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FEMALE PRAIRIE VOLE MATE-CHOICE IS AFFECTED BY THE MALES' BIRTH LITTER COMPOSITION

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Abstract

Experimental testing and retrospective examination of breeding records were used to examine the influence of sex composition and/or size of males' birth litters on female mate-choice. Sexually naïve female prairie voles (*Microtus ochrogaster*) avoided males derived from all-male litters, but showed no preference for, or aversion to, males from single-male litters or from more typical mixed-sex litters. Examination of the pregnancy status of females after two weeks of pairing with a male allowed us to estimate the probabilities of a pups' intrauterine position relative to siblings for various litter sizes. The typical prairie vole pup derived from a mixed-sex litter comprised of 4.4 pups, and had a 13% chance of being isolated from siblings in utero and a 22% chance of being between siblings in utero. Pups from single-sex litters tended to be larger at weaning than did pups from mixed-sex litters; however, male size did not influence female choice behavior. These results suggest that some aspect of the perinatal experience of prairie vole pups from single sex litters can influence social interactions later in life.

Keywords

Microtus; intrauterine position; perinatal development; gonadal steroids

1. Introduction

Selecting a mate may be the most important decision an individual can make. House mice (*Mus domesticus*) that are allowed to choose their mates produce more and higher quality offspring, sooner, than do mice mated with a "non-preferred" individual, and these effects are seen regardless of whether it is male choice, female choice, or mutual choice [1–3]. In species where both members of a pair contribute to care of the offspring, the choice of mate may mean the difference between successful reproduction and failure. Male prairie voles (*Microtus ochrogaster*) have a large investment in their offspring, providing extensive parental care and remaining with the female partner throughout the pups' maturation [4,5]. As such, it might be expected that these males would be "choosy" when it comes to mate selection and, in general, this appears to be true. Male prairie voles are more discriminating when choosing among potential mates than are male montane voles (*Microtus montanus*) [6] which do not participate in pup care. In species in which males provide parental care, the male contributes more than simply a genetic heritage: his continued involvement can

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enhance pup development and/or improve overall reproductive success [7–9]. Given these circumstances, female mate-choice is especially important and it is reasonable to suggest that females of these species might use cues about a male's future suitability as a father, in addition to making an assessment of his immediate fitness.

Monogamy is relatively rare among mammals, but often is found in species for which paternal care is important in rearing young [10–13]. The specific selective pressures that produce monogamous mating systems probably vary among species but, compared to species in which single males monopolize the reproductive efforts of many females, differential access to mates between males from monogamous species may be reduced [14]. This, in turn, may reduce selective pressures that drive the evolution of extreme male-specific traits. Consistent with this idea, sexual dimorphism tends to be subtle in monogamous species [15,16]. Given the absence of drastic outward differences, on what basis do monogamous animals base their mate choices?

Prairie voles are widely considered to display a monogamous mating system [17–19]. Both sexes typically form long-term pair-bonds with their partners that extend beyond the breeding season, share a common nest, vigorously defend their mates against contact with conspecifics, and both sexes provide parental care [20]. Further, females that lose their mates rarely form new pair-bonds [21], although they still may reproduce [22]. The mechanism for mate-choice by female prairie voles is unclear, and more than one criterion may be employed [23]. Since there is little sexual dimorphism in prairie voles [16], especially at the age at which males and females typically form pairs, exaggerated secondary sexual characteristics are unlikely to play a significant role. Rather, more subtle factors likely influence mate selection. Although cues related to the health of the male seems an obvious factor. However, while females from a promiscuous vole species, the meadow vole (*Microtus pennsylvanicus*), distinguished between odors from parasitized and non-parasitized males, female prairie voles did not [24]. Moreover, frequency of scent-marking by males did not influence females' choice [25,26], although females did appear to prefer the top-most scent mark when overmarks were present [27]. Other factors implicated in mate-choice by voles include males' social status, spatial abilities, affiliative behaviors, or previous mating experience; diet; and body size. [23,28–34]. Finally, the level of female-directed aggression exhibited by males may be an indicator of paternal potential in prairie voles [30]. Thus, choosing for appropriate levels of aggression may allow the female to pair with a more paternal male.

Species differences in the central nervous system that are implicated in social behavior are present at birth [35]. Although such differences undoubtedly reflect genetics, prenatal conditions also can strongly influence brain development [36]. It is well-established that perinatal experiences can permanently alter physiology and behavior [37,38], including behaviors that may influence reproductive success [39–42]. In this regard, it previously has been established that the perinatal experience can affect social behavior in prairie voles [43–45]. However, in most studies of perinatal conditions in voles, experimental manipulations have been used [44,46–49], while more natural sources of variation are understudied [16,50–53]. Here, we assess the effects of natural variation in birth litter composition on mate-choice by adult female prairie voles. We hypothesized that the composition of a male's birth litter, and thus the perinatal conditions he experienced, may influence a female's responses to him as a potential mate during adulthood. We combined experimentation with retrospective analyses of breeding records to examine directly the effects of variation in birth litter composition, and indirectly the effects of gestational conditions on social behavior in adult prairie voles.

2. Methods

2.1 Animal husbandry

All animals were sexually-naïve adult prairie voles from a laboratory breeding-colony descended from an Illinois population, and were of the F3 and F4 generations relative to most recent out-crossing with wild stock. The breeding colony is housed in USDA approved facilities with general animal care provided by Laboratory Animal Resources personnel and are monitored daily. Veterinary staff is available for consultation regarding animal health and welfare. All animals are maintained at ~21°C with a 14:10 light:dark cycle (lights on 0800). Breeding pairs are housed in plastic cages (20 × 25 × 45 cm) containing corncob bedding with timothy hay as nesting material. *Ad libitum* food (Purina rabbit chow supplemented with black-oil sunflower seeds) and H₂O are available. After weaning (~20 days of age), pups are housed as same-sex sibling pairs in shoebox style plastic cages (10 × 17 × 28 cm). Pups that are the only individual of a sex in a litter are housed with another pup of the same age and gender, typically also the only representative of its sex in its litter. Females are housed in the same room with the breeders, while males are housed in a separate room until used in experiments. All animals were at least 60 days of age and sexually naïve when used in experiments.

Experimental manipulations, animal handling procedures, and behavior testing were approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee.

2.2.1 Choice test procedures—Females were given a choice between two males derived from birth litters with differing sex ratios. The apparatus for the female choice test consisted of a central cage (10 × 17 × 28 cm) joined by tubes (7.5 × 16 cm) to two identical parallel cages. Each of these latter cages contained an unfamiliar stimulus male from one of three litter classes (litter-classifications are described in section 2.2.2). Each stimulus male was defined as a “target” or a “distractor” prior to the start of testing. The stimulus males were tethered to restrict their movements to their respective cages and thus had no physical contact with each other. All cages contained food and water.

For each test, a female was released into the central cage and had free access to all cages. A customized computer program (R. Henderson, Florida State University) using a series of light beams across the connecting tubes was used to monitor the amount of time the females spent in each cage and the number of transits between cages. In addition, the animals were videotaped throughout the three hour test for later quantification of the time the females spent huddling quietly with each stimulus male. Tests were conducted between the second and sixth hour of the lights-on period.

2.2.2 Litter classification—All animals were assigned to one of three litter classes based on the compositions of their birth litters. Since there were some single sex-litters, the proportion of males (number of males/litter size) in each litter rather than exact sex ratios were used in statistical comparisons. Class 1 included animals from typical mixed-sex litters (≥ one female and ≥ two males). Class 2 included animals from litters containing only one male (one male, ≥ one female). Class 3 included only all-male litters (≥ two males, no females). All females used in behavioral testing were from Class 1 litters.

Groups in the choice test were defined based on the class of the “target” male; the “distractor” always was from a Class 1 litter (Table 1). Thus, in Group 1, females were given a choice between two Class 1 males, with one of the two males designated as the target, the other as the distractor, prior to the test. This group served as a baseline comparison since both males were considered to be equivalent, and no preference was

expected. Females in Group 2 had a choice between a Class 2 male as the target, and a Class 1 male as the distractor. Finally, the third group of females chose between a Class 3 target male and a Class 1 distractor male.

2.3.1 Male size choice—In a separate experiment, females ($n = 8$) were given a choice between two adult Class 1 males that were of similar age (mean age difference = 4.9 ± 1.8 days), but that differed by ≥ 10 grams in mass (large males = 56.8 ± 1.1 g, small males = 46.3 ± 1.1 g; $t = 6.48$, $p < 0.001$). Methods were as described above for the female choice test. The larger of the two males was designated as the “target” male in each pairing.

2.3.4 Statistical treatment of behavioral data—Data from the choice tests were analyzed in three ways. First, paired t-tests were used to compare the amounts of time each group of females spent in contact with each of the stimulus males when given a choice between males of differing litter classes or sizes. Preference for, or avoidance of, a class of males was inferred when there was a statistically significant difference in the amounts of time females spent huddling with the target vs. the distractor males. Second, to test for differential female responses to Class 2 and Class 3 males, the proportions of total contact time that the females in Groups 2 and 3 spent with target males in their respective pairings were compared using an independent t-test. Third, the time spent by the female in each of the males’ cages, the numbers of transits between cages, and the amount of time spent in the neutral cage were evaluated using one-way Analyses of Variance (ANOVA’s).

Litter compositions and birth litter sizes for animals used in the behavioral tests were compared using one-way ANOVA for females, or two-way ANOVA for males with stimulus animal (target or distractor) and group as factors. To compare the ages of all animals used in the choice test, a two-way ANOVA using group and test animal (females, target, or distractor males) as factors was used. Student-Neuman Keuls (SNK) analyses were used to further examine significant main effects or interactions (p -values < 0.05).

2.4.1 Pups in utero—As part of a larger series of experiments, we routinely examine the pregnancy status of females that have been paired with males for two weeks. When females are euthanized, the status of the pregnancy is assigned a score from 0 to 2 (Table 1) and the number of implantation sites/fetuses in each uterine arm also is noted. Grossly smaller fetuses (relative to surrounding fetuses) are noted and are assumed to be evidence of post-implantation mortality [54]. We retrospectively examined these data and calculated the probabilities that pups would be isolated in utero (i.e., the % of total number of pups within each litter size class that were the only pup in one of the uterine arms), adjacent to a sibling in utero (% of pups adjacent only to a single sibling), or between two siblings in utero, for litter sizes from 1 to 8 pups. All pups within each litter were assigned to one of these categories. For example, in a litter with four pups in one uterine arm and one in the other uterine arm, there would one isolated pup, two adjacent pups, and two between-siblings pups. The mean numbers of implantation sites/fetuses were calculated for each of three pregnancy stages: 1) 3–5 days pregnant, evidence of implantations; 2) gestation days 6–9; and 3) gestation days 10–12.

2.4.2 Weaning data—We retrospectively examined the breeding records associated with the first 1000 pups from our captive breeding colony. For each litter, we recorded the number of pups of each sex and the total number of pups at weaning. These data were used to calculate mean litter size and the proportion of litters that contained only one sex of pup. Again, the possibility for single sex-litters necessitated the use the proportion of males (number of males/litter size) in each litter, rather than exact sex ratios, in statistical comparisons. Weights at weaning were available for a sub-set of pups, and these data were used to assess correlations between litter size, litter composition, and mass at weaning.

ANOVA was used to compare weaning masses between mixed-sex, and male- and female-single-sex litters

Results

3.1 Female choice tests

All animals used in the choice tests were of similar age (77.5 ± 2.60 days of age; range 61–150) on the day of testing and there were no age differences between ($F_{2,87} = 1.91$, $p = 0.15$) or within ($F_{2,87} = 0.59$, $p = 0.55$) any of the groups. The compositions and sizes of the birth litters for all animals are shown in Table 2. As expected, the proportions of males ($F_{2,63} = 53.76$, $p < 0.001$) and the litter sizes ($F_{2,63} = 23.17$, $p < 0.001$) were significantly different among the three classes of litters. Among the stimulus males, there was a significant group effect for the proportion of males in each litter class ($F_{2,60} = 20.15$, $p < 0.001$), a marginal effect of stimulus animal type (target or distractor) ($F_{1,60} = 3.46$, $p = 0.07$), and a significant interaction ($F_{2,60} = 32.44$, $p < 0.001$). Moreover, significant litter composition differences between the target and distractor males in Groups 2 and 3 (all p -values < 0.02) were revealed. Among stimulus animals, there were main effects of test group ($F_{2,60} = 11.91$, $p < 0.001$) and stimulus animal type ($F_{1,60} = 17.45$, $p < 0.001$) on litter size, and a significant interaction ($F_{2,60} = 4.42$, $p < 0.02$). Pair-wise comparisons showed that litter sizes differed significantly between the three classes of target males, and that litter sizes were significantly smaller for target males than for distractor males in Groups 2 and 3 (all p -values < 0.002). There were no group differences in either litter composition or litter size among the Class 1 males used as both targets and distractors in Group 1 and as distractors in Groups 2 and 3 (Pair-wise comparisons - all p -values ≥ 0.16). All females were from litters of similar sex ratios ($F_{2,30} = 0.06$, $p = 0.94$) and sizes ($F_{2,30} = 0.13$, $p = 0.88$).

Females' choices depended on the classes of males they were allowed to choose between (Figure 1). Comparisons of contact time spent with the target and distractor males revealed no differences for females choosing between two Class 1 males (Group 1; $t = 0.85$, $p = 0.41$), or between Class 1 and Class 2 males (Group 2; $t = 1.01$, $p = 0.34$). In contrast, females in Group 3 spent significantly less time in contact with Class 3 males than they did with Class 1 males ($t = 2.20$, $p < 0.05$). In terms of absolute numbers, just over half (6/10) of the females in both Groups 1 and 2 preferred the target male. In contrast, 11 of 13 of the Group 3 females preferred the Class 1 male over the Class 3 male. The proportion of contact time spent with the target male was greater ($t = 2.30$, $p < 0.04$) when females were paired with Class 2 males ($0.61 \pm 0.12\%$) than with Class 3 males ($0.30 \pm 0.07\%$) $t = 2.30$, $p < 0.04$). Neither actual time spent in contact ($t = 0.06$, $p = 0.95$) nor the proportion of total contact time ($t = 0.02$, $p = 0.97$) spent by the females with Class 1 and Class 2 males were significantly different.

None of the other behavioral measures were significantly different (Table 3). All females spent at least some time in each male's cage. The number of crossings between cages ($F_{2,29} = 0.54$, $p = 0.59$), and the amounts of time spent in the target cage ($F_{2,29} = 1.38$, $p = 0.27$) and the center cage ($F_{2,29} = 2.11$, $p = 0.14$) did not differ for females in the three groups. In addition, the combined amount of time that females spent in close contact with the males did not differ between groups ($F_{2,29} = 0.66$, $p = 0.52$).

When given a choice between two males of different sizes, females did not display a preference ($t = 0.26$, $p = 0.80$) for contact with either size male (32.9 ± 9.7 min spent with the large male, 27.4 ± 13.2 min with the small male). Females that chose between large and small males did not differ from other females choosing between males from typical mixed litters (Table 3) in any of the non-social behaviors or total contact time (t -value range 0.15 – 1.41, p -value range 0.88 – 0.18).

3.2 Litter demographics

The number of pups in utero was examined in 234 females, each of which had been paired with a male for two weeks but otherwise was unmanipulated (Table 2). The mean number of pups in utero was 4.4 ± 0.1 . Litter sizes ranged from 1 to 8 and were generally normally distributed (data not shown). For each litter size, we calculated the probability that a given pup would be 1) isolated in utero; 2) adjacent to a sibling; or 3) between siblings. These probabilities are presented in Figure 2A. The typical pup (i.e., a pup from an average sized litter) has a 13% chance of being isolated in utero, a 65% chance of being adjacent to a sibling, and a 22% chance of being between two siblings. Consistent with the calculated probabilities, we found that 10.9% of pups were isolated in utero, while the remaining animals were adjacent to at least one sibling. Evidence of resorption was uncommon. We found only six females with what appeared to be firm evidence of pup resorption, involving as many as ten individuals (3 cases with single pups being resorbed, 2 with two pups, and one with three pups). In addition, the mean number of pups weaned from a total of 227 litters was calculated for comparison with the results from in utero examinations (Figure 2B). The mean number of pups weaned per litter was 4.5 ± 0.2 . There were no significant differences in the mean numbers of pups in the uterus at various stages of pregnancy and of pups weaned ($F_{3,409} = 1.32$, $p = 0.27$).

Approximately 20% of weaned litters were single sex (8.8% for females, 10.6% for males). Single-sex litters contained fewer pups (2.6 ± 0.4) than did mixed-sex litters (4.8 ± 0.3) ($t = 4.25$, $p < 0.001$). Overall, mixed-sex litters comprised 63% of all litters. Weaning weight (Figure 2C) also varied depending on the composition of the litter ($F_{2,85} = 12.57$, $p < 0.001$): there was no difference in mass at weaning ($p = 0.75$) between pups from all-male and all-female litters, but pups from mixed sex litters were significantly lighter than pups from single-sex litters ($p < 0.001$ for both sexes). The mass difference was still significant ($t = 3.58$, $p < 0.002$) when we controlled for litter size by including only mixed litters containing 2, 3, or 4 pups to match the range of litter sizes seen for single-sex litters (litters containing a single pup were not included as single-sex litters; likewise a single-sex litter containing 5 pups has only been seen once since the breeding colony was established, thus litter sizes above 4 were not included in these calculations).

4. Discussion

We have found that females' choice between potential mates is influenced by the males' birth litter composition. Females were equally likely to choose males from birth litters that included both brothers and sisters, and males from birth litters in which they had only sisters. In contrast, females appeared to actively avoid males from all-male litters, choosing instead to associate with males from more typical mixed-sex litters.

Naturally occurring variation in gestational and rearing conditions can have important influences on adult behavior [55–57]. Included in such variation are the position of the fetus within the uterus relative to siblings, and the sex ratio and size of the birth litter. The literature regarding the effects of intrauterine position has been reviewed in detail by vom Saal and others [58–60] and the reader is referred to those sources for further information. Briefly, regardless of sex, fetuses positioned between two male siblings in utero experience higher circulating levels of testosterone than do fetuses positioned between female siblings. The reverse is true for estrogens, with fetuses between female siblings having higher levels of estradiol, while fetuses between mixed siblings are intermediate for circulating levels of both hormones. The effects of varying intrauterine environment are manifested not only as morphological, physiological, and behavioral variation among individuals [61–63], but also may influence responses elicited from others [64]. For example, female mice that develop

between sisters in utero are more attractive to males than are females that developed between brothers [65].

4.1 Female choices among males

Male mice that were between brothers in utero are less likely to attack pups [60]. Thus, it might be expected that males from all-male litters would be preferred as mates when paternal care is important for reproductive success. However, sexually naïve female prairie voles appear to avoid males from all male-litters. When given a choice between a male from an all-male litter and a male from a mixed-sex litter, few females spent more time in contact with the male from an all-male litter. In contrast, females spent equivalent amounts of time in contact with males from single-male litters and from other mixed-sex litters. The choice of males was not affected by the backgrounds of the females themselves, since all were from typical mixed-sex litters of similar sizes and sex ratios. Further, the males that comprised the distractor groups provide additional information regarding the attractiveness of males from litters with more balanced sex ratios (overall male proportion = 0.55). Specifically, a clear difference in the amount of time that females spent in contact with one class of males occurred only when one of the choices was a male from an all-male litter, suggesting that males from any mixed-sex litter are equally attractive to females. Thus, the females must have been actively avoiding males from all-male litters, rather than specifically choosing the distractor male. Active avoidance in voles has been noted previously [66], but in that case, pair-bonded males avoided nonestrous females in dyadic encounters.

It is unclear which characteristics females use in choosing between males. One possibility is the relative sizes of the males. Pup growth is inversely related to litter size in several rodent species [67,68]. Thus, one potential confound in interpreting the present results is the fact that litter composition and pup size may be correlated. In the choice tests, litters from which the target males arose differed in size. Not surprisingly, males in the all-male litter class tended to come from the smallest litters since the probability of a single-sex litter decreases as litter size increases. Moreover, at weaning, pups from smaller litters were significantly larger than those from larger litters. If the size advantage at weaning is maintained into adulthood [69], males from all-male litters may be larger as adults as well, and thus more attractive to females. Alternatively, females may avoid larger males, in which case, small litter size may disadvantage males from all-male litters. We found that neither appears to be the case. When given a choice between two males of similar age but that differed in size, females did not display a preference in either direction. Similar behavior was found in house mice [3], but these results contrast with previous findings in voles [32]. However, in that study, females voles were allowed 48h to make their choice between large and small males, while we examined the more immediate time frame pertinent to our experimental design.

A second criterion on which females may base choices between males may be aggression on the part of the male. Mating has been linked to the emergence of aggression behavior in male prairie voles. When given a choice between a recently mated male and a male that had not recently mated, female prairie voles preferred the unmated males [29]. However, the preference for unmated males often waned if the males were anesthetized, suggesting that behavioral differences between mated and unmated males contributed to females' choices. Males from all-male litters obviously are more likely to be adjacent to a brother than are those from litters in which they are the only male and several lines of evidence suggest that such males may be more aggressive (reviewed by [58]). Further, paternal behavior was correlated with female-directed aggression in prairie voles [30], and thus, it might be expected that females would choose males that were more aggressive during initial encounters. However, this expectation must be tempered by the fact that the direction of the choice may depend on the reproductive status of the female.

A third factor that may contribute to females' choice of males is the physiological status of the female herself. In naked mole-rats (*Heterocephalus glaber*) [70] and house mice [71], the reproductive status of the female influences mate choice. "Puberty" in prairie voles does not occur spontaneously, but rather is induced by prolonged, close physical contact with a male [72,73]. This aspect of prairie vole reproductive physiology may provide an important clue in interpreting the females' avoidance of males from all-male litters. The females in the present study had not had prior contact with a male, and thus, essentially, were in permanent diestrous [73]. Zhang et al. [74] found that, although females in estrous preferred dominant males, non-estrous females preferred subordinate males, suggesting that non-estrous female voles may prefer less aggressive males. Since the females in the present study were non-estrous, they may have been attracted more to males that developed in mixed litters in utero and thus may have had less pre-natal exposure to testosterone and its potential effects on adult aggression [58]. It should be noted that Shapiro and Dewsbury [23] also reported preferences for dominant males by female prairie voles, however, there, the females were pharmacologically induced into behavioral estrous prior to testing. As such, the female preference for dominant males in that study would be consistent with the behaviors seen in estrous Brandt's voles [74].

Finally, an intriguing possibility is that some more subtle aspect of behavior on the part of a given male negatively affected the female's choice, in effect driving her toward the more typically behaving male in the choice test. Emotionality has been correlated with several aspects of social behavior [75] and may reflect autonomic activity. A relatively recent line of research suggests that autonomic nervous system (ANS) function may play a prominent role in social behavior history of prairie voles [76]. Interestingly, small litter size [56] and variations in prenatal environment [77] can significantly alter ANS function and aspects of emotionality. Given that the males from single-male litters experience both small litter size and a potentially testosterone rich (or estrogen poor) prenatal environment, they may not display appropriate responses when confronted with a female.

4.2 Litter demographics

As noted previously, males from all male litters were larger at weaning. However, the single-sex litter/weaning size relationship also was seen for all-female litters. This may simply reflect the fact that single-sex litters tended to be smaller and pups from small litters tend to be larger at weaning. However, even when litter size was controlled for, pups from single sex litters still were larger at weaning than were pups from mixed litters. It is probably wrong to assume that all pups experience identical and optimal interuterine conditions. In fact, it is likely that, at least between sexes, there are differences in resource levels and environmental conditions necessary for optimal development [78]. Thus, the size difference actually may reflect the fact that only same-sex siblings were present in utero, and might indicate that mothers (or the fetuses themselves) may be able to "adjust" the intrauterine environment, and that this is better done when only one sex is present. The presence of only one sex may reduce variability between uterine positions or reduce intrauterine competition between siblings. An alternative possibility is that mothers differ in their pup-oriented behaviors when only one sex is present.

In more typical laboratory species, the large number of pups per litter makes studying single-sex litters difficult without manipulation of litter composition. At the other end of the spectrum, litters that are too small may not show enough variation to make studying the effects of single-sex litters feasible. For example, the relationship between uni-sex multiple births and mass does not appear to be found in humans in which same-sex and opposite sex dizygotic twin pairs do not differ in birth weight [79]. Here, the intermediate number of pups per litter produced by voles may provide sufficient numbers of pups per litter to permit inter-litter comparisons, while still having small enough litters that single-sex litters are not too

uncommon. It would be of considerable interest to determine the smallest litter size for which the single-sex effect on weaning masses is seen.

4.3 Gestational conditions

One important consideration that must be addressed is how well the number of weaned pups reflects numbers in utero. The present data on intra-uterine numbers accord well with prior reports on prairie voles. For example, Roberts et al [80] reported about 5 fresh corpora lutea and four embryos in females that had been paired with males for several days. Further, the number of prairie vole pups collected at 20 days after pairing averaged ~4.4 pups/litter [81]. We also found that the numbers of implantation sites/fetuses/pups seen at the various stages of pregnancy and at weaning did not differ. This observation, in conjunction with the very few instances of pup resorption we encountered, would be consistent with previous reports of very low numbers of females displaying evidence of post-implantation losses [54,82]. Since implantation fixes the position of the pups in utero, and there appears to be little post-implantation loss, it is reasonable to conclude that our calculated position ratios for intra-uterine conditions should be quite similar to those previously experienced by weaned pups.

Although prenatal manipulations of gonadal hormones have produced rather subtle effects in male voles [46], such manipulations typically are targeted at defined periods during gestation [44,46]. In contrast, the intrauterine hormonal environment can affect development throughout gestation. This distinction is important since different brain systems may respond differently depending on the onset and duration of prenatal gonadal hormone change [83]. Further, changes associated with prenatal hormone changes may not be apparent from short exposures [84]. Nonetheless, variation similar to that associated with intrauterine position has been seen in prairie voles that have been exposed to perinatal manipulations of gonadal hormones. For example, Lonstein and De Vries [46] showed that prenatal manipulation could alter behavior and development in prairie voles (lengthened anogenital distance in both sexes, marginal differences in some pup-oriented behaviors in males). The present results suggest that more prolonged prenatal manipulations may produce more robust results.

An additional consideration that must be noted is the relatively low probability that a prairie vole pup will be between siblings in utero. Due to the relatively small litter sizes produced by prairie voles, only about one pup in five would be expected to experience such conditions. Thus, the number of pups between two opposite-sex siblings during gestation would be very small, and this would be most likely to happen to pups born of litters of above average size. Interestingly, species differences in litter size may make any effects of the gestational environment on later behavior a larger factor in vole species that routinely produce larger litters than do prairie voles.

4.4 Caveats & Limitations of the study

Retrospective analyses have some important limitations. Overall, adopting this approach permitted examination of data from much larger groups of individuals than could be justified if specific animals had to be sacrificed. We recognize that the litters from which experimental animals descended displayed the normal range of litter sizes and compositions. Unfortunately, artificially adjusting litter sizes and compositions might alter parental behavior, which in turn could alter the pups' later adult behavior [56]. However, this natural variability is unlikely to affect our basic conclusions. In fact, by not attempting to eliminate natural differences in individual perinatal experiences, we might expect that increased variability would mask group differences. Thus, by incorporating natural variation into the study, we actually enhance the strength of our findings.

A second limitation inherent in the present study is the fact that probabilities rather than specific enumerations are used in the analyses. Any assessment of actual intrauterine conditions would require anesthesia and anesthetizing the mother could alter the behavior of pups in later life. Further, caesarian delivery, typically on the penultimate day of gestation, is used in most studies that examine the effects of intrauterine position on behavior. Such an approach would introduce several significant complications. First, female voles that had caesarian deliveries tend toward infanticide [85]. Second, even if the females do not kill the pups, experiencing parturition may be a necessary prelude to appropriate maternal behavior which, in turn, may influence the behaviors of pups later in life. Third, in prairie voles, the father plays an important role in pup rearing, and although they typically are not in the nest during parturition [86], there is the potential for effects of their mates' parturition to alter male behavior [87–89]. Given these potential complications, we did not attempt to identify the exact intrauterine position of individual pups and their siblings in utero. As such, we must rely on probabilities to assess the effects of prenatal conditions. Nonetheless, the present study establishes the potential for male birth litter composition to alter the behavior of potential mates. Although using this method will not provide information regarding specific pups, the probabilities are likely to accurately reflect reality since the average numbers of pup weaned did not differ from the numbers of pups seen in utero at earlier stages of pregnancy. Thus, the current results serve to establish a “baseline” on which subsequent studies may build to examine the effects of intrauterine position on mate choice.

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Fig 1.

Mate choice behavior by female prairie voles differs depending on the composition of the males' birth litter. Females spent significantly less time huddling in close contact with males that were derived from all-male litters (Class 3) than they did with males from typical mixed-sex litters. (Classes 1 and 2) Each pair of bars represents the amounts of time that the females spent in contact with the two stimulus animals. * $p < 0.05$.

Fig 2.

A) probabilities of a pup being isolated, between siblings, or adjacent to a sibling in utero. B) numbers of pups at various stages of development; there were no differences between number of implantation sites and the number of pups weaned. C) pups from single-sex litters were significantly heavier at weaning than were pups from mixed-sex litters. * $p < 0.004$.

Table 1

Descriptions of uterine conditions used in assessing stage of pregnancy of female prairie voles 14 days after pairing with a male.

Pregnancy stage	Description	Number
0	No evidence of sexual activation, uterus white, thread-like	17
0.5	Uterus pink and swollen, no implantation sites evident	20
1	Implantation sites visible, (gestation day ~3–5)	20
1.5	Individual fetuses identifiable, (gestation day 6–9, diameter ~ 5 mm)	36
2	Anterior-posterior axis evident, (gestation days 10–12; length ~ 1 cm)	140

Table 2

Birth litter characteristics for animals used in the female choice tests. (Mean ± SE)

Litter characteristics							
Target male				Distractor male			
Litter class	Proportion male*	Litter size	Litter class	Proportion male	Litter size	Proportion male	Litter size
Group 1 Class 1	0.58±0.05	5.5±0.43	Class 1	0.63±0.05	5.5±0.43	0.41±0.02	5.5±0.48
Group 2 Class 2	0.32±0.08 ^a	4.2±0.57 ^b	Class 1	0.56±0.04 ^a	5.8±0.39 ^b	0.40±0.09	5.4±0.64
Group 3 Class 3	1.00±0.00 ^a	2.7±0.21 ^{b#}	Class 1	0.49±0.05 ^a	4.8±0.30 ^b	0.37±0.07	5.2±0.42

* all groups have significantly different litter compositions; shared letters in a given row indicate significant differences within the row;

all-male litters were significantly smaller than both mixed- and single-male litters;

Females all were from Class 1 litters. Female data were analyzed separately.

Table 3

Non-social behaviours and total contact time for females choosing between males from typical mixed litters, or all-male litters.

	Cage crossings	Cage time (min)		Total contact time (min/3h)
		Target cage	Central cage	
Group 1	250.5±47.5	67.9±8.8	33.1±3.8	77.1±6.3
Group 2	194.7±15.8	77.9±9.1	27.8±4.2	75.3±11.2
Group 3	212.1±36.3	56.9±8.5	45.0±8.3	61.7±11.9
Large v Small males *	260.6±47.4	81.4±14.2	29.4±3.9	60.2±10.8

* data from females used in male-size choice test are included to facilitate comparison. No significant differences from the Group 1 females