995; Woodward et al. 2000; Ferkin et al. in press). In contrast, we found that voles fed either a 9% or 13% protein diet displayed no preference for the scent mark of either the top- or bottom-scent donor of a same-sex over-mark. One possible explanation for this finding is that voles fed a 9% and 13% protein diet are no longer able to discriminate between the top- and bottom-scent marks of a same-sex over-mark. Meadow voles that were exposed to an over-mark and then food deprived for 6 h could not discriminate between the top- and bottom-scent marks of a same-sex over-mark (Pierce et al. 2007b). Likewise, rats fed a ketogenic diet, a high-fat, low-carbohydrate, and low-protein diet, suffered severe impairments in discriminations involving visual-spatial memory (Zhao et al. 2004). Alternatively, this finding may be explained by the fact that male and female voles fed a 9% or 13% protein diet may no longer be motivated to discriminate between the top- and bottom-scent marks of an over-mark, suggesting a decreased interest in mating. Boars and male black field crickets that are fed a low-protein diet and meadow voles that were food-deprived for 6 h displayed fewer behaviors directed at opposite-sex conspecifics than did conspecifics fed a high protein diet or

conspecifics that were not food deprived (Louis et al. 1994; Hunt et al. 2005; Pierce et al. 2005a, b, 2007a). Taken together, these studies support the view that a subject's nutritional state may influence its ability or its motivation to seek mates (Zeh and Zeh 1988; Grafen 1990; Andersson 1994; Johnstone 1995; Ferkin et al. 1997).

In experiment 2, we found that female meadow voles spent more time investigating the scent mark of the top-scent male than that of the bottom-scent male, independent of the protein content of either scent donor's diet. These results are not congruent with the hypothesis that the protein content of the diet of the male scent donors of an over-mark affects how female voles respond to the top- and bottom-scent male donors. The results suggest that how recent a male has been in the female's territory may be more important to the female than how much protein was in his diet. This speculation is supported by the fact that male voles travel through the territories of multiple females (Madison 1980), and the top-scent male may represent the most recent individual that has visited the female's territory (Ferkin et al. 1999, in press; Woodward et al. 2000; Ferkin and Pierce 2007). Our results suggest that a female meadow vole's preference for the top-scent male donor over the bottom-scent male donor of the over-mark is likely due to the temporal association between these males. A preference for the nearby male would be consistent with the natural history of meadow voles. Many males will visit a receptive female (Boonstra et al. 1993). Selecting the top-scent male as potential partner may encourage females to mate with multiple males that are nearby (Berteaux et al. 1999) rather than waiting for a particular male. Multiple mating by females would also encourage sperm competition between males, which could allow females to confuse

paternity, produce litters of mixed paternity, or deliver offspring sired by higher quality males (Boonstra et al. 1993; Birkhead 2000).

Our results suggest that female voles do not respond in the same way to over-marks and non-overlapping scent marks of two male scent donors fed different protein diets. Females first exposed to the single and separate scent marks of two male donors prefer the mark of the male that was fed a diet higher in protein content than that of a male that was fed a lower protein diet (Ferkin et al. 1997, Hobbs et al. 2008). In contrast, in the present study, females exposed first to an over-mark prefer the mark of the top-scent male to that of the bottom-scent male, independent of the protein content of their diets. The difference in the responses of female voles supports and augments the view that voles may view or assess the scent marks encountered first singly and separately as being somehow different than that of the scent marks of the same donors encountered first as part of an over-mark (Hurst and Beynon 2004; Ferkin et al. in press; Vlautin et al. in press). In the present study, it seems that female voles prefer the mark of the top-scent male to that of the mark of the bottom-scent male of an over-mark, independent of relative differences in the protein content of their diets. We cannot rule out the possibility that female voles assess differences between the top-and bottom-scent males by other measures of a males' quality such as their age, familiarity, reproductive condition, or social status (Leonard et al. 2001; Ferkin 2007, 2010; Ferkin et al. in press; Vlautin et al. in press).

The results of experiment 2, however, support the hypothesis that protein content of the diet of the top- and bottom-scent female donors affects how male subjects respond to same-sex over-marks. Male voles spent more time investigating the mark of the top-

scent female than that of the bottom-scent female when the top-scent female was fed either a 22% or 13% protein diet and the bottom-scent donor was fed a 9% protein diet. Interestingly, male voles spent more time investigating the scent mark of the bottom-scent female than that of the top-scent female when she was fed a 22% protein diet and the top-scent female was fed a 9% protein diet. Taken together, these results indicate that male voles responded to relative differences in the protein content of the diet of the top- and bottom-scent female donors and not their relative position in the over-mark. When exposed to an over-mark in which the top-scent donor was fed a 22% protein diet and the bottom-scent donor was fed a 13% protein diet, or vice versa, males spent a similar amount of time investigating each female donor's scent mark. Male voles find the scents of females fed these diets to be equally attractive when exposed to them separately without an over-mark (Ferkin et al. 1997, Hobbs et al. 2008). Taken together, these results suggest that males may perceive females fed a 13% or 22% protein diet to be similar in quality and preferred as mates compared to females fed a diet lower in protein content. Our data support this view in that male voles spent more time investigating the scent marks of a female fed a 22% protein diet to that of a female fed a 9% diet, independent of which scent mark was the top scent of the over-mark. This suggests that male voles may view females fed a 9% protein diet as being of lower quality and not able to bear the nutritional costs associated with reproduction (Jones 1990; Sandell et al. 2007; Warner et al. 2007), such females may be considered by males to be of low quality (Ferkin et al. 1997). It appears that male voles do not simply use the rule of thumb stating that he should prefer the mark of the top-scent female to that of the bottom-scent female of an over-mark, independent of relative differences in the protein content of the diets of

the female donors. Rather, our data provide evidence that male meadow voles are flexible when it comes to distinguishing and responding to the over-marks of female conspecifics that are in different nutritional states. This speculation is in concordance with the natural history of this species, for as a male meadow vole wanders through the territories of females within his home range, he is likely to encounter the scent marks of many females (Madison 1980). A top-scent female whose scent marks signal that she has eaten a 22% protein diet may indicate to males that she resides in a territory that contains or abuts high quality forage (Batzli 1985; Ferkin et al. 1997). Females with access to high quality forage are more attractive and successful in rearing a litter than females that do not have access to such forage (Jones 1990; Sandell et al. 2007; Warner et al. 2007).

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Chapter 5: Effects of food availability on proceptivity: a test of the reproduction at all costs and metabolic fuels hypotheses

Abstract

Proceptive behaviours are used by animals to indicate interest in opposite-sex conspecifics. These behaviours can be affected by an individual's nutritional status. Two mutually exclusive hypotheses have been proposed to account for the effects of food availability on reproduction. These are the metabolic fuels hypothesis and the reproduction at all costs hypothesis. It is not known if food availability affects proceptive behaviours such as scent marking, over-marking, and self-grooming. In this study, we tested the hypothesis that food-deprived and nonfood-deprived meadow voles, *Microtus pennsylvanicus*, differ in the number of scent marks they deposit, the proportion of over-marks they deposit, and the amount of time they spend self-grooming when they encounter the scent marks of opposite-sex conspecifics. We tested this hypothesis by exposing meadow voles that either had continuous access to food or were food-deprived for either 6 or 24 hours to the scent marks of an opposite-sex conspecific. Due to differences in the natural history of male and female meadow voles, we predicted that female voles will follow the metabolic fuels hypothesis whereas males will follow the reproduction at all costs hypothesis. We found that both male and female voles deprived of food for either 6 hours or 24 hours spent less time self-grooming compared to nonfood-deprived voles. However, food availability did not affect the scent marking and over-marking behaviour of male and female voles. Differences in the effects of food

availability on these proceptive behaviours are discussed within the context of the natural history of meadow voles.

Proceptivity is one of three components of sexual behaviour as defined by Beach (1976). Individuals perform proceptive behaviours to indicate their interest in an opposite-sex conspecific as a potential mate, thereby facilitating sexual interactions between male and female animals (Beach 1976). As such, proceptivity is particularly important since it provides the transition from attracting mates to the act of copulation itself (Beach 1976; Stopka & Macdonald 1998, 1999; Vaughn et al. 2010). In terrestrial mammals, two behaviours that have a proceptive function are over-marking and self-grooming. These behaviours allow individuals to signal their presence in an area and indicate their interest or willingness to mate with nearby, opposite-sex conspecifics (Johnston 1983; Brown & Macdonald 1985; Ferkin & Leonard 2010).

The behaviours that male and female terrestrial mammals use to show interest in the opposite sex may be affected by the actor's nutritional status (Pierce & Ferkin 2005; Pierce et al. 2005; Hobbs & Ferkin 2011), which can be influenced by its access to food (Bronson 1989; Wade et al. 1996). Two hypotheses have been put forth to explain the effects of an individual's nutritional status on reproduction. The metabolic fuels hypothesis states that individuals may forgo reproduction when sufficient food is not available for them to offset the high energetic costs of pregnancy and lactation and parenting with their survival and that of their potential offspring (Wade & Schneider 1992; Wade et al. 1996). Alternatively, the reproduction at all costs hypothesis states that animals will continue to reproduce when faced with low food availability (Boonstra et al.

2001). Tests of these two hypotheses have examined the effects of food abundance on aspects of the physiological processes underlying reproduction (Wade et al. 1996; Gill & Rissman 1997; Boonstra et al. 2001). Pierce and colleagues (2005, 2007) have augmented these two hypotheses to explain the effects of food availability on sexual behaviours. Thus, the metabolic fuels hypothesis would state that food-deprived individuals would be less likely than nonfood-deprived individuals to display behaviours associated with securing a mate. In contrast, the reproduction at all costs hypothesis would state that food-deprived individuals and nonfood-deprived individuals would be equally likely to display behaviours associated with securing a mate. Pierce et al. (2005) tested these hypotheses and found that female meadow voles, *Microtus pennsylvanicus*, that were food deprived for 6 hours or longer spent similar amounts of time investigating the scent marks of a male and female vole. They also found that female voles that were food deprived for 24 hours no longer produced scent marks that were attractive to males (Pierce et al. 2005). Unlike female voles, male meadow voles that were food deprived for 24 hours continued to produce odours that were attractive to females (Pierce et al. 2005). These findings showed that the attractiveness of female voles' odours to males followed the predictions of the metabolic fuels hypothesis whereas the attractiveness of male voles' odours to females followed the predictions of the reproduction at all costs hypothesis. Pierce et al. (2005, 2007) attributed these sex differences to the constraints placed on the fitness of male and female voles, differences in their space use and life history patterns, and the energetic demands placed on female and male meadow voles during the breeding season.

We do not know whether food availability affects the expression of scent marking, over-marking, and self-grooming in rodents (Johnston 1979; Harriman & Thiessen 1985; Ferkin & Pierce 2007). Over-marking the scent marks of an opposite-sex conspecific allow individuals to indicate an individual's interest or willingness to mate with an opposite-sex conspecific (Hurst 1990; Woodward et al. 2000; Ferkin & Pierce 2007). Another behaviour that mammals may use to signal their presence in the area to nearby opposite-sex conspecifics is self-grooming (Steiner 1974; Thiessen 1977; Ferkin et al. 1996). Self-grooming increases the attractiveness of an animal's odours to opposite-sex conspecifics (Ferkin et al. 1996), increases the active space surrounding the groomer, making it easier to be detected (Bossert & Wilson 1963; Thiessen 1977), and stimulates the groomer to become more motivated to find nearby opposite-sex conspecifics (Vaughn et al. 2010). Although both over-marking and self-grooming have similar functions, the context in which these behaviours are displayed differs. Self-grooming produces an ephemeral signal, and as such, is only used when opposite-sex conspecifics are present or nearby (Hobbs et al. 2008; Ferkin & Leonard 2010; Vaughn et al. 2010). Scent marks and over-marks, however, are often deposited when opposite-sex conspecifics may or may not be nearby (Ferkin & Pierce 2007).

In this study, we tested the hypothesis that food-deprived voles and nonfood-deprived voles differ in the number of scent marks they deposit, the proportion of over-marks they create, and the amount of time they spend self-grooming when they encounter the scent marks of opposite-sex conspecifics. Pierce et al. (2005) suggested that female voles may be more sensitive to changes in food availability whereas male voles may be less sensitive to such changes in food availability. Thus, we predict that the proceptive

behaviour of female meadow voles will follow the predictions of the metabolic fuels hypothesis, whereas the behaviour of male meadow voles will follow the predictions of the reproduction at all costs hypothesis. We base these predictions on the natural history of meadow voles. A female vole's fitness may be more dependent on securing a territory and obtaining the necessary food to support both her pregnancy and the demands of lactation than it is on attracting or showing interest in and finding multiple mates (Keller 1985; Wolff 1993). More importantly, female voles are territorial and are limited to the amount of food in their territory (Madison 1980; Lindroth & Batzli 1984; Batzli 1985). In contrast to females, a male meadow vole's fitness depends mainly on the number of offspring he can sire (Boonstra et al. 1993), which would be increased by securing multiple mates (Trivers 1972; Birkhead 2000). Male voles occupy large home ranges and can likely find suitable amounts of forage (Madison 1980; Lindroth & Batzli 1984; Batzli 1985).

Methods

Animals

Meadow voles used in this study were descendants of field-caught voles caught in Ohio and Pennsylvania, USA. All voles used in this study were born and raised under a long photoperiod (14 L: 10D, lights on at 0700 hours CST and off at 2100 hours CST). This photoperiod is representative of the amount of light present during the breeding season. Voles were weaned at 19-21 days of age, housed with littermates until 33-35 days of age, and then housed singly in clear plastic cages (30.5 x 35.5 x 22.8 cm). Cages contained woodchip bedding and cotton nesting material. Voles were provided with

continuous access to food (rodent diet 8640, Harlan Teklad, Madison, WI, USA) and water, with the exception of the food-deprived subjects and scent donors (see below). We followed Animal Care Protocol 0647, which was approved by the IACUC at The University of Memphis. We adhered to the ‘Guidelines for the use of animals in research’ as published in *Animal Behaviour* (1991, 41, 183-186) and the laws of the country where the research was conducted.

Experiment 1: Scent marking and over-marking

Subjects and scent donors

Subjects (top-scent donors) were 72 voles (36 male and 36 female voles) that were divided into three groups, a group with continuous access to food, and two food-deprived groups: 6 hours and 24 hours. Each group consisted of 12 male and 12 female voles. Bottom-scent donors were selected at random from a pool of 50 voles (25 males and 25 females) that were not food-deprived and had continuous access to food. Female voles do not undergo regular oestrus cycles (Milligan 1982). Rather, female voles are sexually receptive when housed under a long photoperiod (Zucker et al. 1980). Subjects were each tested once for their scent marking and over-marking behaviour in response to the scent marks of an opposite-sex conspecific that had continuous access to food. The top-scent donor and the bottom-scent donor were unfamiliar and unrelated to one another. We used unique combinations of top-scent donors and bottom-scent donors for each trial.

Arena

We have measured scent marking and over-marking previously by using this arena (Ferkin et al. 2004a, b; Ferkin 2010). The top-scent donors were allowed to scent mark in a T-shaped arena. This arena was constructed of green acrylic sides. The stem and each arm of the arena was 25 cm (length) x 13 cm (width) x 16 cm (height). The color, shape and size of the arena simulate an intersection of two natural vole runways (Ferkin et al. 2004a, b). White photocopy paper served as the substrate and was placed on the floor of the arena. The experimenter wore disposable nitrile gloves when handling the paper to prevent the introduction of human scents.

Procedure

Faeces from bottom-scent donors were collected fresh for each trial. Voles typically deposit faeces when scent marking (Ferkin et al. 2004a, b). Briefly, the bottom-scent donors provided a fixed number (16 marks) of scent marks that were placed in one arm of the arena. We chose to use sixteen marks as it was approximately the mean number of marks deposited by meadow voles while exploring an empty arena for 10 minutes (Ferkin 2010). We placed the 16 faecal scent marks (two equidistant rows of 8 marks) from a male or a female vole (the bottom-scent donor) onto the substrate; each faecal scent mark was approximately 0.4-0.5 cm in length and 0.1-0.2 cm in width. As in other studies (Ferkin et al. 2004a, b; Ferkin 2010), this method of scent deposition allowed the investigator to control for the size and the number of marks deposited by the bottom-scent donor and to prevent a bias in the scent marking behaviour of the bottom-scent donor. Under UV light (Blak-Ray Longwave UV lamp, UVP Model B100 AP,

Upland CA, USA), we circled each of these 16 marks using a pencil to distinguish them from marks that would be deposited by the top-scent donor. Previous tests indicated that the presence or color of the ink used in tracing has no effect on the marking behaviour of voles (Ferkin et al. 2004a, b; Ferkin 2010). The resulting configuration of the T-arena was such that it contained an arm that contained the scent marks of the bottom-scent donor (the scented arm) and an arm that was not marked by that donor (the unscented arm).

We placed the top-scent donor into the stem of the arena 15 min after the marks of the bottom-scent donor were placed in the arena. We allowed the top-scent donor to explore and scent mark within the T-maze for 10 minutes and then returned it to its home cage. The paper substrate was allowed to dry for 5 minutes. Under UV illumination in an otherwise dark room, we recorded the number and location of scent marks that the top-scent donor deposited in the T-shaped arena (Ferkin et al. 2004a, b; Ferkin 2010). Scent marks deposited included faeces, urine, and anogenital area secretions. Under UV light, faeces appears as brown streaks or boli, urine appears as blue spots, and anogenital area secretions appear as silver-white slivers. We assumed their signal values to be similar (Ferkin et al. 2004a, b). Thus, one urine mark, two anogenital area marks, and two faecal boli were considered to represent five marks. The three types of scent marks convey sexually discriminable information to conspecifics (Ferkin & Johnston 1995). We used red ink to trace the outline of the scent marks deposited by the top-scent donor. This allowed the observer to distinguish between the marks of the top-scent donor from those of the bottom-scent donor, whose scent marks were previously traced in pencil.

We considered over-marks to be marks by the top-scent donor that overlapped or touched those deposited by the bottom-scent donor (Ferkin et al. 2004a, b; Ferkin 2010). We counted the total number of scent marks deposited in each arm by the top-scent donor. We also counted the number of the bottom-scent donor's marks that were over-marked by the top-scent donor. We used this number to calculate the proportion of the bottom-scent donor's marks that were over-marked by the top-scent donor. We also calculated the proportion of the top-scent donor's scent marks that were used to over-mark the scent marks of the bottom-scent donor.

We determined whether significant differences existed in the number of scent marks deposited in the scented or unscented arm using a 2-way analysis of variance (ANOVA) for each arm with sex of the top-scent donor (male or female) and food availability (not food deprived, food deprived for 6 hours, or food deprived for 24 hours) as main effects. We used separate 2-way ANOVA's with the same main effects (sex of the top-scent donor and food availability) to determine if significant differences existed in either the proportion of bottom-scent donor's marks that were over-marked by the top-scent donor or the proportion of marks used by the top-scent donor to over-mark the marks of the bottom-scent donor. In both cases, we arcsine-square root transformed the proportions prior to analysis. We used *a posteriori* multiple pairwise comparisons (Holm-Sidák method) to assess statistical differences across groups. Significant differences were accepted at $P < 0.05$ for all statistical analyses. The non-transformed data are presented in the figures.

Experiment 2: Self-grooming

Subjects and scent donors

Subjects were 72 voles (36 male and 36 female voles that were divided into three groups: a group that was not food deprived, a group that was food deprived for 6 hours, and a group that was food deprived for 24 hours)). Each group consisted of 12 male and 12 female voles. Scent donors were selected at random from a pool of 50 voles (25 males and 25 females) that were not food deprived and had continuous access to food. Subjects were each tested once for their self-grooming behaviour in response to the scented bedding of an opposite-sex conspecific that was not food deprived. The subject and scent donor were unfamiliar and unrelated to one another. We used unique combinations of subjects and scent donors.

Procedure

We followed the methods used to measure self-grooming in voles (Ferkin et al. 1996; Ferkin & Leonard 2010). Self-grooming tests occurred in the subject's home cage. We removed the nesting material of the subject from its cage and replaced it with 8 g of nesting material scented by an opposite-sex conspecific. The nesting material remained in the cage of the scent donor for 10 days prior to being used. Cotton nesting material was used for only one test and then discarded. Ten seconds after placing the scent donor's nesting material in the subject's home cage, we continuously recorded the amount of time that the subject vole self-groomed during a 5 minute testing period. An animal was considered to be self-grooming when it licked, scratched, or rubbed its face, over the ears, flank, ventrum, anogenital region, or tail; these areas contain sexually discriminable

odours (Ferkin et al. 1996). At the end of the self-grooming test, the scent donor's nesting material was removed from the subject's cage and discarded, and the subject's own nesting material was returned to its cage. We determined if significant differences existed in the amount of time meadow voles spent self-grooming between groups using a 2-way ANOVA with sex of the subject and food availability as main effects. We used *a posteriori* multiple pairwise comparisons (Holm-Sidák method) to assess statistical differences across groups ($P < 0.05$).

Results

Experiment 1- Number of scent marks

Food availability did not affect the number of scent marks the top-scent donor placed in the scented arm of the arena ($F_{2,66} = 0.0169$, $P = 0.983$). However, the top-scent donor's sex did affect the number of scent marks it deposited ($F_{1,66} = 9.595$, $P = 0.003$). There was no significant interaction between food availability and the top-scent donor's sex ($F_{2,66} = 0.651$, $P = 0.525$). Male meadow voles deposited a greater number of scent marks in the scented arm relative to female voles (Figure 5.1a).

The number of scent marks placed in the unscented arm of the arena by the top-scent donor was not affected by food availability ($F_{2,66} = 0.703$, $P = 0.499$). The top-scent donor's sex affected the number of scent marks it deposited in the unscented arm ($F_{1,66} = 6.387$, $P = 0.014$). There was no significant interaction between the top-scent donor's sex and food availability ($F_{2,66} = 0.044$, $P = 0.957$). Male meadow voles deposited more scent marks in the unscented arm relative to female voles (Figure 5.1b).

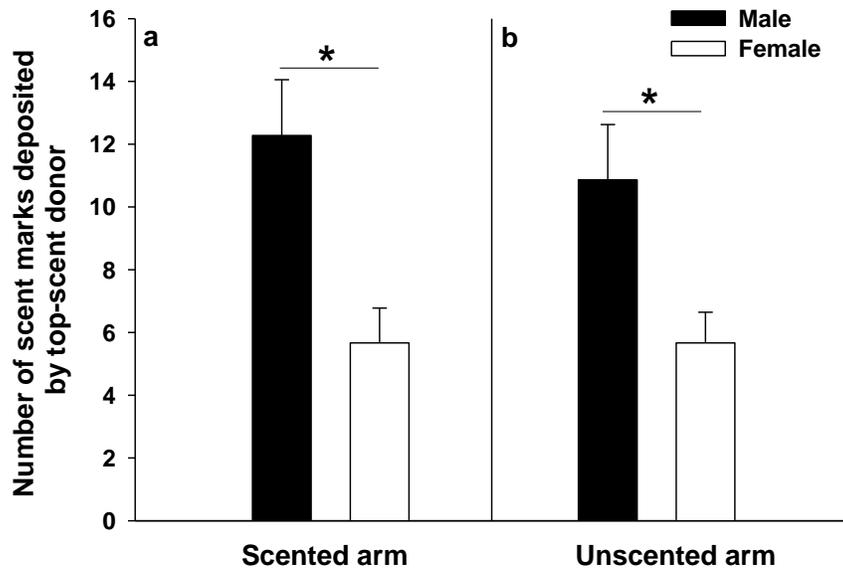


Figure 5.1: The mean number (\pm SE) of scent marks deposited by male and female meadow voles in the a) scented and b) unscented arm of an arena containing the scent marks of an opposite-sex conspecific. * indicates significant differences across groups at $p < 0.05$.

Proportion of top-scent donor's scent marks used as over-marks

Food availability did not affect the proportion of the top-scent donor's scent marks used to over-mark the scent marks of the bottom-scent donor ($F_{2, 66} = 0.953$, $P = 0.391$). The sex of the top-scent donor affected the proportion of its scent marks used as over-marks ($F_{1, 66} = 5.616$, $P = 0.021$). There was no significant interaction between food availability and sex ($F_{2, 66} = 0.118$, $P = 0.889$). Male meadow voles used a greater proportion of their scent marks to over-mark the bottom-scent donor relative to female voles (Figure 5.2a).

Proportion of bottom-scent donor's scent marks over-marked by the top-scent donor

Food availability did not affect the proportion of the bottom-scent donor's marks over-marked by the top-scent donor ($F_{2, 66} = 0.251$, $P = 0.779$). The top-scent donor's sex affected the proportion of the bottom-scent donor's scent marks it over-marked ($F_{1, 66} = 15.766$, $P < 0.001$). There was no significant interaction between food availability and the top-scent donor's sex ($F_{2, 66} = 2.998$, $P = 0.057$). Male voles over-marked a greater proportion of the bottom-scent donor's scent marks than did female voles (Figure 5.2b).

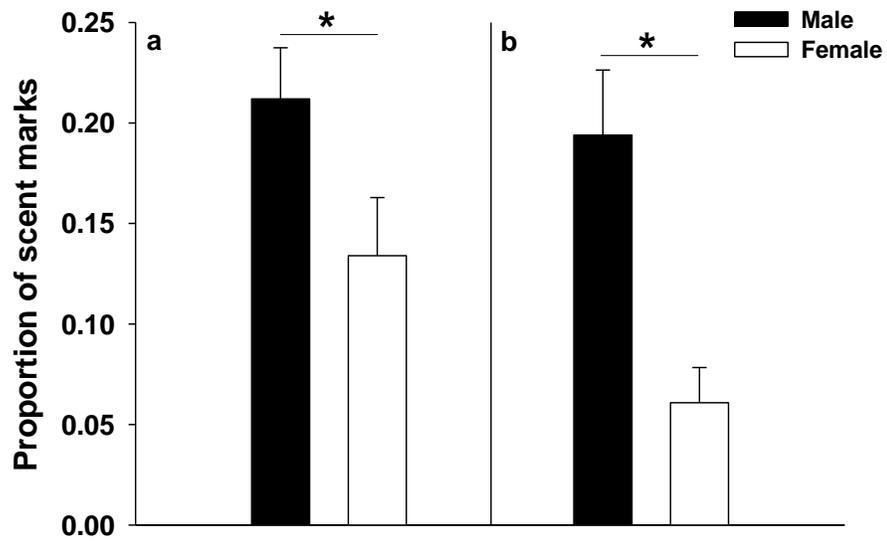


Figure 5.2: Proportion (\pm SE) of a) scent marks used by the top-scent donor as over-marks and b) bottom-scent donor's marks over-marked by the top-scent donor. * indicates significant differences across groups at $p < 0.05$.

Experiment 2-Self-grooming

Food availability affected the amount of time meadow voles spent self-grooming in response to the odour of an opposite-sex conspecific ($F_{2,66} = 12.539, P < 0.001$). The subject's sex also affected the amount of time it spent self-grooming ($F_{1,66} = 4.352, P = 0.041$). There was no significant interaction between food availability and the subject's sex ($F_{2,66} = 1.183, P = 0.313$). Meadow voles that were not food deprived spent more time self-grooming compared to individuals that were deprived of food for either 6 hours or 24 hours (Figure 5.3). Meadow voles that were food-deprived for 6 hours or 24 hours spent a similar amount of time self-grooming (Figure 5.3). Female voles spent more time self-grooming than did male voles in response to the scented bedding of an opposite-sex conspecific (Figure 5.3).

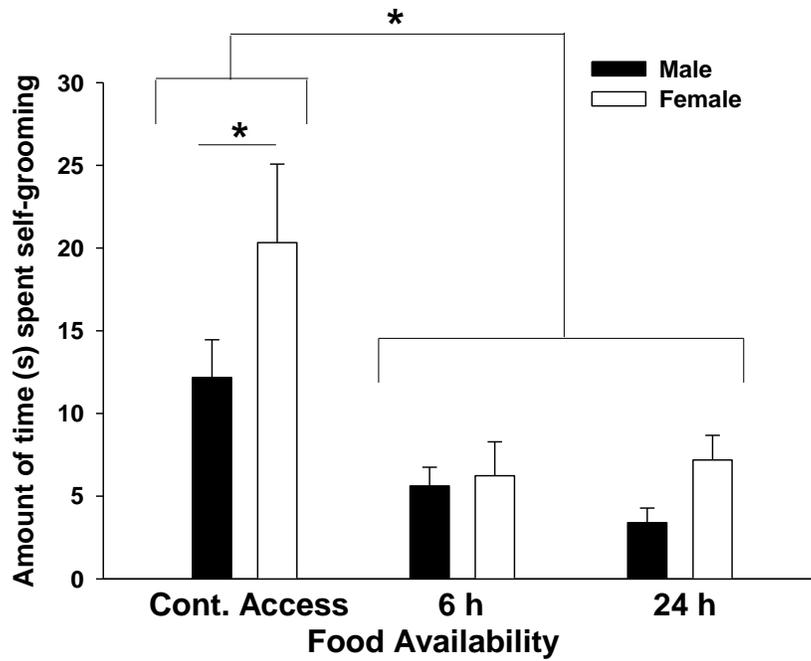


Figure 5.3: Mean (\pm SE) time (s) that male and female meadow voles with continuous access to food (Cont. Access) or that were food-deprived for 6 hours or 24 hours spent self-grooming in response to the scented bedding from an opposite-sex conspecific. * indicates significant differences across groups at $p < 0.05$.

Discussion

In this study, we assessed whether modified versions of the metabolic fuels hypothesis or the reproduction at all costs hypothesis (Wade & Schneider 1992; Boonstra et al. 2001; Pierce et al. 2005) best explained the effects of food availability on the amount of time meadow voles spent self-grooming and over-marking when they encountered the odours of opposite-sex conspecifics, via either scented bedding (self-grooming) or scent marks (over-marking). We did so by conducting two experiments that compared the scent marking, over-marking, and self-grooming behaviours of male and female voles that either had continuous access to food or that were food deprived for 6 hours or for 24 hours and then exposed to the scent marks of a sexually receptive, opposite-sex conspecific. We found that food availability did not affect the scent-marking and over-marking behaviour of male and female meadow voles when they encountered the scent marks of opposite-sex conspecifics. These results suggest that the reproduction at all costs hypothesis (Boonstra et al. 2001; Pierce et al. 2005) best explains the effects of food availability on the over-marking behaviour of male and female meadow voles. Thus, the results were not in agreement with our prediction that female voles would follow the metabolic fuels hypothesis (Wade & Schneider 1992). One possible explanation for these findings is that both male and female meadow voles need to continue to signal their presence in an area to opposite-sex conspecifics. Thus, female voles can indicate that they occupy a territory, which would increase their likelihood of finding a mate, producing a litter, and reduce intrusions by female conspecifics (Wolff 1993; Ferkin et al. 2004a). Similarly, as the reproductive success of male meadow voles is associated with the number of females with which he mates (Boonstra et al. 1993),

male voles should continue to attract and indicate interest in female conspecifics by scent marking and over-marking the marks of female voles. Another explanation, which is not mutually exclusive, is that the frequency of scent marking and over-marking by male and female meadow voles is not sensitive to changes in food availability. It is also possible that for voles, the benefits of scent marking and over-marking outweigh the costs of providing information to conspecifics about the former's nutritional status. However, this possibility does not account for the fact that food availability does affect the attractiveness and receptivity of female meadow voles (Pierce et al. 2005).

We did find, however, that food availability does affect the amount of time male and female meadow voles spend self-grooming in response to the odours of opposite-sex conspecifics. Specifically, male and female meadow voles that were food deprived for either 6 hours or 24 hours spent less time self-grooming compared to voles that had continuous access to food. These results suggest that the metabolic fuels hypothesis (Wade & Schneider 1992; Wade et al. 1996) best explains the effects of food availability on the self-grooming behaviour of male and female meadow voles. As such, the results support our prediction that female voles would follow the metabolic fuels hypothesis (Wade & Schneider 1992), but fail to support our prediction that male voles would follow the reproduction at all costs hypothesis (Boonstra et al. 2001). One possible explanation for this is that food-deprived females may be less interested in increasing their odour field and attracting nearby male voles because they do not have the energy to meet the costs associated with gestation and lactation. By not self-grooming at high rates when they encounter the scents of a nearby male, food-deprived female voles may delay mating until food is more plentiful and they have sufficient energy to reproduce (Wade &

Schneider 1992; Wade et al. 1996; Pierce & Ferkin 2005; Pierce et al. 2005, 2007). The current results differ from those of a previous study by Hobbs et al. (2008), who found that the protein content of a female vole's diet did not affect the rate at which females self-groomed in response to male odours. As such, female voles fed a low protein diet have enough energy to reproduce and will self-groom to attract nearby male voles whereas food-deprived females may not have the necessary energy needed for reproduction and are therefore less likely to attract or signal their interest in nearby males. This suggests that female voles may be more sensitive to changes in food availability relative to changes in the protein content of their diet. It is unclear why food-deprived males spent less time self-grooming compared to nonfood-deprived males, as up to 24 hours of food deprivation does not affect the attractiveness of a male vole's scent mark to female conspecifics (Pierce et al. 2005).

Taken together, the results of Experiments 1 and 2 suggest that it is the behaviour of a vole rather than its sex that determines which of the food availability hypotheses it follows. Specifically, a vole's scent marking and over-marking behaviour follows the reproduction at all costs hypothesis, whereas its self-grooming behaviour follows the metabolic fuels hypothesis. This difference may be explained in part by the context in which each of these behaviours is used. Over-marking the scent marks of an opposite-sex conspecific, and indicating one's interest in that individual as a potential mate can be accomplished without the top-scent donor and bottom-scent donor being present at the same time (Johnston 2003; Hurst & Beynon 2004; Ferkin & Pierce 2007). As such, the top-scent donor may not immediately encounter the bottom-scent donor. However, by scent marking and over-marking, an individual can still signal its presence in an area,

which may allow females to indicate ownership of a territory and males an opportunity to announce their recency in a location (Woodward et al. 1999; Ferkin & Pierce 2007). The benefits of depositing scent marks may outweigh the costs of producing scent marks that may not be attractive to the opposite sex. Thus, how scent marks and over-marks are responded to would depend less on the condition or identity of the donor who created that mark, and more on that of the individual that encounters it (Hobbs & Ferkin 2011). Moreover, a previously deposited scent mark may no longer convey accurate information about that scent donor's current nutritional status. An older scent mark may not indicate that the donor has recently found food and consequently increased its attractiveness to opposite-sex conspecifics (Ferkin et al. 1997; Pierce & Ferkin 2005). An individual would have to deposit fresher scent marks or encounter the opposite-sex conspecific that may investigate those scent marks. An individual is likely to encounter an opposite-sex conspecific if it recently self-groomed (Steiner 1974; Thiessen 1977; Harriman & Thiessen 1985; Ferkin & Leonard 2010). Self-grooming creates an ephemeral signal, and can be used to attract and signal interest in opposite-sex conspecifics that are nearby (Ferkin et al. 1996; Hobbs et al. 2008; Ferkin & Leonard 2010). Thus, self-grooming conveys accurate and more up-to-date information regarding an individual's condition relative to a scent mark that had been deposited earlier. Our results support this speculation and suggest that food-deprived voles spent less time self-grooming because by doing so they would transmit odiferous substance that would have indicated that they were of lower quality compared to nonfood-deprived voles. The differences in self-grooming and over-marking behaviour in response to food deprivation can also be considered from the physiological level of analysis. It is possible that the neural and

endocrine substrates that underlie self-grooming and over-marking (Leonard et al. 2001, 2005; Pierce et al. 2007) differ in their sensitivity to food availability.

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Chapter 6: The response of male meadow voles, *Microtus pennsylvanicus*, to same- and mixed-sex over-marks depends on the reproductive state of the top- and bottom-female scent donors

Abstract

We determined if male meadow voles, *Microtus pennsylvanicus*, respond differently to the top- and bottom-scent marks of females in different reproductive states after being exposed to same-sex and mixed-sex over-marks. When females differed in their reproductive state, male voles spent more time investigating the mark of the female scent donor that was in postpartum estrus (PPE) relative to a female that is not pregnant or lactating (REF), independent of the position of the former's mark in the over-mark. However, male voles spent more time investigating the mark of the top-scent donor relative to that of the bottom-scent donor when the female donors were in the same reproductive state. Males spent more time investigating the mark of a novel PPE female to that of a top-scent female of a mixed-sex over-mark independent of her reproductive state. Males, however, spent similar amounts of time investigating the mark of a top-scent PPE female and that of a novel REF female. Males spent a greater amount of time investigating the mark of a novel female to that of a bottom-scent female, independent of either female's reproductive state. Our results are discussed within the context of the natural history of male meadow voles.

1. Introduction

Male mammals use scent marks to assess the quality of female conspecifics and their suitability as a potential mate (Johnston, 2003; Ferkin and Pierce, 2007). These scent marks provide accurate information regarding the donor's current reproductive state (Johnston, 1983; Brown and Macdonald, 1985; Roberts, 2007). Typically, male mammals are more attracted to the scent marks of females that are sexually receptive than to those of females that are not sexually receptive (Johnston, 1979; Brown and Macdonald, 1985; Leonard et al., 2001). Similarly, male mammals spent more time investigating the scent marks of females that are in a heightened state of sexual receptivity, postpartum estrus (PPE) (Gilbert, 1984), compared to those of females that are not in postpartum estrus (Ziegler et al., 1993; Ferkin and Johnston, 1995; Vaughn and Ferkin, 2011).

However, male mammals alter their response to the scent marks of female conspecifics depending on if they first encounter them separately or as part of a same-sex over-mark, the overlapping scent marks of two or more individuals of the same sex (Hurst and Beynon, 2004; Ferkin and Pierce, 2007; Ferkin et al., 2011). Male meadow voles, *Microtus pennsylvanicus*, and golden hamsters, *Mesocricetus auratus*, spent a similar amount of time investigating each scent mark after they encountered the separate, non-overlapping scent marks of two female conspecifics in a similar reproductive state (Johnston, 1979, 1983; Ferkin and Seamon, 1987; Ferkin and Johnston, 1995). Male meadow voles, prairie voles, *Microtus ochrogaster*, and golden hamsters, however, distinguished between the two scent marks if they overlapped one another. These rodents spent more time investigating the scent mark of the top-scent donor relative to that of the bottom-scent donor (Ferkin et al., 1999, 2001; Woodward et al., 1999, 2000) or behaved

as if they had a better selective memory for the mark of the top-scent donor (Johnston et al., 1994, 1995; Johnston, 2003) after they encountered these same scent marks as part of a same-sex over-mark. These findings suggest that the information conveyed in the mark of the top-scent donor is either more salient or devalues the information conveyed in the mark of the bottom-scent donor (Woodward et al., 1999, 2000; Ferkin and Pierce, 2007). However, these studies examined the responses of males to over-marks in which the top- and bottom-scent females were similar in condition. When male meadow voles were exposed to an over-mark of two female scent donors that differed in either their gonadal hormone status or protein content of their diet, they spent more time investigating the mark of the female with higher levels of estradiol or that was fed a diet higher protein content, respectively, independent of the position of the female's scent mark in the over-mark (Leonard et al., 2001; Hobbs and Ferkin, 2011). Taken together, these results suggest that a male vole's preference for a particular female conspecific may depend on the position of her scent mark in the over-mark or her condition as conveyed by the scent mark (Ferkin and Pierce, 2007).

Male mammals may also encounter the scent mark of a female conspecific associated with the scent mark of a male conspecific as part of a mixed-sex over-mark. By placing their scent mark over that of a female conspecific, male meadow voles create a male/female mixed-sex over-mark. It has been proposed that a male/female over-mark indicates the top-scent male's interest in the bottom-scent female as a potential mate to nearby conspecifics or serves as a form of mate guarding (Woodward et al., 2000; Ferkin and Pierce, 2007; Ferkin et al., 2010). After encountering a male/female over-mark, male meadow voles later spent more time investigating the mark of a novel female whose mark

was not part of the over-mark relative to that of the female that provided the bottom-scent mark of the over-mark (Woodward et al., 2000). Male voles may not have preferred the bottom-scent female because she may have mated with the top-scent male (Ferkin and Pierce, 2007). Similarly, female voles may over-mark the scent marks of males to indicate interest in the bottom-scent male, creating a female/male mixed-sex over-mark. After encountering a female/male over-mark, male meadow voles later spent more time investigating the mark of the top-scent female relative to that of a novel female (Woodward et al., 2000; Ferkin et al., 2010). Male voles may be more interested in the top-scent female because by over-marking a male's scent mark, the top-scent female may be seeking out mates (Ferkin and Pierce, 2007).

Male voles, however, may encounter the scent marks and over-marks of females in different reproductive states (Ferkin and Johnston, 1995; Vaughn and Ferkin, 2011). The reproductive state of female voles varies and during the breeding season adult female voles could be in PPE, pregnant, lactating, pregnant and lactating, or not pregnant or lactating (Keller, 1985). Male voles may use the reproductive state of a female vole as an indicator of her willingness to mate (Keller, 1985). PPE female voles are most receptive and will mate within a few minutes of being paired with a male, whereas females that are not in PPE are moderately receptive, such as those that are not pregnant or lactating (reference female, REF; Ferkin and Johnston, 1995). A REF female may mate within a few hours of being paired with a male (Meek and Lee, 1993; delBarco-Trillo and Ferkin, 2004). PPE females are preferred mates relative to females that are not in PPE, and not surprisingly produce scent marks that are more attractive to males than those of females not in PPE (Ferkin and Johnston, 1995; Vaughn and Ferkin, 2011). Ferkin et al. (2004)

previously examined how the reproductive state of meadow voles affects their scent marking and over-marking behaviors. Briefly, these PPE females deposit more scent marks and over-marks than do females that are not in PPE and males deposit more scent marks and over-marks in response to areas containing the scent marks of PPE females than they do in response to areas containing those of females not in PPE (Ferkin et al., 2004). We do not know how male mammals respond to over-marks in which the top- and/or bottom-scent donors are in PPE.

In this study, we determined whether the amount of time male meadow voles spent investigating the scent marks of female voles was affected by the reproductive state of female donors or the relative position of their scent marks in same-sex and mixed-sex over-marks. In experiment 1, the top- and bottom-scent female scent donors were in the same reproductive state. We hypothesized that males use the position of the marks in the over-mark when distinguishing between the top- and bottom-scent female donors. Thus, we predicted that male voles would spend more time investigating the scent mark of the top-scent donor than that of the bottom-scent donor. In experiment 2, the top- and bottom-scent female scent donors were not in the same reproductive state. We hypothesized that male voles would use differences in the condition of the female donors when distinguishing between the top- and bottom-scent female scent donors. Thus, we predicted that male voles would spend more time investigating the scent mark of the PPE female than that of the REF female. In experiments 3 and 4, male voles were exposed to a mixed-sex over-mark. We hypothesized that male voles distinguish between the scent mark of the top- or bottom-scent female donor and that of a female whose mark was not part of the over-mark. In experiment 3, we predicted that male voles would spend more

time investigating the scent mark of the top-scent female than that of a novel female donor, independent of either female's reproductive state; the top-scent female is likely to be searching for a mate (Ferkin and Pierce, 2007; Ferkin et al., 2010). In contrast, in experiment 4, we predicted that male voles would spend more time investigating the scent mark of a novel female donor than that of the bottom-scent female, independent of either female's reproductive state; the bottom-scent female is likely to have already located a mate, in this case the top-scent male donor (Ferkin and Pierce, 2007; Ferkin et al., 2010).

2. Materials and methods

2.1. Animals

Voles used in the current study were descendants of those captured in Pennsylvania, Kentucky, and Ohio, USA. All voles were weaned at 19 days of age, housed with littermates until 33-35 days of age, and then housed singly in clear, plastic cages (30.5 x 35.5 x 22.8 cm). Cages contained woodchip bedding and cotton nesting material. Water and food (Laboratory Rodent Diet #8640, Harlan Teklad, Madison, WI, USA) were provided *ad libitum*.

All voles used in this study as subjects and scent donors were born and raised under a long photoperiod (14L: 10D, lights on at 0700 hours CST and off at 2100 hours CST) in the animal facility at the University of Memphis. This long photoperiod simulates the typical amount of light present during the breeding season. Adult female voles born and reared under a long photoperiod are sexually receptive (Meek and Lee, 1993). Female meadow voles are induced ovulators that do not undergo regular estrus

cycles (Milligan, 1982; Keller, 1985). Subjects and scent donors were between 70-110 days of age at the start of the study. Subjects within each experiment were from different litters.

2.2. Reproductive state of the scent donors

We used females that were in postpartum estrus (PPE) and females that were not pregnant or lactating (reference, REF females) to represent different levels of female receptivity. Immediately after parturition, female meadow voles enter PPE, a heightened state of sexual receptivity, lasting 8-12 hours (Keller, 1985; Ferkin et al., 2004; delBarco-Trillo and Ferkin, 2007). The PPE females used in the current study had delivered pups 4-6 hours prior to testing and were in postpartum estrus. The litter size of the PPE females did not differ and were approximately 4.3 ± 0.3 pups. REF females were sexually mature and experienced, but were not pregnant or lactating at the time of the current study. The males used as scent donors in experiments 3 and 4 were sexually mature and experienced, having sired at least one litter. Scent donors for the experiments were selected from a pool of 25 PPE females, 25 REF females, and 25 male voles. Subjects in all experiments were exposed to a unique combination of scent donors. In each trial, the subjects and the two scent donors were unfamiliar and unrelated to one another.

2.3. Experiment 1: Male voles exposed to a same-sex over-mark in which the top- and the bottom-scent female donors are in the same reproductive state

We used 24 male voles as subjects in this experiment. Males were exposed to one of two possible over-mark combinations: 1) the top-scent donor was a REF female and

the bottom-scent donor was another REF female (REF/REF) or 2) the top-scent donor was a PPE female and the bottom-scent female was another PPE female (PPE/PPE). We exposed 12 different male subjects to each over-mark combination.

2.3.1. Experimental design

Our testing methods were similar to those detailed in other studies that examined the response of voles to over-marks (Ferkin et al., 1999, 2010; Woodward et al., 1999, 2000; Hobbs and Ferkin, 2011). Our design involved two phases: the exposure phase and the test phase, both of which took place in the subjects' home cages. All testing was carried out between 0900 and 0200 hours CST.

Table 2: Exposure phase and Test phase layout for each of the 4 experiments.

Experiment	Exposure phase	Test phase	Fig.
1	REF/REF	REF _{Top} vs. REF _{Bottom}	1
1	PPE/PPE	PPE _{Top} vs. PPE _{Bottom}	1
2	REF/PPE	REF _{Top} vs. PPE _{Bottom}	2
2	PPE/REF	PPE _{Top} vs. REF _{Bottom}	2
3	REF/Male	REF _{Top} vs. PPE _{Novel}	3a
3	PPE/Male	PPE _{Top} vs. PPE _{Novel} PPE _{Top} vs. REF _{Novel}	3b
4	Male/REF	REF _{Bottom} vs. PPE _{Novel}	4a
4	Male/PPE	PPE _{Bottom} vs. PPE _{Novel} PPE _{Bottom} vs. REF _{Novel}	4b

2.3.2. Exposure phase

To create the over-mark, we pressed the anogenital region of the bottom-scent donor and dragged it across the center of a glass microscope slide (2.5 x 7.6 cm) such that the resulting scent mark was 1.5-2.0 cm long and 0.3 cm wide. Two min later, we rotated the glass slide 90⁰ and pressed the anogenital region of the top-scent donor against the slide and dragged it across the slide; the resulting scent mark was 1.5-2.0 cm long and 0.3 cm wide. The two scent marks formed a “+” configuration. Voles typically drag their anogenital regions against the substrate when depositing scent marks (Ferkin et al., 2001).

After the second scent mark was placed on the slide, we waited 2 minutes before placing the slide into the subject’s cage. The slide was placed against the wall opposite the subject’s nest and suspended 2 cm above the substrate by a clean metal clip and hook. Subjects were exposed to this slide for 5 minutes. This slide was placed in the cage of only one subject and then discarded. In all observations, the observer was blind to the identity of the top- and bottom-scent mark donors. All subjects investigated the slide during the exposure phase. We used different male subjects in each experiment.

2.3.3. Test phase

The test phase began 1 minute after the completion of the 5-minute exposure phase. We presented males (subjects) with a glass slide (2.5 x 7.6 cm) that contained the anogenital scent marks of the top- and bottom-scent donors from the over-mark they were presented with during the exposure phase. The scent marks were not overlapping and

placed separately on opposite ends of the test slide. We divided the glass test slide into three equal sections (each 2.5 cm in length); the left side of the slide contained the anogenital scent mark of one scent donor, the middle contained no scent marks, and the right side of the slide contained the anogenital scent mark of the other female scent donor. We created the scent marks by following the methods detailed in the exposure phase; the two scent marks were similar in length and width (1.5 -2.0 cm x 0.3 cm). The placement of a particular donor's scent mark on the left or right side of the slide was random. After both scent marks were placed on the slide, we waited 1 minute before we suspended the slide in the subject's cage with a clean clip and hook. We recorded the amount of time that males licked or sniffed (the subject's nose came within 2 cm) each scent mark on the slide continuously for 3 minutes. The observer was blind to the position of the donors' scent marks on the slide. Each test slide was used once and discarded.

2.3.4. Statistical analysis

We analyzed the data using a two-way (exposure over-mark x donor's position in the over-mark) analysis of variance (ANOVA) with repeated measures to determine whether significant differences existed in the amount of time male voles spent investigating the scent marks of the two scent donors during the test phase (Sigmaplot 11.0). If the ANOVA revealed any significant effects, we used the Holm-Sidák method to determine significant differences between pairwise comparisons. Significant differences were accepted at $p < 0.05$. To be included in the data analysis, subjects had to have investigated the scent marks of both donors and spent more time investigating the scent

marks of the two donors than they did investigating the clean portion of the slide (Ferkin et al., 1999; Hobbs and Ferkin, 2011). No subjects were excluded from the data analysis.

2.4. Experiment 2: Males exposed to same-sex over-mark in which the top- and the bottom-scent female donors differed in their reproductive state

The methods followed those detailed in experiment one with these notable exceptions. We used 24 males as subjects. Subjects were exposed to one of two possible over-mark combinations: 1) the top-scent donor was a REF female and the bottom-scent donor was a PPE female vole (REF/PPE) or 2) the top-scent donor was a PPE female and the bottom-scent donor was a REF female (PPE/REF).

2.4.1. Exposure phase

The preparation of the exposure slide followed the methods detailed in experiment 1, with the exception that one scent donor was a PPE female and the other scent donor was a REF female.

2.4.2. Test phase

The preparation of the test slide followed the methods detailed in Experiment 1. Statistical methods were the same as those described in Experiment 1.

2.5. Experiment 3: Males exposed to mixed-sex over-mark in which the top-scent donor was a female conspecific and the bottom-scent donor was a male conspecific (female/male over-mark)

The methods used in Experiment 3 are similar to those of the previous experiments. Male voles were exposed to a mixed-sex over-mark in which the top-scent donor was a female vole. We used 36 males as subjects. Subjects were exposed to one of two over-mark combinations: 1) the top-scent donor was a REF female and the bottom-scent donor was a male vole (REF/Male) or 2) the top-scent donor was a PPE female and the bottom-scent donor was a male vole (PPE/Male). Twelve males were exposed to the first over-mark and 24 males were exposed to the second over-mark.

2.5.1. Exposure phase

The preparation of the exposure slide followed the methods detailed in experiment 1, with the exception that the top-scent donor was a female vole and the bottom-scent donor was a male vole.

2.5.2. Test phase

The preparation of the test slide followed the methods detailed in Experiment 1. Males exposed to the REF/Male over-mark were given a test slide made up of the scent mark of the REF female from the over-mark and that of a novel PPE female whose scent mark was not part of the over-mark. Twelve males exposed to the PPE/Male over-mark were given a test slide in which one scent donor was the PPE female from their exposure over-mark and the other was a novel PPE female. The other twelve males were given a

test slide in which one scent donor was the PPE female from the over-mark and the other was a novel REF female. We used a two-way ANOVA with repeated measures (exposure over-mark x familiarity of female scent donor) to determine if significant differences existed in the amount of time male voles spent investigating the females' scent marks during the test phase. We used Holm-Sidák method to determine if significant differences were found among the pairwise comparisons.

2.6. Experiment 4: Males exposed to mixed-sex over-mark in which the top-scent donor was a male conspecific and the bottom-scent donor was a female conspecific (male/female over-mark)

The methods used in Experiment 4 are similar to those of the previous experiments. Male voles were exposed to a mixed-sex over-mark in which the bottom-scent donor was a female vole. We used 36 males as subjects. Subjects were exposed to one of two over-mark combinations: 1) the top-scent donor was a male vole and the bottom-scent donor was a REF female vole (Male/REF) or 2) the top-scent donor was a male and the bottom-scent donor was a PPE female vole (Male/PPE). Twelve males were exposed to the first over-mark and 24 males were exposed to the second over-mark.

2.6.1. Exposure phase

The preparation of the exposure slide followed the methods detailed in experiment 1, with the exception that the top-scent donor was a male vole and the bottom-scent donor was a female vole.

2.6.2. Test phase

The preparation of the test slide followed the methods detailed in Experiment 1. Males exposed to the Male/REF over-mark were given a test slide made up of the scent mark of the REF female from the over-mark and that of a novel PPE female whose scent mark was not part of the over-mark. Twelve males exposed to the Male/PPE over-mark were given a test slide in which one scent donor was the PPE female from their exposure over-mark and the other was a novel PPE female. The other twelve males were given a test slide in which one scent donor was the PPE female from the over-mark and the other was a novel REF female. Statistical methods followed those described in Experiment 3.

3. Results

3.1. Experiment 1

We tested whether the amount of time males spent investigating the scent marks of the top- and bottom-scent donors of a same-sex over-mark in which the top- and bottom-scent females were in the same reproductive state was affected by the females' reproductive state. There was no significant interaction between the type of over-mark was exposed to and the position of the female's scent mark in the over-mark ($F_{1,47} = 1.208, p = 0.284$). The type of over-mark that males were exposed to did not affect the amount of time that males spent investigating the marks of the female scent donors ($F_{1,47} = 3.414, p = 0.078$). However, the position of the female's mark in the over-mark affected the amount of time he spent investigating the scent marks ($F_{1,47} = 13.110, p = 0.002$). Male voles spent a greater amount of time investigating the mark of the top-scent

donor relative to that of the bottom-scent donor, independent of the females' reproductive state (Fig. 6.1).

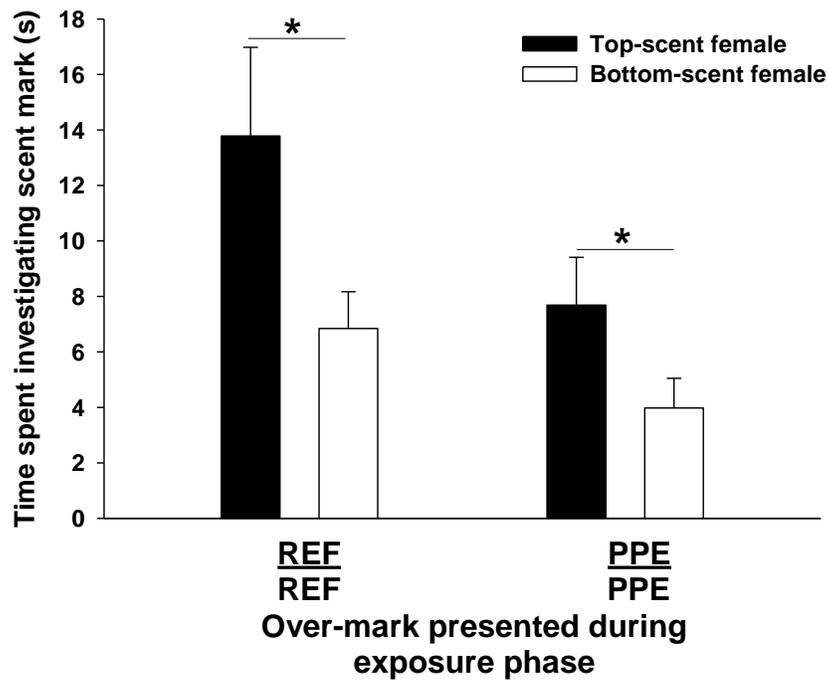


Fig. 6.1: Mean (\pm SE) time (s) that male meadow voles spent investigating the scent marks of the top- and bottom-scent donors of a same-sex over-mark in which both scent donors were in the same reproductive condition. * indicates a significant difference between scent donors at $p < 0.05$ (Holm-Sidák paired comparisons).

3.2. Experiment 2

We determined whether the amount of time male voles spent investigating the scent donors of an over-mark was affected when the top- and bottom-scent donors varied in their reproductive state. We found a significant interaction between the type of over-mark the male was exposed to and the position of a female's mark in the over-mark ($F_{1,47} = 13.299, p = 0.001$). As such we ran two separate one-way ANOVAs with repeated measures, one for each type of over-mark. When exposed to a REF/PPE over-mark, the position of the females' marks in the over-mark affected the amount of time males spent investigating their scent marks ($F_{1,23} = 9.584, p = 0.010$). Males spent a greater amount of time investigating the mark of the bottom-scent PPE female relative to that of the top-scent REF female (Fig. 2). Similarly, the position of the females' marks in an over-mark affected the amount of time males spent investigating their scent marks after being exposed to a PPE/REF over-mark ($F_{1,23} = 6.507, p = 0.027$). Male voles spent a greater amount of time investigating the mark of the top-scent PPE female relative to that of the bottom-scent REF female (Fig. 6.2).

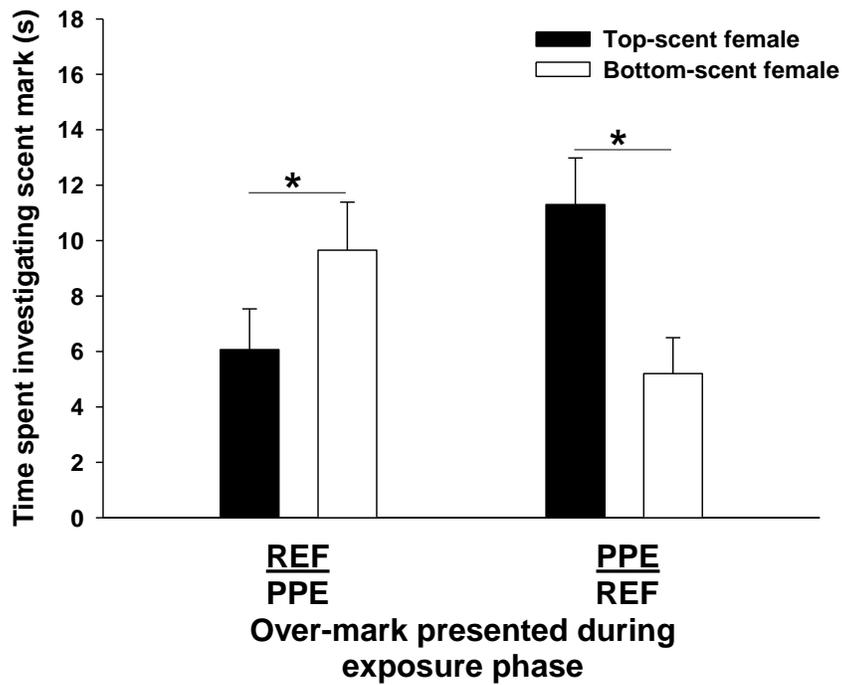


Fig. 6.2: Mean (\pm SE) time (s) that male meadow voles spent investigating the scent marks of the top- and bottom-scent donors of a same-sex over-mark in which scent donors differed in their reproductive condition. * indicates a significant difference between scent donors at $p < 0.05$ (Holm-Sidák paired comparisons).

3.3. Experiment 3

There was a significant interaction between the type of over-mark males were exposed to and the familiarity of the female ($F_{2, 71} = 11.746, p < 0.001$). As such, we ran a one-way ANOVA with repeated measures for each test phase. The amount of time males spent investigating the scent marks of a top-scent REF female and that of a novel PPE female was affected by the females' familiarity ($F_{1, 23} = 45.578, p < 0.001$). Males spent a greater amount of time investigating the mark of the novel PPE female relative to that of the top-scent REF female (Fig. 6.3A). Similarly, after being exposed to a PPE/male over-mark, familiarity affected the amount of time males spent investigating the scent marks of females ($F_{1, 23} = 25.457, p < 0.001$). Males spent a greater amount of time investigating the mark of a novel PPE female relative to that of the top-scent PPE female (Fig. 6.3B). Males spent a similar amount of time investigating the mark of a top-scent PPE female and that of a novel REF female ($F_{1, 23} = 1.127, p = 0.311$, Fig. 6.3B).

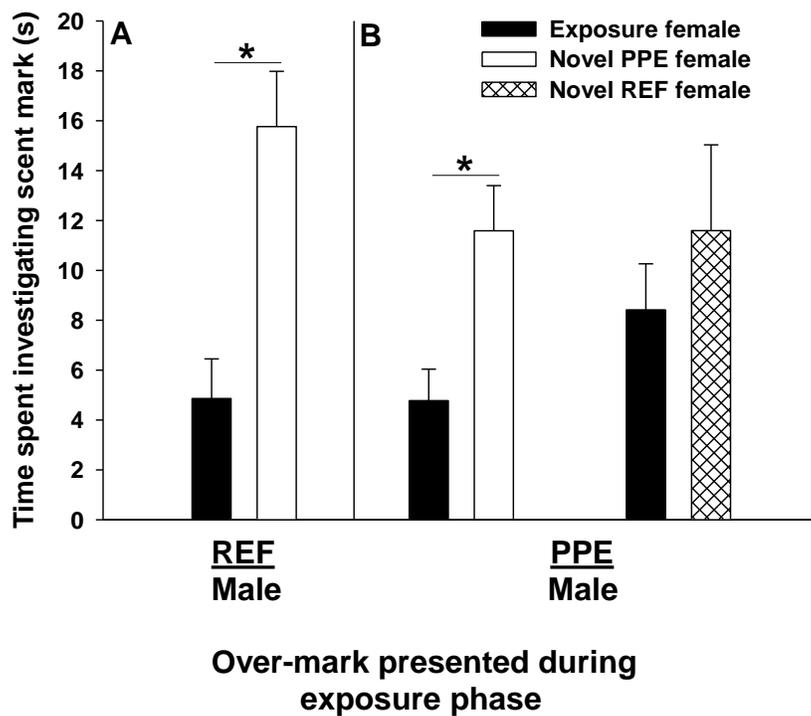


Fig. 6.3: Mean (\pm SE) time (s) that male meadow voles spent investigating the scent marks of the top-scent female and a novel female after being exposed to a female/male over-mark in which A) a REF female was the top-scent donor or B) a PPE female was the top-scent donor. * indicates a significant difference between scent donors at $p < 0.05$ (Holm-Sidák paired comparisons).

3.4. Experiment 4

There was no interaction between the type of over-mark males were exposed to and the familiarity of the scent donors during the test phase ($F_{2,71} = 3.085, p = 0.059$). The type of over-mark that males were exposed to did not affect the amount of time they spent investigating the scent marks of the two female donors ($F_{2,71} = 0.862, p = 0.432$). There was an effect of the females' familiarity on the amount of time male voles spent investigating the females' marks ($F_{1,71} = 30.966, p < 0.001$). Male voles spent more time investigating the mark of a novel female to that of a female whose mark he first encountered as the bottom-scent mark of a mixed-sex over-mark, independent of either female's reproductive state (Fig. 6.4A, B).

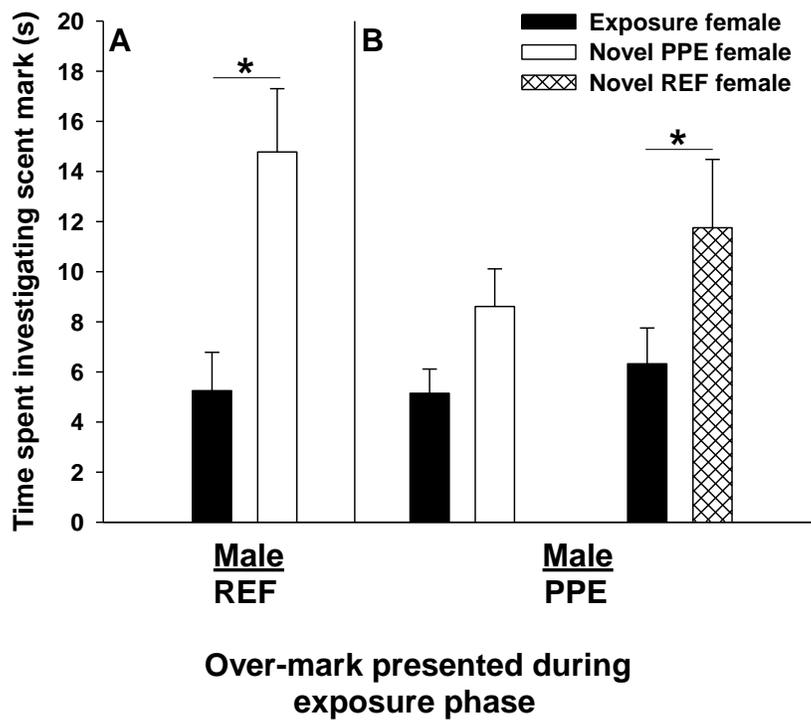


Fig. 6.4: Mean (\pm SE) time (s) that male meadow voles spent investigating the scent marks of the bottom-scent female and a novel female after being exposed to a male/female over-mark in which A) a REF female was the bottom-scent donor or B) a PPE female was the bottom-scent donor. * indicates a significant difference between scent donors at $p < 0.05$ (Holm-Sidák paired comparisons).

4. Discussion

Male meadow voles spent more time investigating the scent mark of the top-scent female of a same-sex over-mark to that of the bottom-scent female when the females were in the same reproductive state. As such, the results of Experiment 1 support our hypothesis that males would prefer the scent mark of the top-scent female to the bottom-scent female of a same-sex over-mark when both females are in the same reproductive state. Our results are consistent with previous studies in which male meadow voles and prairie voles spent more time investigating the scent mark of a top-scent female relative to a bottom-scent female when both females are of similar condition (Ferkin et al., 1999, 2001; Woodward et al., 1999, 2000; Hobbs and Ferkin, 2011). However, if male voles and golden hamsters first encountered the scent marks of two female donors of similar quality separately and not as an over-mark, they spent similar amounts of time investigating the scent marks of the two females (Johnston, 1979, 1983; Ferkin and Seamon, 1987; Ferkin and Johnston, 1995). Over-marks likely provide additional information about the two scent donors that is not available to investigators that encounter the marks of these two donors separately (Hurst and Beynon, 2004; Ferkin et al., 2010, 2011). Individuals may use such information to distinguish between potential mates (Rich and Hurst, 1998; Johnston, 2003; Ferkin and Pierce, 2007). For example, male meadow voles may prefer the top-scent female of a same-sex over-mark because he is more likely to encounter that female than he is the bottom-scent female. Male voles may view the top-scent female as the resident in that territory (Ferkin and Pierce, 2007).

In experiment 2, male meadow voles spent more time investigating the mark of the PPE female relative to that of the REF female, independent of the position of the

former's scent mark in the over-mark. Similar results were obtained in a study in which the estradiol titer of the top- and bottom-scent female scent donors differed. Briefly, male voles later spent more time investigating the mark of the female with higher estradiol titer than that of the female with the lower estradiol titer. This preference by the male voles was independent of the position of the former female's mark in the over-mark (Leonard et al., 2001). Female voles with higher levels of estradiol produce scent marks that are more attractive to male conspecifics because these females are in heightened state of sexual receptivity (Keller, 1985; Ferkin and Zucker, 1991; Ferkin and Johnston, 1993). Likewise, PPE females have high estradiol titers (Knobil and Neill, 2006), and also produce scent marks that are more attractive to males than those of females not in PPE (Ferkin and Johnston, 1995; Vaughn and Ferkin, 2011). It is likely male voles respond preferentially to PPE females because these female have shorter latencies to mate, allow males to have fewer ejaculations, reducing his sperm investment, and are more likely to get pregnant relative to females that are not in PPE, such as REF females (Gilbert, 1984; Keller, 1985; delBarco-Trillo and Ferkin, 2007).

The results of Experiment 3 failed to support our prediction that male voles would spend more time investigating the scent mark of a top-scent female from a mixed-sex over-mark to that of a novel female whose mark he did not encounter as part of the over-mark. Specifically, male meadow voles spent more time investigating the scent mark of a novel PPE female relative to that of the top-scent female, independent of the latter's reproductive state. When the top-scent female was a REF female, males may have preferred the novel PPE female because she was in a heightened state of sexual receptivity relative to the top-scent female, and such a state provides males with benefits,

such as a decreased sperm investment due to fewer ejaculations needed to successfully mate with the female (delBarco-Trillo and Ferkin, 2007). Males spent more time investigating the mark of a novel PPE female when the top-scent female was another PPE female. Presumably, the male's preference for the scent mark of the novel female was because that scent mark was not associated with another male's in a mixed-sex over-mark. It is possible because of the short-term nature of postpartum estrus, PPE females may only mate with 1-2 males. As top-scent females may have already mated with the bottom-scent male (Ferkin and Pierce, 2007), male voles may prefer a novel PPE female whose scent mark he did not first encounter as part of a female/male over-mark.

Males spent similar amounts of time investigating the mark of a novel REF female and that of a top-scent PPE female. However, if the top-scent female was also a REF female, males spent more time investigating its scent mark relative to that of a novel REF female (Woodward et al., 2000; Ferkin et al., 2010). Taken together, this suggests that males may devalue the information conveyed by the scent mark of a PPE female that places her scent mark over that of another male. However, because she is in PPE, males may still gain benefits by mating with her relative to a novel REF female, even if the former has already mated with the bottom-scent male (Dewsbury et al., 1979; Gilbert, 1984; delBarco-Trillo and Ferkin, 2007; Vaughn and Ferkin, 2011). While males may be more certain of paternity if they choose to mate with a novel REF female, such females take longer to mate with and require more ejaculations relative to PPE females. By selecting the top-scent PPE female, male voles may sire a smaller percentage of the female's pups relative to the bottom-scent male, but require fewer ejaculations to do so, saving sperm for future females (delBarco-Trillo and Ferkin, 2007). This may be

important because a male meadow vole's reproductive success is directly proportional to the number of females with which he mates (Boonstra et al., 1993; Berteaux et al., 1999). For example, males that copulate with a mated PPE female may have to increase his sperm allocation relative to that of males that copulate with an unmated REF female (delBarco-Trillo and Ferkin, 2004), but the former males may have to spend less time mating with that female than he would spend mating with a REF female (delBarco-Trillo and Ferkin, 2007). The net result would be a tradeoff between being able to potentially mate with more females at the cost of a smaller chance of paternity versus a higher chance of paternity at the cost of increased investment, potentially reducing the number of females the male can mate with in the future.

The results of Experiment 4 supported our prediction that male voles would spend a greater amount of time investigating the mark of a novel female whose scent mark he had not first encountered in a male/female over-mark relative to that of a bottom-scent female from such an over-mark. Male voles spent a greater amount of time investigating the mark of a novel female relative to that of a bottom-scent female, independent of either female's reproductive state. Because a bottom-scent female may have already mated with the top-scent male (Ferkin and Pierce, 2007), male voles may prefer the novel female as a potential mate. Male voles may select this female because her scent mark was not encountered with that of a male conspecific as well as the benefits previously mentioned that are associated with mating with a PPE female. Woodward et al. (2000) found that male voles spent a greater amount of time investigating the mark of a novel REF female compared to that of a bottom-scent REF female. Taken together, these results suggest that male meadow voles devalue the scent marks of females whose scent marks have been

over-marked by other males. However, male voles spent a similar amount of time investigating the mark of a bottom-scent PPE female and that of a novel REF female, suggesting a tradeoff between mating with a PPE female that may have mated with another male and mating with an unmated REF female (Ferkin et al., 2010).

The results of the current study support the view that how male meadow voles evaluate over-marks depends on the condition of their female scent donors, as well as their relative position in a same-sex and in a mixed-sex over-mark (Ferkin and Pierce, 2007; Ferkin et al., 1999, 2010, 2011). Males prefer the mark of a top-scent PPE female to that of a bottom-scent REF female. Males also preferred the mark of the bottom-scent PPE female to that of a top-scent REF female. These two results suggest that male voles are using differences in the reproductive condition of the top- and bottom-scent donors to display a preference for the female that is in PPE. Hobbs and Ferkin (2011) also found that the condition of the top- and bottom-scent female donors, specifically their relative nutritional states, affected how male voles responded to female/female over-marks. Males spent more time investigating the mark of the top-scent female if she was fed a diet higher in protein relative to that of the bottom-scent female but not vice versa (Hobbs and Ferkin, 2011). Taken together, these two findings suggest that condition of the female donors as well as their position in the over-mark affects the male's responses to the top- and bottom-scent females in a same-sex over-mark. Interestingly, male voles exposed to a mixed-sex over-mark prefer the mark of a novel female relative to that of a bottom-scent female, independent of either female's reproductive state. In this case, males appear to be responding to whether the female's scent mark was encountered as part of an over-mark with another male. However, males prefer the top-scent female to the bottom-scent

female when both females are similar in quality (this study, Ferkin et al., 1999). Overall, it appears that male voles use different rules when evaluating same-sex and mixed-sex over-marks (Ferkin et al., 1999, 2010, 2011; Woodward et al., 2000). In addition, the results are consistent with the view that because males respond differently to the top- and bottom-scent donors of same- and mixed-sex over-marks that these over-marks provide somewhat different information to males (Ferkin and Pierce, 2007; Ferkin, 2011).

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Chapter 7: Conclusion

We tested the hypothesis that the amount of time that individuals self-groom to opposite-sex conspecifics is affected by the amount of protein in their diet and that of the scent donor. We found that the protein content of the diet of male and female groomers did not affect the amount of time they self-groomed. However, the protein content of the diet of male odor donors affected the amount of time that female voles spent self-grooming. Female voles self-groomed more in response to male odor donors fed a 22% protein-content diet than to those produced by male odor donors fed either a 9% or a 13% protein-content diet. Interestingly, the amount of time males self-groomed was not affected by the protein content of the diet of the female odor donor. The results, therefore, provide partial support for our hypothesis that the amount of time that individuals self-groom to opposite-sex conspecifics is affected by the amount of protein in their diet and that of the scent donor.

Two other behaviors voles may use to attract or indicate interest in potential mates are scent marking and over-marking (Brown & Macdonald 1985). In Chapter 3, we tested the hypothesis that the protein content of the top- and bottom-scent donors of an over-mark's diets affects the scent marking and over-marking behavior of the top-scent vole. Protein content of the top-scent vole's diet did not affect the number of scent marks and over-marks it deposited. Likewise, the protein content of the bottom-scent vole did not affect the number of scent marks and over-marks deposited by the top-scent vole. This suggests that protein content of the diet does not affect the act of scent marking even though it does affect information about the donor's diet that is contained in the scent mark.

In Chapter 4, we determined if a meadow vole's response to the scent donors of an over-mark was affected by the protein content of their diet or that of the top- and bottom-scent donors' diets. Male and female voles fed a 22% protein diet spent more time investigating the scent mark of the top-scent donor than that of the bottom-scent donor; voles fed 9% and 13% protein diets spent similar amounts of time investigating the top- and bottom-scent donors. Upon altering the protein content of the scent donors' diets we found that female voles spent more time investigating the mark of the top-scent male than that of the bottom-scent male, independent of the differences in protein content of the diets of the two scent donors. Male voles, however, only showed a preference for the mark of the top-scent female when she was fed a diet higher in protein content than the bottom-scent female. This sex difference may be due to differences in the natural history of male and female meadow voles.

In Chapter 5, we tested the hypothesis that food-deprived and nonfood-deprived meadow voles differ in the number of scent marks they deposit, the proportion of over-marks they deposit, and the amount of time they spend self-grooming when they encounter the scent marks of opposite-sex conspecifics. We tested this hypothesis by exposing meadow voles that either had continuous access to food or were food-deprived for either 6 or 24 hours to the scent marks of an opposite-sex conspecific. Due to differences in the natural history of male and female meadow voles, we predicted that female voles will follow the metabolic fuels hypothesis whereas males will follow the reproduction at all costs hypothesis (Wade & Schneider 1992; Boonstra et al. 2001; Pierce et al. 2005). We found that both male and female voles deprived of food for either 6 hours or 24 hours spent less time self-grooming compared to nonfood-deprived voles.

However, food availability did not affect the scent marking and over-marking behavior of male and female voles. Differences in the effects of food availability on these proceptive behaviors may be due to the context in which each behavior is used.

In Chapter 6, we determined if male meadow voles respond differently to the top- and bottom-scent marks of females in different reproductive states after being exposed to same-sex and mixed-sex over-marks. When females differed in their reproductive state, male voles spent more time investigating the mark of the female scent donor that was in postpartum estrus (PPE) relative to a female that is not pregnant or lactating (REF), independent of the position of the former's mark in the over-mark. However, male voles spent more time investigating the mark of the top-scent donor relative to that of the bottom-scent donor when the female donors were in the same reproductive state. Males spent more time investigating the mark of a novel PPE female to that of a top-scent female of a mixed-sex over-mark independent of her reproductive state. Males, however, spent similar amounts of time investigating the mark of a top-scent PPE female and that of a novel REF female. Males spent a greater amount of time investigating the mark of a novel female to that of a bottom-scent female, independent of either female's reproductive state. Overall, proceptive behaviors such as scent marking, odor preferences, and self-grooming, depend on the nutritional status of the actor.

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Appendix A

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