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ORIGINAL ARTICLE

# Odour preferred males led to a higher offspring number in the common vole

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## Abstract

Very recently, an interesting phenomenon was described in the common vole; vole parents with similar locomotor ability produced significantly larger litters. Positive assortative mating is a tendency to prefer individuals with similar phenotypes. We tested whether this also applies to smell similarity. Odour preference was tested in a T-maze, where each female was presented with two male odours, i.e. shavings together with feces and urine from home boxes. After female preference was established, the female was either paired with a preferred male (chosen) or paired with a non-preferred male (opposite choice). For analysis of the relationship to odour preference, genotyping of major histocompatibility complex (MHC) Class II DRB was done using amplicon sequencing. In the set of 45 individuals from two populations, we recovered 38 nucleotide haplotypes (alleles). Similarity of alleles in parent pairs according to the indexes of Sørensen–Dice (S–D) and Jaccard were calculated. Values of these indexes in parental pairs with preferred males were significantly higher (more similar) than in not preferred. The number of offspring in parental pairs with preferred males were significantly higher than in not preferred males. However, there is no correlation between the mentioned indexes and the number of offspring. The relationship between the success of reproduction and alleles is not clear-cut, this may be influenced by the measure of similarity we used, or by something that we could not detect.

**Keywords** *Microtus arvalis* · T-maze · Odour preference · MHC Class II DRB · Sørensen–Dice index · Offspring number

## Introduction

When animals, especially vertebrates, mate, the selection of a partner takes place based on somatic or behavioural manifestations. However, which manifestations will be preferred depends on social system or a successful strategy in specific population conditions. Behavioural or genetic analysis can indicate positive or negative assortative mating. Positive assortative mating is a tendency to prefer individuals with similar phenotypes (Jiang et al. 2013). Thiessen et al. (1997) argued that positive assortative mating may be a successful strategy since couples sharing a similarity are likely to pass more than 50 % of their genetic information onto their offspring. Negative assortative mating, also called disassortative mating, favours pairs formed by individuals with different phenotypes or genotypes, i.e., different sizes, different colours or with different major histocompatibility complex (MHC) genes. Probably the most extensive occurrence of disassortative mating was found in the white-throated bunting in connection with colour polymorphism. In the overwhelming majority of cases, pairs were formed from differently coloured individuals (Hedrick et al. 2018). In mammals, we can encounter this phenomenon too, for example, in wolves in Yellowstone National Park (Hedrick et al. 2016).

The most frequent occurrence of negative assortative mating is associated with a preference for MHC genotype dissimilarity, which is presented by odour (see e.g. Penn

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and Potts 1999; Yamazaki et al. 1999). The more diverse the MHC, the better an organism can defend against a greater number of pathogens or parasites. Therefore, it is important to maintain this diversity. Zufall et al. (2005) and Boehm and Zufall (2006) found that MHC class I molecules are important for MHC genotype signalling, precisely in the epithelium of the vomeronasal organ, and are also necessary for the proper functioning of the adaptive immune system. MHC gene products are found in various secretions, which create a specific odour also aided by bacterial decomposition. This smell then conveys information about its owner to a mating partner (Heath and Carbone 2001). Beyond this genetic distance, odour can inform, for example, about an individual's sex, age, reproductive condition and general viability (Manzini and Korsching 2011). All these components contribute to the constancy of an individual odour (Penn et al. 2007). The smell is even able to reveal whether the wearer is in a stressful or restful state (Cecchetto et al. 2019) and indirectly indicate their social status (Manzini and Korsching 2011).

Since females tend to invest more in gamete formation and care for the young, they are more sex-selective. Therefore, they choose sexual partners which have some direct or indirect advantages, for example good genes (Kirkpatrick 1996), which are then inherited by the offspring. Several studies show that the urine of sick individuals has a different smell and animals can identify these smells. In female house mice (*Mus musculus domesticus*), it was found that out of three samples, where one sample was the urine of a sick male, the second was the urine of a healthy individual and the third control sample was pure water, the females spent the least time with the urine of the infected male (Hurst 1990). Similar studies were also carried out with the prairie vole (*Microtus ochrogaster*) and the Pennsylvania vole (*Microtus pennsylvanicus*). The male voles were infected with the spiralworm, which it is not transmissible through contact between individuals. As expected, Pennsylvania voles preferred healthy individuals, but prairie voles did not. One possible explanation is the different mating systems of these two species, Pennsylvania voles are polygynous, while prairie voles are monogamous, so the value of health status may not have been decisive for them (Klein et al. 1999). In our latest study on the common vole (*Microtus arvalis*), it was found that the similarity of a behavioural personality trait in a parent pair increases the number of offspring produced (Urbánková et al. 2023). This raises the question; Does odour based negative assortative mating occur in the common vole as a counterbalance to the observed positive assortative mating, in order to sustain genetic polymorphism?

Assuming that the ultimate goal of reproduction is to transfer one's own genes to the next generation, it is well understood that females will prefer similar males who also have a similar genome with many of the same genes (Thiessen et al. 1997). However, it is argued that more isolated populations can lead to inbreeding if there is no preference for diversity. In the case of voles, however, there is no such isolation effect, due both to the widespread zoogeographic distribution and continuity of the primary and secondary habitats in the agricultural landscape, and significant cycles of abundance followed by emigration to new environments (Gauffre et al. 2014). Trait similarity in a pair could be greater probably during high population densities (e.g. Andreassen et al. 2013), when females can easily choose and achieve an increased number of pups with a preferred male, and conversely, can afford to reject an unpreferred male. In this case, it could be a strategy for the foreseeable future with enough males (Stamps and Krishnan 2014) and corresponds with laboratory preference tests, where females prefer known males over unknown ones (Řičánková et al. 2007). It is also possible that a male's odours and behavioural manifestations which are more similar to those of a female will be more likely accepted by that female than distinct ones (Jiang et al. 2013). Females are even able to show aggression against dissimilar individuals (Řičánková et al. 2007). A more similar acceptable male can induce the oestrus phase and ovulation in the female due to the time spent together (Sawrey and Dewsbury 1985). Induced ovulation is very useful with accidental contact of partners at low population densities (Katandukila and Bennett 2016). Clulow and Mallory (1970) suggested that induced ovulation may be a general feature of the genus *Microtus*. Therefore, mating of partners with similar traits (genes) could be successful during the whole population cycle.

Study of mating in voles and their population interactions with the environment is important not only for fundamental science (Lantová et al. 2011; Eccard and Herde 2013; Herde and Eccard 2013; Gracceva et al. 2014; Urbánková et al. 2020), but also for applied science such as pest management and conservation (Jacob et al. 2014, 2020; Heroldová et al. 2021). The variation of reproduction is a very important subject of study to better understand the dynamics of population growth. Here, we focused on odour preference, which is linked to MHC. In order to gain knowledge about the mentioned variation, we formulated the following working hypothesis: a female with a preferred male according to his smell will have a greater number of offspring than with a non-preferred male. First, we tested the odour preference for male common voles by females in the T-maze. We then paired both odour-preferred and non-preferred males with females. After that, we related the number of offspring to odour preference and finally, we attempted to explain the results by similarity or dissimilarity of MHC alleles in the vole parents.

## Material and methods

### Vole individuals

Wild common voles (*Microtus arvalis*) were caught on agriculturally managed meadows from April to September 2021 using Sherman live traps for small mammals. The parental pairs came from two distant localities (about 30 km apart), locality B: České Budějovice, 48.977821 N, 14.441390 E, locality V: Veselí nad Lužnicí, 49.080373 N, 14.755786 E). In total, 80 adult animals were captured, of which 78 individuals, 39 males and 39 females, were used for testing. To ensure that the animals were adults, sexual maturity was determined in males according to the scrotal position of the testes and in females immediately after the odour preference test, the state was verified according to the vaginal smear (Cora et al. 2015; Nubbemeyer 1999). Results are presented in Table S1.

### Breeding conditions

Voles were kept individually in polycarbonate breeding boxes 35 × 20 × 15 cm (T3, VELAZ Prague) with wood shavings, hay, and a plastic tube as a shelter (l = 15 cm, d = 4 cm). Commercial pellets for rats and mice, as well as pellets for guinea pigs and rabbits (VELAZ Prague), fresh carrots and water were available ad libitum. All individuals were individually marked on the breeding boxes. The laboratory conditions were stable, with room temperature about 19 °C and humidity about 50% under a L:D 16:8 photoperiod.

The voles were bred and tested in accordance with the principles of animal welfare and guidelines of the Departmental Commission for Animal Protection of the Ministry of Education, Youth and Sports in Czechia (permit number 7945/2010-30). These guidelines on animal treatment also conform to the journal's ethics guidelines. After the experiments, the voles were euthanized by inhalation anesthetic Isoflurane for DNA extraction from spleen.

### Test T-maze

Odour preference was tested in the T-maze (Fig. 1), which was made up of three parts: the main arm and two side arms–right and left. These arms were made of Plexiglas and had a square cross-section of 8 × 8 cm. The length of the main arm was 10 cm and the length of the side arms together was 30 cm. A starting box (35 × 20 × 15 cm) was attached to the main arm, into which the vole in the home tube was inserted. After insertion, this box was covered with a glass plate so that the animal could not possibly escape. Small boxes (20 × 15 × 14 cm) were placed at the end of the side

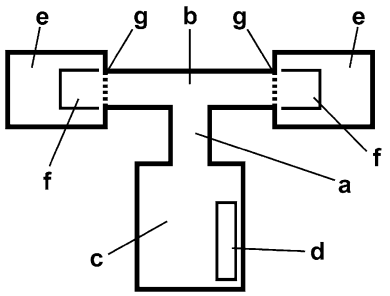


**Fig. 1** Scheme of the T-maze: the main arm (**a**), two side arms right and left (**b**), starting box (**c**) with the home tube (**d**), small boxes (**e**) with containers (**f**) with odour sources, separated from the side arms by metal grids (**g**)

arms, into which the container with the odour was placed. These small boxes were separated from the side arms by a metal grid. The small odour boxes were covered with opaque Plexiglas sheets so that the dark shade of the scent shavings does not interfere with the monitoring of the dark vole by the tracking program (see the EthoVision program). The resulting length of the assembled labyrinth was 90 cm and width 110 cm. The entire labyrinth was placed on a pedestal at a height of 80 cm. A camera was installed above the labyrinth to record the trials. The computer used by the experimenters to monitor the entire experiment was located in another room so that the voles would not be disturbed.

### Odour preference test

In order to manage the whole experiment, we had several trapping rounds throughout the season. In each round we trapped 13 voles which were processed completely before the next trapping round took place. The first series of experiments with 13 males and 13 females took place in June 2021. The second series, again with 13 males and 13 females, took place in August 2021, and the third series with the same number of individuals took place in October 2021. The test was always carried out at the same time of day in all phases and under the same conditions. Each individual was used just once throughout the experiment.

Odours were collected from all males. The shavings together with feces and urine were collected in a jar with a closable lid. Each female was presented with two odours of the opposite sex. The jars were placed in small boxes at the end of the side arms and were separated from the labyrinth by a grid that prevented the animal from entering the odour jar directly, but at the same time allowed for the best presentation of the odour. Before the start of each trial, all parts of the labyrinth were cleaned with an alcohol solution and rinsed with water. The test animal was placed in a tube in the starting box of the labyrinth. After inserting the transport tube with the vole and covering the starting

box with a cover glass, recording was started in the EthoVision program. The trial duration was set to 3 min. This time limit was evaluated based on pilot tests as sufficient for the manifestation of odour preference. In four cases, the individual did not climb out of the tube after 3 min, and then the interval was extended to 6 min, without the animal being removed from the tube. In this way, the animal received an additional 3 min of testing. The sample of odor shavings was voluminous enough to provide odour for a much longer period then 6 min. Then, like the other individuals, female had again 3 min of a new test ahead. In the T-maze, the animals moved cautiously and switched sides several times. For the evaluation of odour preference, the decisive parameter was the total time spent on the right and on the left segment of the T-maze. The odour with which the individual spent more time was marked as preferred. Critical level for recognizing a preference was generated from 10 odourless tests. The spontaneous difference between the sides was $5.4 \pm 1.9$ s. To the least odour side difference (7 s) the t-value was 2.67 ($p = 0.026$). The side difference of 7 s was considered odour preference. After the experiment, the individual was returned to the breeding box and moved to the breeding room. After testing all individuals in a given phase, pairs were formed on the basis of female selection, see Tables 1 and S1.

## Formation of pairs

After female preference was established, the female was either paired with a preferred male (chosen) or paired with a non-preferred male (opposite choice). Parental pairs were created by adding a male to the female's larger breeding box $52 \times 31 \times 19$ cm (T4, VELAZ Prague) according to the test result, Table 1 and S1. They were left together for 6 days. During this interval, we watched to see if any of the partners tried to escape systematically, two times (7 a. m. and 5 p. m.) a day personally for 15 min. After this time, the males were removed from the females' boxes, and it was noted whether they were found together in the tube or shared a nest. After mating, males were returned to their original breeding boxes ($35 \times 20 \times 15$ cm). A litter of young was expected after approximately 19–22 days. Most of the young were born exactly 21 days after the female and male were put together. Pups were counted and weighed after birth their weight was checked weekly for three weeks. After three weeks, the female was separated from her offspring and placed back into her breeding box.

## Genetic analysis of vole individuals

The voles tested in the summer season and partially also in the spring and autumn season (22 pairs in total, equally represented between the two localities) were subjected to DNA analysis. For analysis of the relationship to odour preference, genotyping of MHC Class II DRB was done (Meléndez-Rosa et al. 2018) using amplicon sequencing. A piece of tissue from toe and spleen from each individual after euthanasia was dissected and kept in pure ethanol. All DNA samples were extracted using DNeasy Blood and Tissue kit (QIAGEN) following the manufacturer's instructions. Then a dual-indexed amplicon library was created to multiplex all samples in one sequencing run. Amplicon PCR was done for all samples in duplicates to control for amplification bias and included negative controls following the Illumina protocol (Illumina 16S metagenomic sequencing library preparation, 2017). DNA template concentration for every sample was at minimum 4 ng/µl for the first PCR step. Reactions contained: 2.5 µl of the DNA sample, 12.5 µl of KAPA HotStart ReadyMix (Roche), 5 µl of 1 µM forward primer and 5 µl of 1 µM reverse MioeL primers (Kloch et al. 2012) extended with Illumina overhang at the 5′ end (compatible with the indexing primers below). Thermocycler setup was as follows: initial denaturation at 95 °C for 3 min, 25 cycles of: denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, with final elongation at 72 °C for 5 min.

PCR reactions were cleaned using AMPure XP beads (Beckman) and all samples were measured on a Qubit

**Table 1** Odour test algorithm for creation of parental pairs

| Test order | Odour of male ID | Female ID preference | Odour of male ID | Time diff between arms (s) | For pair chosen male | ID of pairs put together |
|---|---|---|---|---|---|---|
| 1 | M1 | ←F1 | M2 | 42.6 | Preferred | M1F1 |
| 2 | M2 | F2→ | M3 | − 56.6 | Opposite | M2F2 |
| 3 | M3 | F3→ | M4 | − 18.3 | Preferred | M4F3 |
| 4 | M3 | ←F4 | M5 | 128.4 | Opposite | M5F4 |
| 5 | M3 | ←F5 | M6 | 16.9 | Preferred | M3F5 |

Information provided in this table is an excerpt of the whole data set in Table S1

← = heading to the preferred male from the two exposed odours, values show time differences between the two T-maze arms, positive value means vole spent more time in the left arm, negative value in the right arm, *ID* animal identity (male M1, female F1)

fluorometer using a dsDNA High Sensitivity kit. Gel electrophoresis was done using 1.5% agarose gel with GelRed (Biotium), GeneRuler 100 bp Plus DNA (ThermoFisher) ladder, and 6 × Loading Dye (ThermoFisher). All samples produced visible bands, and concentrations above 0.223 ng/µl and were used for the second, indexing PCR step.

The indexing PCR mixture included: 5 µl of the Amplicon PCR product, 25 µl of KAPA HotStart ReadyMix (Roche), 10 µl of $H_2O$, 5 µl of the 5 µM forward index primer (S5: AATGATACGGCGACCACCGAGATCTACAC(indexS5) TCGTCGGCAGCGTC), and 5 µl of the 5 µM reverse index primer (N7: CAAGCAGAAGACGGCATACGAGAT (indexN7)GTCTCGTGGGCTCGG).

PCR was run for 8 cycles using the same thermal profile as above. Also, the PCR reaction clean-up, measurement on a Qubit fluorometer, and gel electrophoresis procedures were identical. All samples were of high quality. They were adjusted to the same 30 nM concentration, pooled, and sent to Novogene (Cambridge, UK) for sequencing on the Illumina NovaSeq machine (1.5 million of 250 bp paired-end reads).

Adaptors and low-quality bases were removed from sequence reads by cutadapt (Martin 2011). Forward and reverse reads were assembled by PEAR (Zhang et al. 2014) and only reads with 169 bp length were retained. Unique reads were collapsed and their frequency within an amplicon was used to distinguish error reads from true MHC alleles. We retained the first three to nine alleles per individual with frequency at least 1000, as we observed a clear bimodal distribution of coverage (Fig. 2). This straightforward filtering strategy was validated by comparison of genotypes between replicates that showed 100% repeatability. Population level analyses of nucleotide diversity, haplotype diversity and Pxy and Fst distances were done in DNA SP v 6 (Rozas et al. 2017). Significance of Pxy and Fst values was tested using 5000 permutations.

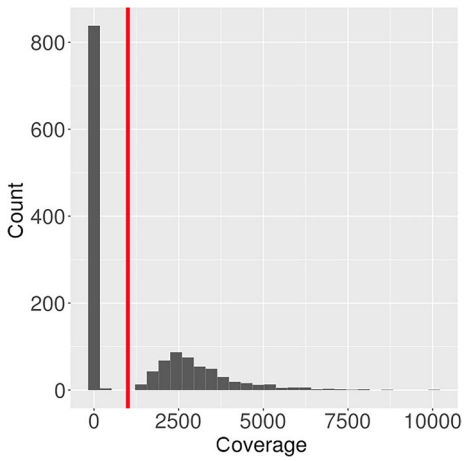


**Fig. 2** Distribution of coverage of unique DNA sequences per individual amplicon. Red line marks threshold used to distinguish between errors with low coverage and true alleles

### Statistical processing

The odour preference of females was based on a difference of the total time spent at the left and right T-maze arms. We used the t-test for independent samples and the Shapiro–Wilk test for normality to determine the voles' overall bias toward a particular side (Fig. 3). For comparison of two groups of offspring number and similarity indexes, we used one-way ANOVA and Tukey HSD post-hoc test in Statistica 13 (TIBCO Software Inc. 2017). Generalized linear models (GLM) were set up to evaluate the effect of odour preference, annual phase (season), body weight, and the parent origin on the number of offspring using R 3.6.3 software program (R Core Team 2020). Since the response variable are counts, the models were set up with a Poisson distribution. Different models were tested according to the AICc, ΔAICc and Akaike weights (Burnham and Anderson 2007). For calculation, we have used "AICcmodavg" package in R. Partial effects of selected predictors were tested using chi-square (likelihood-ratio) tests comparing full model with a model where tested predictor is omitted (in R: drop1(model, test = "Chisq"). To compare similarity of allele sets between parents, we used indexes according to Jaccard (Levandowsky and Winter 1971) and Sørensen–Dice (Ondov et al. 2016).

## Results

### Odour preference parameter in the T-maze

Evaluation of the odour preference was based on the total duration(s) of the difference between prevailing exposition to the right or left odour sources (Tables 1 and S1). Absolute difference between the left and right side in this parameter ranged from 7 to 118 s, preference of the left side was with the mean = 41 s, of the right side with the mean = 43 s, t-value = 0.193, p = 0.848. T-test does not show that there was a one-sided deviation. The distribution does not show deviation from the normal distribution, see Fig. 3.

### Relationship between the offspring number and the predictors

The best relationship was characterized by the following GLM: offspring ~ season + female choice + female weight (ΔAICc = 0, Table 2). The predictor season (a—spring, b—summer, c—autumn) had the highest influence (Chi-square tests, $p < 0.001$, Table 3). Vole parents had significantly higher number of offspring in spring than in summer or autumn ($F_{(2,36)} = 8.970$, $p < 0.001$, see Fig. 4). A female

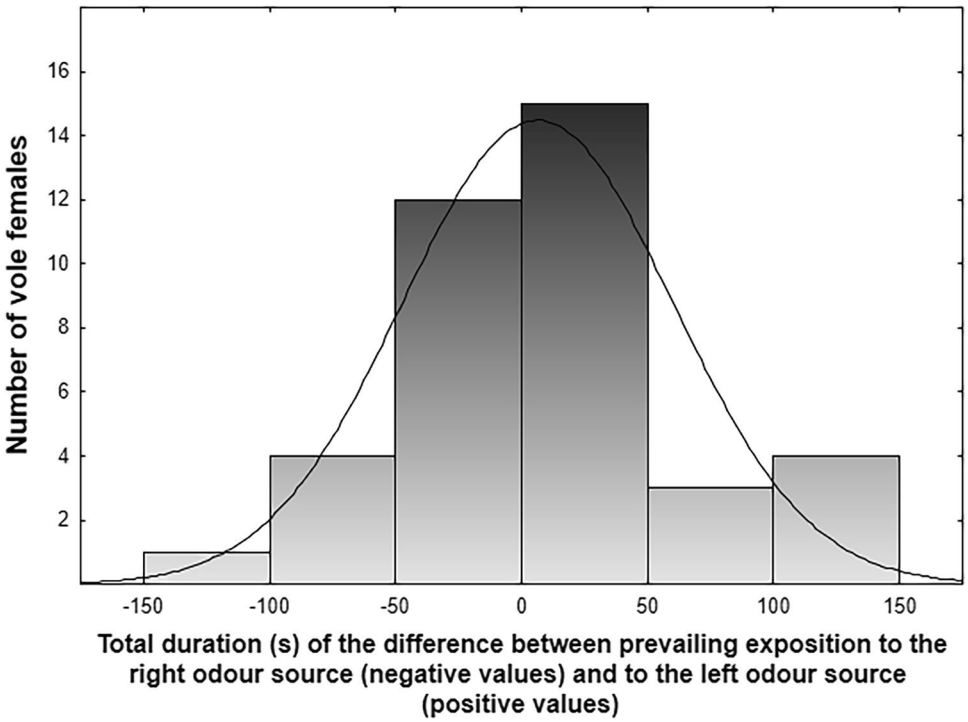


**Fig. 3** Histogram of the total duration of the difference between exposition to the odour on the right or left side of the T-maze. The graph shows on the y axis the number of vole females and on the x axis the difference (s) resulting in preference of the left side (positive values) and on the right side (negative value). Shapiro–Wilk test for normality W = 0.973, p = 0.465, expected normal curve is shown

**Table 2** Set of models (GLM) explaining offspring number by different combinations of predictor variables

| Response variable | Predictor variables | Δ AICc | K | wi |
|---|---|---|---|---|
| Offspring | ~ season + f_choice + f_weight | 0 | 5 | 0.42 |
| " | ~ season + f_choice | 0.47 | 4 | 0.33 |
| " | ~ season + f_choice + f_weight + parent_loc | 2.02 | 6 | 0.15 |
| " | ~ season | 3.86 | 3 | 0.06 |
| " | ~ season + f_choice + f_weight + parent_loc + m_weight | 4.95 | 7 | 0.04 |
| " | ~ f_choice*parent_loc | 10.22 | 4 | 0.00 |

Delta AICc, the number of estimable parameters (K) and Akaike weights (wi) are given

*Season* (spring, summer, autumn), *f_choice* female choice–male preferred or not preferred, *f_weight* female body weight, *parent_loc* pairs from the same or different localities, *m_weight* male body weight

with a preferred male (p), based on smell, had a significantly larger litter than with a not preferred male (n) (Chi-square tests, p = 0.018, Table 3). The difference between the mentioned groups was significant too ($F_{(1,37)} = 4.484$, p = 0.041, see Fig. 5a). Following this, female body weight no longer had a statistically significant effect (Chi-square tests, p = 0.078, Table 3). The GLM with season and female choice predictors (offspring ~ season + female choice, ΔAICc = 0.47, Table 2) was a weaker model, as well as, the GLM with season, female choice, female body weight and parent localities (offspring ~ season + female choice + f_weight + parent locality, ΔAICc = 2.02, Table 2) and the GLM with season only (offspring ~ season, ΔAICc = 3.86, Table 2). The models where the ΔAICc is less than 2 should be considered too, however, where the ΔAICc is more than 3 the models have less support (Burnham and Anderson 2007).

**Table 3** Detailed description of the top model as given in Table 2 (Δ AICc = 0) with coefficients and likelihood ratio test (LRT)

| Predictors | Coefficient estimate | Standard error | Predictors | Chi-squared | P |
|---|---|---|---|---|---|
| Intercept | 0.049 | 0.652 | | | |
| Season b | − 0.944 | 0.264 | Season | 19.678 | **< 0.001** |
| Season c | − 1.066 | 0.284 | | | |
| f_choice p | 0.484 | 0.209 | f_choice | 5.594 | **0.018** |
| f_weight | 0.060 | 0.034 | f_weight | 3.110 | 0.078 |

Bold font indicates statistical significance

*Season b* data from summer, *season c* data from autumn, *f_choice p* preferred male, *f_weight* female body weight

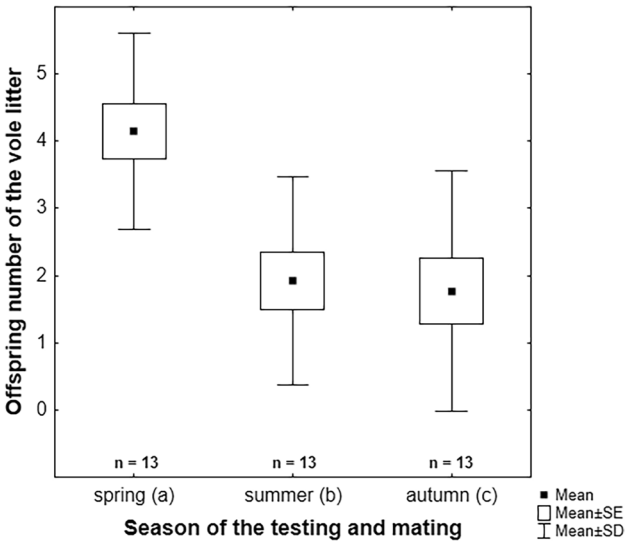


**Fig. 4** Comparison of the offspring number (mean ± SE) from three seasons. Thirteen pairs were tested in each season: spring (**a**) 4.15 ± 0.45, summer (**b**) 1.92 ± 0.45, autumn (**c**) 1.77 ± 0.45. Post-hoc test a, b: p = 0.003, a–c: p = 0.002, b, c: p = 0.968

Using GLM, the effect of female preference and parents' origin on the number of offspring was also assessed separately (ΔAICc = 10.22, Table 2). The interaction of levels female with preferred male (f_choice p) and parents from the same locality (parent_loc s) was not significant (p = 0.105). In another form of evaluation (ANOVA), if the not-preferred male came from the same locality as the female, this pair had not significantly larger litter than not-preferred male from another locality than the female ($F_{(1,16)} = 3.802$, p = 0.069).

## MHC allelic set

In the set of 45 individuals (analytical laboratory only a portion of samples has examined, see supplementary information) from two populations, we recovered 38 nucleotide haplotypes (alleles). Number of alleles per individual ranged from three to nine, indicating several paralogous loci being amplified. The majority of individuals carried 6 alleles and the extremes of three and nine alleles were rare (two and two individuals, respectively). Most alleles—18 alleles—were shared while 13 were unique to Veselí (V) and 7 unique to Budějovice (B). The unique alleles were low in frequency and the genetic differentiation between the populations estimated either by Fst (− 0.004) or Pxy (− 0.109) was statistically non-significant in both cases (p > 0.05). Total nucleotide diversity was 0.162 and was very similar for both populations. The same applies for haplotype diversity with values around 0.944 (Table 4).

Similarity of alleles in parental pairs according to the indexes of Sørensen–Dice and Jaccard are presented in Table S2. Their values ranged between 0.0 and 1.0 and were higher (more similar allele sets) in parents with preferred male (S–D: $F_{(1,20)} = 8.489$, p = 0.009, see Fig. 5b; Jaccard: $F_{(1,20)} = 6.563$, p = 0.019). The number of offspring and the mentioned indexes did not correlate (S–D: r = 0.09, p = 0.690; Jaccard: r = 0.07, p = 0.750).

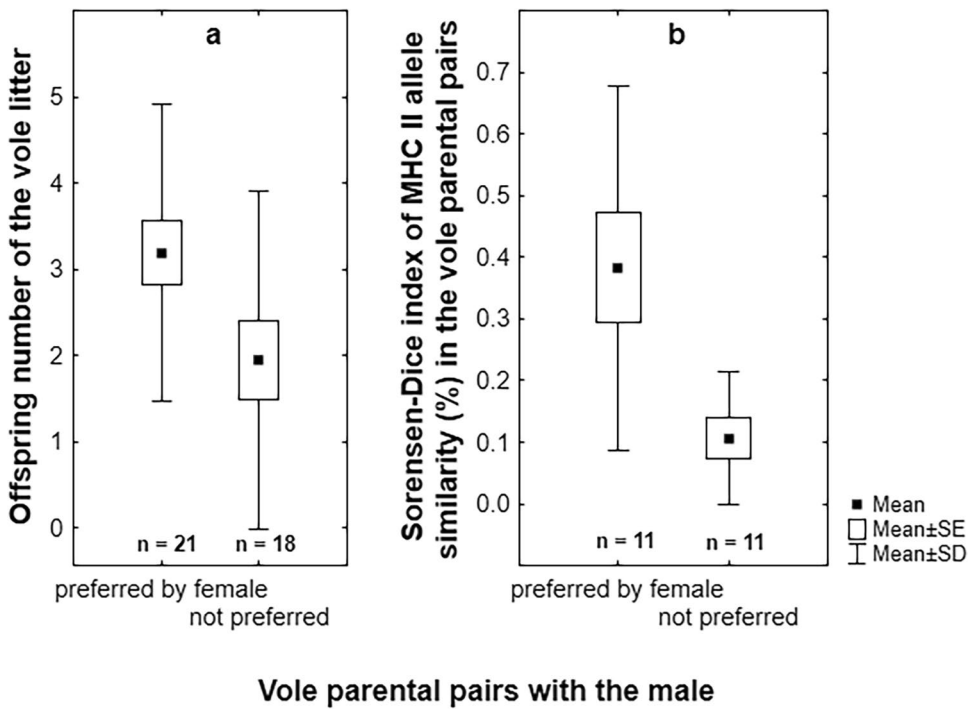


**Fig. 5** **a** Comparison of the offspring number from the pairs with a preferred (n = 21) and not-preferred male (n = 18). Mean ± SE in preferred: 3.19 ± 0.40, not-preferred: 1.94 ± 0.43. **b** Comparison of the Sørensen–Dice index of MHC II allele similarity from the pairs with a preferred (n = 11) and not-preferred male (n = 11). Mean ± SE in preferred: 0.38 ± 0.07, not-preferred: 0.11 ± 0.07

**Table 4** Comparison of the population level MHC statistics

| Statistical parameters | Locality Budějovice (B) | Locality Veselí (V) | Total |
|---|---|---|---|
| Number of individuals | 23 | 22 | 45 |
| Number of alleles | 24 | 32 | 38 |
| Number of polymorphic sites | 76 | 84 | 85 |
| Nucletide diversity JC corrected | 0.169 | 0.167 | 0.162 |
| Haplotype diversity | 0.923 | 0.953 | 0.944 |

*JC* Jukes-Cantor correction

## Discussion

### Behavioural testing of odour preference

The hypothesis, "a female with a preferred male according to his smell will have a greater number of offspring than with a non-preferred male", means both the offspring number increase with preferred male as well as, number decrease with not preferred male. In the first case, it is possible to talk about a genetically fixed behavioral strategy for choosing a partner for higher fitness. In the second case, the neuro-humoral system comes into play, which limits less perspective reproduction by perceived pheromones or stress stimuli. If we start with the idea that the goal of reproduction is to pass on one's own genes to subsequent generations, it is generally accepted that females may be inclined to select males whose genomes are closely aligned with their own (Thiessen et al. 1997; Jiang et al. 2013). However, it is also necessary to consider that in this way, more isolated populations may tend to reduce heterozygosity and could be threatened by inbreeding depression (Charlesworth and Willis 2009), if there was also no preference for differences primarily represented by different MHC and mediated by smell (Yamazaki et al. 1999). Our experiment of pairing females with an odour-preferred male resulted in a higher number of young, by about one pup. These results suggest that common vole females are able to choose a male with which she will have greater reproductive success based on odour preference. Conversely, females are also able to avoid, if possible, an unsuitable male with whom she may have lower fitness.

Partner preference has already been studied in several further small rodents, e.g., house mice (*Mus musculus*). In this polygynous species, higher litter numbers were found in pairs with preferred individuals (Drickamer et al. 2000). Also, in the monogamous California hamster (*Peromyscus californicus*), females with a preferred male produced litters faster and had higher reproductive success than females with a non-preferred male (Gleason et al. 2012). Similarly in mound-building mice (*Mus spicilegus*), pairs with preferred individuals based on behavioral similarity were more reproductively successful (Rangassamy et al. 2015). This species lives in monogamous pairs where the father helps raise the offspring. In such a social system, it is quite understandable that similarity in behavior is useful for reproduction. Despite the promiscuous common vole lives under completely distinct social conditions, higher parental behavioral similarity was also associated with increased reproductive success. (Urbánková et al. 2023).

When considering the proximate mechanism of the influence of female odour preference, it is necessary to consider both the positive effect of the preferred male, as well as the negative effect of the non-preferred male. An important part of the mechanisms of significant influence could be induced ovulation, which is convenient for random contact of partners at low population densities (Katandukila and Bennett 2016). Induced ovulation is apparently a general trait of voles of the genus *Microtus*. Proximity of a male behind mesh can lead to ovulation in females, and the exchange of the male behind the barrier can further promote the effect (Clulow and Mallory 1970; Milligan 1974). In the female vole, this neuro-hormonally controlled process could be supported by the positive perception of the male, but on the contrary also delayed or stopped by a negative odour stimulus. When testing sexual odour preference in females, it is important that they are in estrus. Since provoked ovulation should be considered here, proestrus was also included in the odour-sensitive state. In the females in the experiment, both the mentioned phases were observed. On the contrary, in the case of metestrus or even diestrus, some deviation of preference should be expected (Egid and Brown 1989).

Female rodents are very sensitive to reproductive odour communication and there are several interactions that need to be considered. The Vandenberg effect is a situation where chemo-signals from male mice accelerate the onset of puberty in females and influence the onset of ovulation (Vandenbergh 1973). The Lee-Boot effect is observable if females are placed in a larger group without the presence of a male, the estrous cycle is lengthened to the point of complete suppression of estrus (van der Lee and Boot 1955 in Kelliher and Wersinger 2009; Stopka et al. 2007). The Whitten effect is mentioned when a male is placed next to the females or they are exposed only to his smell, then they experience a shortening of the estrous cycle, induction and usually synchrony of estrus (Whitten 1958 in Bronson and Whitten 1968). Bruce effect is observed if a female after mating is exposed to a male other than the one, she mated with, or just his smell. Pregnancy is interrupted and within a week the female returns to the estrus phase (Parkes and Bruce 1961).

Physical contact with a foreign male can cause failure of the egg to implant in the uterus or after implantation by interrupting the development of the embryo. In contrast, the

physical presence of a known male, who may not even be the father, can act as a prevention of this blockage or disruption of pregnancy. However, the separation of such a male, who is not the father, can again disrupt the reproduction process (Bartoš et al. 2021). For the tested pairs, it could be applied in the sense that after copulation both preferred and non-preferred males were taken from the females after several days. Owing to induced ovulation, implantation could occur soon, but non-preferred males could have a negative effect, e.g. through implantation failure.

Behavioral-physiological mechanisms leading to infanticide must also be included in the possible proximate mechanisms that influence the number of young. They are widely distributed in the genus *Microtus* (Blumstein 2000). After comparing the two smells in the preference test, the experience of mating with a non-preferred (perhaps perceived as foreign) male could lead to increased concern for the young born (Breedveld et al. 2019). Females perceive familiar and new males crucially, they are forced to distinguish them not only because of the similarity of phenotype/genotype, preservation of MHC gene variation, but also because of the danger of infanticide from new foreign males (Heise and Lippke 1997; Eccard et al. 2018). Females could perceive a conflict between a preferred and then mated male during pairing. At this point, mechanisms involved in the prevention of infanticide, i.e. increased vigilance, locomotor activity and aggression could be activated. In addition, it is also probably accompanied by stress reaction.

As mentioned above, females are able to remember the odour of males and respond accordingly (Kelliher and Wersinger 2009). It is important for our study that the females became familiar with the odour of two males during the test and the female was subsequently paired with one of them. Thanks to the odour test, the females received information that there are several males, a higher population density, and can afford male selectivity. Subsequently the females meet a non-preferred male and try to avoid mating or minimize investment in the upcoming litter. Thus, in the case of a non-preferred male, mating likely took place differently than with a preferred male. When interacting with a non-preferred male, activation of the hypothalamic–pituitary–adrenal (HPA) axis and glucocorticoids, as well as the hypothalamic-pituitary (gonadal) axis (HPG) and sex steroids, especially testosterone could apply (Ryan et al. 2014). Specifically, it was found in the ground squirrel (*Urocitellus richardsonii*) that as the total level of cortisol increases, the number of young in the litter decreases, and similarly, as the level of testosterone increases, the size of the litter decreases too. On the other hand, the proportion of males in the litter increases with the level of bound cortisol.

The regulation of reproduction in voles is directly linked to the population dynamics. The number of offspring is influenced by the population density almost directly (neuro-hormonally) via food supply. Physical condition of the female and the perspectives of the litter is also a part of this regulation. This was shown in this study on the marginal effect of the predictor weight of the female. During artificial enlargement of the bank vole litter, the survival and fertility of the mothers decreased. Litter enlargement did not increase the number of pups weaned per mother and significantly reduced the size of pups weaned (Koivula et al 2003). A negative phenotypic (and genotypic) correlation between the number and size of offspring at birth was also found (Mappes and Koskela 2004).

## Ofspring numbers from field and laboratory conditions

Common vole females produce about four litters of 1–13 young each year, averaging 5.5 young (Reichstein 1957, 1960 ex Niethammer and Krapp 1982). In laboratory conditions, it is an average of 4.2 young per litter. The stated decrease in value is explained by less suitable rearing conditions and embryonic mortality (Reichstein 1964 ex Niethammer and Krapp 1982). These authors calculated the average value based on the number of pups born only. In our case, the average value for all pairs was 2.6 offspring per litter, respectively 3.5 offspring for all fertile females. This shift could only be due to the fact, that in our organized mating, roughly half of the pairs consisted of females with non-preferred males.

The period in which the animals were caught and tested had a more significant influence on the offspring number than female choice, pairing with preferred or non-preferred males. In the first test round in May–June, all pairs were reproductively successful with an average number of 4.2 young per litter. In the second test round, which took place in August, nine pairs were reproductively successful, and the average value was 2.8 young per litter. In the third test round, only seven pairs were successful, with an average of 3.3 young per litter. The observed trend is consistent with published data on the breeding intensity of the field vole in Central Europe during the growing season (Reichstein 1957, 1960, 1964 ex Niethammer and Krapp 1982; Tkadlec and Zejda 1998). It is not entirely clear whether the biological changes are controlled completely by the circadian endogenous rhythm, or whether the circannual endogenous rhythm is also involved. For a critical reassessment, see an inspiring overview of the issue by Kumar and Mishra (2018).

## Localities, genetic differences and similarities

To assess the influence of genetic differences on odour preference and reproductive success in the common vole, the tested individuals were captured in two locations, 30 km apart. It already follows from earlier findings that a 20 km

distance in the Central European landscape generates a different frequency of neutral microsatellite alleles (Rico et al. 2009). Despite the lack of genetic differentiation between the populations B and V in MHC, there were still 13 alleles unique to V and 7 unique to B population. Although the localities are not different from the population-genetic point of view, the mentioned allelic set difference between localities was probably important for the behavioural test, because the GLM with the predictor parental locality (same/different) was not significantly weaker than the best model without parental locality. Selection of only not-preferred males that came from the same locality as the female had only not significantly larger litters than not-preferred males from another locality than the female. This is likely to be evidence for an effect, albeit it rather weaker. Řičánková et al. (2007) showed that female common voles clearly prefer certain males, specifically known ones over unknown ones. In addition, they showed that females can be clearly agonistic against unknown males. This could very well correspond to the fact that non-preferred males from a location 30 km away represent very different individuals for females and mating with them is riskier.

The samples taken for MHC analysis yielded rather surprising results. It was quite clearly shown that preferred males had significantly higher allele composition similarity with females than non-preferred males. However, the studies published so far in this area show a prevalent disassortative pairing, i. e. a preference for a different MHC allelic composition (Penn et al. 2002; Radwan et al 2008). There is also a considerable number of studies that do not show straightforward maintenance of higher MHC allele variability, but a response to local pathogen load (Meléndez-Rosa et al. 2018) or a significant influence of genetic background, sexual differences, or early life experience (Jordan and Bruford 1998). Higher allele similarity, preferred by female voles, corresponds with behavioral personality similarity and correlated positively with offspring number (Urbánková et al. 2023). So, in the common vole, odour preference corresponds with behavioral preference, however, allele similarity was not related to offspring number. This is understandable because these genes are mainly involved in pathogen defense and not directly in reproduction. But the question remains whether adequate parameters of allelic similarity were chosen. The indices according to Sørensen–Dice and Jaccard, although originally derived for the assessment of biological/ecological communities, are also used in genetic analyzes (Levandowsky and Winter 1971; Ondov et al. 2016). The procedure chosen by us does not evaluate molecular similarity of individual alleles, but considers them as an indicator of complex odour similarity.

In conclusion, odour preference was driven by MHC similarity and subsequently litter size was influenced by preference. The relationship between the success of reproduction and alleles is not clear-cut. This could be influenced by the measure of similarity we used. The genes analysed are, of course, not directly involved in the reproduction or maybe the number of tested voles was too small.

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**Data availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on request.

**Code availability** Not applicable.

## Declarations

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.

**Ethical approval** The voles were bred and tested in accordance with the principles of animal welfare and guidelines of the Departmental Commission for Animal Protection of the Ministry of Education, Youth and Sports, permit number 7945/2010-30. After the experiments, the voles stayed in the laboratory and were used for further breeding and behavioural testing.

**Consent to participate** All authors agreed to participate in the study on the influence of behavioural traits on reproduction.

**Consent for publication** All authors agreed with the content and gave explicit consent to submit this paper.

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