Male prairie voles with different *avpr1a* microsatellite lengths do not differ in courtship behaviour

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Keywords:

Anogenital sniffing

Courtship behaviour

Mate choice

Rodents

Voles

ABSTRACT

Females are generally expected to be selective when choosing their social and sexual partners. In a previous laboratory study, female prairie voles (*Microtus ochrogaster*) showed significant social and sexual preferences for males with longer microsatellite DNA within the *avpr1a* gene encoding the vasopressin 1a receptor, as predicted if females select mates whose parental behaviour should increase female reproductive success. We tested the hypothesis that males with short versus long *avpr1a* microsatellite alleles exhibit differences in courtship behavior, which could act as cues for female mate preference. The only behavioural difference we detected between males with short versus long *avpr1a* microsatellite alleles in mate preference trials was that males with short *avpr1a* microsatellite alleles sniffed the anogenital region of females more frequently during the first two days of the trials. Our results did not strongly support the hypothesis that a male's *avpr1a* genotype predicts the courtship behaviours we measured and suggests that other courtship behaviours or traits, such as odour and vocalizations, may be more important to female prairie voles when choosing mates. Additional studies using a wider array of species are needed to assess the degree to which male mammal courtship behavior provides information on mate quality to females.

1. Introduction

In most animal species, females are expected to be more selective in choosing mates than males, displaying social and sexual preferences for males with particular phenotypes (Kokko et al., 2003; Trivers, 1972). Females may select particular males because they receive direct benefits, such as access to a high quality territory, protection from predators, or future paternal investment in offspring (Hoezler, 1989; Møller and Jennions, 2001). Females may also choose males that provide them with an indirect benefit, such as 'good genes' (Fisher, 1930; Hamilton and Zuk, 1982). Types of benefits from choice of particular males are not mutually exclusive and may have worked in combination in the evolution of traits in mate choice by females.

When choosing a mate, females may be unable to directly assess fitness benefits obtained by mating with a particular male, such as future paternal care. Nonetheless, several models have shown that mate choice can evolve if females use 'indicator' traits that are correlated with the benefits associated with the preferred traits (Michod and Hasson, 1990; Wolf et al., 1997). Numerous empirical studies have documented that females display mating biases based on male variation in morphological traits such as coloration, body size, and body symmetry (Møller and Thornhill, 1998; Møller and Jennions, 2001 and references therein). These results support the hypothesis that morphological traits can be indicator traits, however, these traits do not always correlate with fitness benefits (Møller and Jennions, 2001). Although not as extensively studied as morphological traits, behaviours, particularly those that occur during courtship, may also serve as indicators of male quality. For example, specific components of courtship behaviour were correlated with increased paternal behaviour and hatching of young

insticklebacks (*Spinachiaspinachia*; Östlund and Ahnesjö, 1998), suggesting that females use these behaviours to select mates.

Rodents do not typically have conspicuous courtship rituals, but the behaviour of males may still provide cues useful in a female's selection of a social or sexual partner. Prior to copulation, males approach females and typically engage in anogenital investigation, which is often followed by mutual naso-nasal sniffing (Gavish et al., 1983; Gleason and Marler, 2010; Maken and Hennessey, 1999). Other male behaviours that typically occur before copulation include allogrooming of females, sitting next to females, and following and mounting them (Young and Grunt 1951; Ewer 1971; Fernandez-Vargas et al., 2011).

In prairie voles (*Microtus ochrogaster*), most males form a pair bond with one adult female and display social monogamy (Getz et al., 1993). Social partners defend their territory from potential competitors and provide high levels of biparental care (Carter and Getz, 1993; Getz et al., 1993; Solomon, 1993a). However, in natural populations, as well as when maintained in semi-natural enclosures, some males occupy home ranges that overlap with the ranges of multiple females, and these "wanderer" males appear to lack a strong pair bond with a single female (Getz et al., 1993; Solomon and Jacquot, 2002; Streatfield et al., 2011). The continued presence of the female's male partner at the nest postmating can significantly increase offspring production, development and survival (Mahady and Wolff, 2002; McGuire et al., 1992; Wang and Novak, 1992). Thus, if a female is able to discriminate among males and select a male partner that is likely to remain with her throughout pregnancy and the rearing of offspring, she could increase her overall reproductive success.

Some intraspecific variation in male prairie vole sociosexual behaviour (e.g. pair-bonding) has been attributed to differences in environmental or demographic conditions (e.g., vegetation, adult density, Streatfeild et al., 2011) and individual characteristics (e.g., body size, Chesh et al., 2012). Additionally, neurobiological mechanisms have been found that influence variation in male sociosexual behaviour (Hammock and Young, 2005; McGraw and Young, 2010). Differences among males in the neural expression of the vasopressin 1a receptor (V1aR) in specific brain regions are correlated with the length of microsatellite DNA within the regulatory region of the gene (*avpr1a*) encoding the V1aR (Hammock and Young, 2005). Variation in V1aR expression levels within specific regions of the brain appears to affect differences in partner preference by males and paternal behaviour in laboratory tests (Lim and Young, 2004). Males with longer *avpr1a* microsatellite alleles display significantly greater partner preference, and lick and groom pups more often than males with shorter *avpr1a* alleles (Hammock and Young, 2005). These behaviours are correlated with offspring production and survival (Mahady and Wolff, 2002; McGuire et al., 1992; Wang and Novak, 1992) and suggest a female could maximize her reproductive success by selecting a male with longer *avpr1a* microsatellite alleles as a partner because he will be more likely to form a stronger social bond with her and provide greater paternal care.

This hypothesis, that female prairie voles display social and sexual preferences for males with longer *avpr1a* microsatellite alleles, has been supported by results of a laboratory partner preference test (Castelli et al., 2011). In these trials, females cannot assess male fidelity and paternal behaviour directly, thus, they must use some indicator trait(s) that correlates with male *avpr1a* genotype when choosing a partner. However, the

trait(s) females use to discriminate between males with different *avpr1a* genotypes has yet to be assessed. Females could use any combination of behavioural, olfactory, and/or auditory cues associated with male *avpr1a* genotype to discriminate between males. For example, females showed significant mating preferences for males displaying more affiliative behaviour, but the affiliative behaviours (male allogrooming and side-by-side contact) were not correlated with male V1aR expression (Ophir et al., 2008).

In our study, we reexamined videotapes from the preference trials conducted by Castelli et al. (2011) to test the hypothesis that males with different *avpr1a* genotypes exhibit differences in courtship behaviour. If female social and sexual preferences for males with longer *avpr1a* microsatellite alleles (Castelli et al., 2011) were influenced by male courtship behaviour, we predicted that at least one affiliative, investigatory or agonistic behaviour would differ between males with long versus short *avpr1a* microsatellite alleles.

2. Materials and methods

2.1 Ethics statement

All of the research procedures we used in this study that involved live prairie voles were approved by Miami University's Animal Care and Use Committee (protocol #689).

2.2 Subjects

In 2006 and 2007, we collected prairie voles from natural populations located within the Phillips Tract, University of Illinois, Urbana, IL (40°08'03"N, 88°08'58"W)

and the Nelson Environmental Study Area (39°03'07"N, 95°11'32"W) at the University of Kansas, Lawrence, KS. After collection, we transported all voles to Miami University's animal facilities in Oxford, OH where we established separate breeding colonies for the Illinois and Kansas voles. We maintained voles under the following conditions: 14:10 h light: dark cycle (lights on at 0800 h), 19 ± 1 °C, and 55% humidity with ad lib food (Rodent Breeder Diet no. 5013, PMI Nutrition International) and water (for details on collection and housing see Castelli et al., 2011).

The Illinois and Kansas breeding colonies were used to generate voles for the female partner preference trials. We genotyped all field-caught voles and their offspring at their *avpr1a* microsatellite locus, according to the procedures described in Solomon et al. (2009). For the partner preference trials, we designed a breeding scheme to produce F₂ males that possessed two *avpr1a* microsatellite alleles at least one standard error (SE) shorter or longer than the mean allele length, measured in base-pairs (bp), within the population from which the stimulus males descended (Illinois: mean = 743 ± 3 bp; Kansas: mean = 742 ± 2 bp). For the trials involving Illinois voles, mean *avpr1a* microsatellite allele length was 724.7 ± 1.9 bp among stimulus males we defined as having short *avpr1a* microsatellite alleles, and mean *avpr1a* microsatellite allele length was 765.7 ± 1.8 bp among stimulus males we defined as possessing long *avpr1a* microsatellite alleles. The mean allele length among the Kansas stimulus males with short and long *avpr1a* microsatellite alleles was 719.7 ± 1.4 bp and 767.4 ± 2.4 bp, respectively. The breeding scheme we used to generate the F₂ stimulus males for the preference trials was designed to maximize the difference in *avpr1a* microsatellite allele lengths between males,

while also uncoupling genes at other loci that might co-segregate with the *avpr1a* locus and that also could influence partner preference (for details see Castelli, et al., 2011).

All the voles we used in the preference trials were sexually naïve, F₂ generation animals, and all individuals within a trial were unfamiliar and unrelated to each other (Castelli et al., 2011). The two stimulus males we selected for each preference trial were derived from the same population as the subject female in each of the trials (either Kansas or Illinois). Females, with the exception of two siblings from Illinois born in separate litters, were unrelated and selected without regard to their *avpr1a* genotype, since there is no evidence that their vasopressin genotype affects female partner preference (Cho et al., 1999). The stimulus males and subject females were between 38 and 301 days and 45 and 264 days of age, respectively, so all individuals were sexually mature (Solomon, 1991; Mateo et al., 1994). We also controlled for male body mass to ensure that there were no systematic differences between males with long versus short *avpr1a* microsatellite alleles, because females prefer heavier males (Solomon, 1993b; Aschmeier et al., 2008). No vole was used in more than one preference trial.

2.3 Test apparatus

We conducted partner preference trials in 60 X 20 X 20cm (L x W x H) clear Plexiglas arenas divided into three equal sized compartments (20 x 20 x 20cm) by Plexiglas dividers that had a 3.5 cm circular opening at the base through which a vole could pass (Castelli et al., 2011). Each compartment was provisioned with processed paper bedding (Cell Sorb Plus, A & W Products, Inc., New Philadelphia, OH, U.S.A.), and ad libitum food and water. Approximately one day prior to testing, we placed cable-

tie collars on the necks of the stimulus males. About 1 hour before the start of a trial, we tethered a stimulus male in each of the end compartments of the arena to allow them to become acclimated to the testing apparatus. Males were tethered using a fishing leader (Panduit Corp., Tinley Park, IL, U.S.A.) attached to the cable-tie collar and the wire-screen lid on the top of the arena via a swivel clip. The two stimulus males were always from the same population with one male possessing two long *avpr1a* microsatellite alleles and the other two short *avpr1a* microsatellite alleles. Stimulus males could freely move within their own compartment but could not leave the compartment in which they were tethered. This prevented male-male interactions from influencing female preference. Male *avpr1a* microsatellite allele length was counterbalanced to prevent position effects.

2.4 Behavioural assay

We conducted the trials in a room isolated from the breeding colony rooms with a separate airflow and with the same photoperiod, humidity, and temperature as the breeding colony (Castelli et al., 2011). During the lights off portion of the light cycle, the arena and voles were illuminated with red light bulbs mounted on the ceiling.

At the start of each preference trial ($N = 16$ for Illinois voles; $N = 16$ for Kansas voles), we placed females in the central compartment and they were free to roam in all three compartments during the trials. All preference trials lasted 72 h and were videotaped using a time-lapse VCR at either 0.2 s intervals (Panasonic AG-TL950) or 0.6 s intervals (Panasonic AG-6040; Castelli et al., 2011). A 72-h duration for the trials was selected because a previous preference trial using sexually naïve prairie voles found that 52 h was the median time for oestrus females to reach oestrus and mate (Witt et al.,

1988). After each trial, arenas were cleaned with water and Quatricide (Pharmaceutical Research Laboratories, Inc., Waterbury, CT, U.S.A.) and subsequently rinsed with 70% ethanol.

To evaluate the behaviour of males, we randomly selected one 30-min period from each light and dark period from each day ($N = \text{six } 30\text{-min periods per trial}$). In each period, we recorded the number of separate occurrences of naso-nasal sniffing and anogenital sniffing by males. We recorded the duration of side-by-side contact, allogrooming by males, active exploratory contact by males (male climbing on, sniffing anywhere other than naso-nasal and anogenital, and/or mounting the female), and male recipient of exploratory contact (allowing the female to climb on and/or sniff the body of the male). Aggression was originally included in the list of behaviours recorded but it occurred so rarely (0.30 ± 0.12 [$\bar{x} \pm \text{SE}$] bouts of aggression per trial) that it was eliminated from analyses. We recorded duration of behaviour only if a single bout exceeded 10 seconds. If a behaviour was interrupted by a different behaviour or if 5 seconds elapsed between performing the same behavior, we scored this as a new bout of the same behavior. The observer (BMG) was blind to the *avpr1a* microsatellite allele lengths of males until after all videotapes were scored.

2.5 Statistical analysis

For each trial, we summed the number of bouts or duration of each behaviour separately for the male with long versus short *avpr1a* microsatellite alleles during each of the six 30-min observation periods. Because the amount of time that a female spent with a male could influence the frequency or duration of behaviours with a specific male, we

divided the number of bouts or duration of each behaviour by the total amount of time the female spent in the compartment of that male during a particular observation period. We then used these ratios (number of bouts or duration/total time in male compartment) to calculate a difference score for each behaviour during each 30-min period of a trial by subtracting the ratio observed in the male with the long *avpr1a* microsatellite alleles from that observed in the male with the short *avpr1a* microsatellite alleles. The difference scores between the short allele value and the long allele value (Short-Long) for each trial were analyzed using a repeated-measures ANOVA in JMP Pro 11.2.0. Population (Kansas or Illinois), period (1=Day1Light, 2=Day1Dark, 3=Day2Light, 4=Day2Dark, 5=Day3Light, 6=Day3Dark), population* period, and TrialID nested within population (random effect) were the factors incorporated into the analysis. After the analyses were completed, we adjusted the rejection criteria for each comparison using a sequential Bonferroni adjustment, with α initially equal to 0.05, to reduce the probability of committing a Type I error due to conducting multiple comparisons.

3. Results

There were no differences between voles from Kansas versus Illinois in any of the behaviours we measured (all $P > 0.05$), so we removed population and the interaction with population as factors in the models. Males with short versus long *avpr1a* microsatellite allele lengths did not differ in any behaviour except anogenital sniffing ($F_{(5, 155)} = 4.24$, $P = 0.0012$, sequential Bonferroni adjusted $P = 0.0083$ [0.05/6]; Table 1). Males with short *avpr1a* microsatellites sniffed the genital region of females significantly more often than did males with long *avpr1a* microsatellites during light

period of days one and two of the 72-h trials (Fig. 1). The frequency of anogenital sniffing during the other time periods was indistinguishable from the dark period on day three.

4. Discussion

The results of our study do not strongly support the hypothesis that males with different *avpr1a* genotypes exhibit differences in courtship behaviour and that a male's courtship behaviour is indicative of his *avpr1a* genotype. The only difference that we found in the behaviour of males with different *avpr1a* microsatellite allele lengths was in anogenital sniffing during the first two lights-on periods of the trials. Males with short *avpr1a* microsatellites sniffed females more often than did males with long *avpr1a* microsatellites, suggesting that initially males with the short *avpr1a* microsatellite alleles spent more time investigating unfamiliar females. Our result is consistent with the finding that males with shorter *avpr1a* microsatellite alleles display greater interest in novel females relative to males with longer *avpr1a* microsatellite alleles (Hammock and Young, 2005). Since male rodents can use anogenital or vaginal odors to assess the species, sex and reproductive condition of females (Johnston, 2003; delBarco-Trillo et al., 2009), males with shorter *avpr1a* microsatellite alleles may take longer to make these assessments or are being more cautious before engaging in the next phase of courtship.

In Castelli et al. (2011), females were sexually naïve and displayed a significant mating bias toward males that engaged in less anogenital sniffing. Since oestrus is induced in prairie voles, females need to come into physical contact with chemical cues from an unfamiliar adult male to become reproductively activated (Carter et al., 1980).

Females can come into contact with the necessary chemical cues through anogenital sniffing the unfamiliar male when males and females engage in mutual anogenital sniffing. Females could perceive excessive anogenital sniffing as a type of harassment or they could be ready for males to progress to the next step in courtship. Females may also take longer to become behaviorally receptive when courted by males with short *avpr1a* microsatellite alleles.

None of the other behaviours we measured differed between males with different *avpr1a* genotypes, suggesting that these behaviours did not influence female mate selection. In a similar preference test by Ophir et al. (2008), females were significantly more likely to mate with males that groomed the females more and spent more time in side-by-side contact with females, but the neural expression of V1aR in five regions of male brains was not correlated with these behaviours. In our study we found that these same two behaviours were not different between males with long versus short *avpr1a* microsatellite alleles. There were some differences in the methods used in these two studies that could explain the inconsistencies in the results between them. For example, in Ophir et al.'s (2008) study, females had been exposed to soiled male bedding and thus, may have already been in oestrus by the time the trials started, and all males were sexually experienced. These differences may have altered the behaviour of males and females, as well as which behaviours females preferred in these studies. Although the studies differ in regards to the role that affiliative behaviours can play in mate choice, both studies indicate that neither male *avpr1a* genotype nor V1aR neural density was related to variation in these two particular affiliative behaviours.

The results of the Castelli et al. (2011) study are consistent with the hypotheses that females show social and sexual preferences for males with long *avpr1a* microsatellite alleles because the behaviour of these males should increase female reproductive success. However, the phenotypic cues females used to discriminate among males with different *avpr1a* genotypes during mate selection remain unidentified. One interpretation of our results and those of Ophir et al. (2008) is that male behaviour may not be the primary cue used by females to discriminate among males with different *avpr1* genotypes. Alternatively, females may be assessing subtler aspects of the behaviours we measured or are influenced by male behaviours that were not measured in these studies.

Olfactory cues are important in mate selection by female rodents (Roberts, 2007); some studies suggest that they may function to allow females to recognize males of the correct species (e.g., eastern chipmunks, *Tamias striatus*, Keevin et al., 1981), sex of the scent donor, if they have a compatible genotype (e.g., house mice, *Mus musculus*, Potts et al., 1991), and whether scent donors are kin, are dominant, or are territory holders (Ropartz, 1977; Halpin, 1986; Tregenza and Wedell, 2000). Male and female prairie voles can distinguish among conspecifics using odour cues, and these cues can influence behaviours such as nest building (Newman and Halpin, 1988). However, there is no empirical evidence demonstrating that male *avpr1a* genotype is correlated with differences in odour cues, or that females can discriminate among males on the basis of these odour differences.

Some rodents use audible or ultrasonic vocalizations (USVs) in mate choice (Clutton-Brock and McAuliffe, 2009; Fernandez-Vargas et al., 2011; Monticelli and Ades, 2013; Musolf et al., 2010). Adult male prairie voles produce significantly more USVs than

females and these calls may be important in attracting mates since males produce more calls in response to unfamiliar females than to female siblings or conspecific males (Lepriet et al., 1988; Ma et al., 2014). The male calls are also more structurally complex relative to female calls and could be used to transmit information about male quality (Lepriet et al., 1988; Ma et al., 2014).

In socially monogamous mammals that exhibit biparental care, females may use phenotypic cues indicative of mate fidelity and parental behavior when selecting a mating partner (Hoelzer, 1989). In rodents, there is evidence that males produce olfactory cues and vocalizations that convey information about male quality (Roberts, 2007; Musolf et al., 2010); females respond selectively to these cues. Males investigate females before attempting to mount them and may engage in courtship behavior such as seen in the rumba movements (hip-swaying) of the male guinea pig, which is often accompanied by purring or rumbling vocalizations (King, 1956; Cohn et al., 2004). However, it is not clear whether the behavior alone, the vocalizations alone or a combination of the two cues provide the best information to conspecific females. A growing number of studies indicate that females of some species use multiple cues when selecting a mate (Candolin, 2003). If male courtship behavior is an honest indicator of the males' quality, these behaviors could also be a target of sexual selection. Additional studies using a wider array of species are needed to assess the degree to which male mammals display courtship behavior that provides information to females that is not provided by auditory or olfactory cues or whether courtship behavior reinforces the information provided by other sensory modalities.

Acknowledgements

We thank Dean Kettle and Steve Buck for providing logistical support at the Kansas University and University of Illinois field sites. Funding for this study was provided by the National Science Foundation (IOS-0614015 to BK, and NGS), and a Howard Hughes summer internship (BG) from Miami University. The content of this paper is solely the responsibility of the authors and does not represent the official views of the National Science Foundation or Miami University.

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Table 1

Results of repeated measures ANOVA to examine the effects of the length of the *avpr1a* microsatellite allele and time on male prairie vole (*Microtus ochrogaster*) behavioural responses in a choice test ($N = 32$ trials). Table contains the P -values for the main effects of allele length (difference scores between the short allele value and the long allele value; Short-Long) and period.

Behaviour	Short-Long	Period
Naso-nasal sniffing	0.274	0.567
Anogenital sniffing by male	0.309	0.001*
Side by side contact	0.081	0.314
Allogrooming by male	0.286	0.407
Active exploratory contact by male	0.038	0.141
Male recipient of exploratory contact	0.056	0.102

* The asterisk indicates that there is a significant difference after using a sequential Bonferroni adjustment ($P = 0.0083$), with α initially equal to 0.05.

Fig. 1. Mean difference scores(+ SE) for the frequency of male anogenital sniffing behaviour by male prairie voles (*Microtus ochrogaster*) during each of the six observation periods during the 72-h trial ($N = 32$ trials). Difference scores for a period were calculated by dividing the number of bouts of anogenital sniffing for a particular male by the time the female spent in the male's compartment and subtracting the ratio observed in the male with the long *avpr1a* microsatellite alleles from that observed in the male with the short *avpr1a* microsatellite alleles. * indicates a significant difference in anogenital sniffing compared to the last observation period (Day3 Dark).