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TANYA ZORENKO
tatjana.zorenko@lu.lv

LARISA PETRA KAIJA
larisakurr@gmail.com

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Inbreeding tolerance in two isolated populations of Harting's vole Microtus hartingi (Rodentia, Arvicolinae)

Tanya ZORENKO 🆔**, Larisa Petra KAIJA**[*] 🆔
Department of Zoology and Animal Ecology, University of Latvia, Riga LV, Latvia

Abstract: The adaptive effects of inbreeding are understudied, but accumulating evidence suggests that it plays a major adaptive role in speciation, as populations with a history of inbreeding are more prone to it. Differences in inbreeding tolerance in two populations of *Microtus hartingi* voles were studied through a series of experiments on reproductive behaviour and partner choice. Modelling an artificial polygyny via the formation of trios composed of a brother, sister, and an unrelated female has shown an uneven attitude towards inbreeding, which was closely linked to the reproduction strategy: voles of the Rhodopean population (RP) would reproduce communally with only a moderate decrease in reproduction success, while in monogamous *M. hartingi ankaraensis* voles from Central Anatolia (CAP), the reproductive success dropped almost to zero due to severe social stress resulting in heavily antagonistic behaviour between females; aggression levels rose with maturation. Studies of reproduction in pairs of relatives revealed the absence of inbreeding avoidance in the polygynous population (RP) and a sharp decrease in reproduction probability in the monogamous subspecies (CAP). Olfactory three-choice behavioural tests have proven that voles do not choose the smell randomly, juveniles do not have partner preferences, and adults choose the unrelated individual's odour. RP voles prefer to mate with kin first, whereas CAP voles mate with non-kin first. The differences in reproductive strategy might have evolved due to habitat fragmentation and restriction of natal dispersal in the RP which has been isolated from the ancient Anatolian *Microtus hartingi* population since the Pleistocene. The adaptation to communal reproduction via kin selection resulted in increased inbreeding tolerance.

Key words: *Microtus hartingi*, inbreeding tolerance, kin selection, polygyny, reproductive strategy, partner preference

1. Introduction

Studies in recent decades show that inbreeding avoidance strategies have evolved in many animal taxa, with natal migration and kin recognition being the most important (Lehmann et al., 2003). Consanguineous reproduction increases offspring homozygosity and promotes the expression of deleterious recessive alleles, which determines the negative consequences of inbreeding depression (Charlesworth and Charlesworth, 1987). Homozygosity, combined with a reduction in major histocompatibility complex (MHC) gene diversity, results in impaired immunity (Potts et al., 1994).

In nature, inbreeding is often unavoidable, which results in mechanisms that enable effective coping with reduced gene diversity (Tabadkani et al., 2012). Lineages that have undergone generations of consanguineous reproduction often show a decrease in the rate of inbreeding depression, as natural selection enhances the rapid 'purging' of deleterious alleles and increases inbreeding tolerance (Kristensen et al., 2005). In 'founder effects', inbreeding is a necessary stage in a population's

growth and development. Periodical inbreeding can help the population to become more resistant to environmental changes, which makes it an essential tool for evolution (Joly, 2010; Matute, 2013).

Evidence shows that inbreeding has a substantial positive impact on inclusive fitness, as it increases the representation of genes identical by origin (Kokko and Ots, 2006). An increase in gene diversity does not always bring additive genetic effects (Hughes et al., 2008). When migration opportunities are scarce and population density is high, inbreeding tolerance becomes beneficial, as it increases inclusive fitness by kin selection and enhances sociality levels in the population (Tabadkani et al., 2012). Inbreeding reduces the accumulation of harmful alleles in genomes through 'purging', while outbreeding induces it, sometimes causing reduced fitness or even the extinction of the population (Joly, 2010). According to Joly's hypothesis, inbreeding is the most important microevolution and speciation factor in the case of new species divergence via isolation from the ancestral population, as it develops positive recessive mutations. In

rare occasions, the synergy between bottleneck effects, founder effects and inbreeding can accelerate speciation (Matute, 2013; Golenischev et al., 2022).

Mammal species with limited natal dispersal are more prone to inbreeding; however, lowered offspring fitness can be compensated by kin selection (Kokko and Otts, 2006). The connection between inbreeding, migration and partner choice in rodents is not yet fully understood. Up to 30% of *Microtus* genus females remain philopatric after reaching maturity (Boonstra et al., 1987). Both in polygynous and monogamous arvicoline species, males are the dispersing sex, while females tend to be territorial. Males that are born late and become mature only by the end of the breeding season are more likely to stay in the nest (Boonstra et al., 2002). The opposite situation is also possible: males of *Microtus socialis* (Pallas, 1773), stay in the natal nest and form pairs with migrating females (Kasatkin et al., 1998). The higher the density of the population, the earlier young polygynous *Mynomes pennsylvanicus* (Ord, 1815) voles leave the natal deme; females migrate twice as often as males (Myllymäki, 1977). Philopatry is connected with the quality of the habitat, but also with the group structure. For example, *Mynomes townsendii*'s (Bachman, 1839) choice between migrating and helping depends on population density and intrasexual aggression (Lambin, 1994). In species exhibiting pronounced sociality, inbreeding rates are dramatically higher, which makes inbreeding both a cause and a consequence of the evolution of sociality (Tabadkani et al., 2012). It is known that oxytocin plays a role in kin recognition, parental care, and mate choice and has many social functions (Kendrick et. al, 1997). It is likely that epigenetic markers such as methylation or acetylation of histone oxytocin played a significant role in the evolution of regulation of oxytocin-vasopressin in animals, as they can be heritable (Harony-Nicolas et al., 2014; Wang et al., 2013). Olfactory memory can be an effective tool for kin recognition and inbreeding avoidance and is a basis of incest taboos (Penn and Potts., 1998; Wilson and Stevenson, 2006; Zorenko, 2013). The essential role of MHC (which determines body odour) in behaviour was studied on mice: specimens preferred the least genetically related partner (Wilson and Stevenson, 2006). Similar odours indicate similar genes, and olfactory recognition helps animals avoid inbreeding (Penn and Potts., 1998). Rates of incest avoidance in voles fluctuate, and attitude towards inbreeding correlates with reproductive strategy (Zorenko and Kaprale, 2003; Smorkatcheva, 2021). Thus, monogamous vole species such as *Mynomes ochrogaster* (Wagner, 1842) (McGuire and Getz, 1981), *My. pinetorum* (Le Conte, 1830) (Schadler, 1983) and *M. socialis* (Zorenko and Kaprale, 2003; Zorenko, 2013) tend to avoid inbreeding,

while polygynous species *My.pennsylvanicus* (Pugh and Tamarin, 1988), *My. montanus* (Peale, 1848) (Berger et al., 1997), *Lasiopodomys brandtii* (Radde, 1851) (Zorenko and Kaprale, 2003) and *Eolagurus luteus* (Eversmann, 1840) (Streltsov et al., 2022) have less pronounced incest-taboo. However, the Zaisan mole voles *Ellobius tancrei* (Blasius, 1884) are apparently exclusively monogamous, but their inbreeding avoidance mechanisms are weakened (Smorkatcheva, 2021).

Harting's vole *Microtus hartingi* (Barret-Hamilton, 1903) is an effective model object for studying incest taboo and inbreeding tolerance. The European and Asian populations diverged as they were isolated from each other by the Sea of Marmara and narrow Bosporus and Dardanelles straits in the Pleistocene and Holocene, as evidenced by the data on morphology, hybridization and behaviour (Yiğit et al., 2012; Zorenko et al., 2016). We studied *M. h. ankaraensis* from Central Anatolia and *M. hartingi* from the Rhodope Mountains. In previous studies, this population was sometimes marked as '*M. hartingi hartingi*'; however, its subspecies status has not been defined yet, and in the current research the form is marked as the Rhodopean population (Golenischev et al., 2022). Rhodopean voles have a strong tendency to communal reproduction, while *M. h. ankaraensis* voles are monogamous (Zorenko, 2022). Unlike the Central Asian subspecies (Çolak et al., 1998), European Harting's voles live in fragmented habitats (Markov and Dimitrov, 2010; Kryštufek et al., 2018), with limited dispersal possibilities, which might have led to high inbreeding tolerance, exhibited as the absence of inbreeding avoidance mechanisms and lower susceptibility to inbreeding depression. Previous research found evidence that Harting's vole could have gone through a 'bottleneck' in the Rhodopes (Golenishchev et al., 2022). However, the population adapted to living in adverse environments and maintained high reproduction rates. Our hypothesis is that communal reproduction rates in the population increase together with inbreeding tolerance rates. Inbreeding might have played an important role in the Rhodopean *Microtus hartingi* adaptation to lower migration rates in fragmented habitats. To compare the inbreeding tolerance levels, we used Harting's vole subspecies from Asia Minor, which preserved the initial reproductive strategy and lifestyle of this species.

The aim of the research was to study the inbreeding tolerance of *M. hartingi* under laboratory conditions. It includes the ability for olfactory kin recognition (i), reproduction of animals with different degrees of relatedness (ii), possibility of competition between females related or unrelated to a male in trios (iii), and the tendency of kin and non-kin females to reproduce and successfully wean offspring in trios (iv).

2. Materials and methods

2.1. Model object

M. hartingi laboratory population founders were sampled from two populations: the Rhodopean population (RP) (10 individuals, Eastern Rhodopes, Mandrica, Bulgaria: 41°41′N 26°12′′E) and the Central Anatolian population *Microtus hartingi ankaraensis* (CAP) (8 individuals, Kırşehir, Central Anatolia, Turkey: 39°08′N 34°09′E). Animals were sampled at different locations of each region, which made their initial inbreeding coefficients close to 0. Seven F5 males and 7 F5 females from CAP were obtained from the Zoological Institute of the Russian Academy of Sciences (Saint Petersburg, Russian Federation). The research was carried out on F2–F4 *RP* and F8–11CAP at the Ethology Laboratory, University of Latvia. In breeding of laboratory lineages, the maintenance of animal genetic variability is possible by using the 'random mating of unrelated individuals' technique. It is known that in F25 of laboratory mice, the inbreeding coefficient remains at 20% (The laboratory mouse, 2004). Six to ten founders are enough to maintain the wide spectrum of fitness-associated trait variability in the isolated population (Markova, 2020).

F15–F25 social voles exhibit high reproduction rates, and behaviour during mating or open field tests does not differ from F1 voles (Zorenko, 2013). Therefore, we assume that the difference in generations does not influence the results. Specimens were held in cages with sawdust used as litter and hay as nesting material. Their diet consisted of vegetables, apples, and a mixture of grain and hay, supplemented with grass during spring and summer. Experiments were conducted between March and December. During the year, the temperature on the premises was maintained at 20 ± 2 °C, and the light mode was 12 hours of light and 12 hours of darkness.

Experiment 1: Reproduction of related individuals in experimental pairs.

The study, based on data obtained in 2017–2018, invloved specimens from F1–F2 RP generations and F8 CAP generations. Juveniles were kept with their parents until reaching 30 days of age, after which one or two offspring were left with their parents, while the rest were placed together in a separate cage until 40 days of age. Afterwards, specimens were separated from their siblings for 2 more days. They were randomly assigned to one of four social conditions: with a sibling male littermate (SIBS, N = 20 CAP and N = 18 RP); with an unrelated sexually inexperienced male of the same age (NSIBS, N = 12 CAP and 10 RP); a daughter with a father (DF, N = 10 CAP and RP), adult male with young female (NDF, N = 5 CAP and 10 RP), a son with a mother (SM, N = 18 CAP and 13 RP) and young male with adult female (NSM, N = 5 CAP and 6 RP). The total experiment number was 137. Animal age

(mean and standard deviation): SIBS: 53 ± 7 days; NSIBS: females 59 ± 12 days; males 65 ± 10 days; DF: fathers 125 ± 15 days; daughters 51 ± 10 days; NDF: males 122 ± 26 days; females: 60 ± 10 days; SM: mothers 124 ± 20 days, sons 61 ± 11 days; NSM: females 128 ± 28 days; males 63 ± 15 days. Duration of the experiment was at least 60 days. After the end of the experiment, animals that had not reproduced were placed with unrelated individuals to test their fertility. The period between pair formation and first birth, the quantity of born and successfully weaned offspring were registered in pairs. Offspring mortality was noted until 20 days of age, but surviving pups were observed till the very end of the experiment.

Experiment 2: Polygynous reproduction and inbreeding.

The research, conducted in 2021, involved voles from F4–F5 RP generations and F11 generations. The test duration was 60 days; in case of late parturition, it was prolonged in order to gain data on offspring mortality. Juveniles were kept with their parents up to 30 days of age; afterwards they were put into separate cages (400 × 350 × 200mm) and kept there until reaching the age of 45–60 days. There were 10 RP and 10 CAP groups in the first treatment of the experiment: brother, sister, and nonkin female aged 60–70 (64.0 ± 1.82; mean, SE) days (older, O-groups). Difference between the age of sisters and non-kin was 0–3 days. In the second treatment, 8 CAP and 5 RP groups of animals aged 40– 52 (46.9 ± 1.39) days were formed (younger, Y-groups). The second period (45–60 days) can be connected with the possibility of inbreeding as it is when sexual maturation starts, which explains the choice for experimenting with two age groups. In the first three days of the experiment, the group formation was observed (three hours in the first day, then two hours a day), and the following behaviour was recorded: outbursts of ritualised aggression in form of squeaks and aggressive poses, avoidance of physical contact, nest building, and first huddling. If one of the females in the groups showed signs of pronounced aggression (chasing and biting) and the second female was unable to resist her and became severely weakened and wounded, it was removed from the group, marked as 'dead' and removed from the following analysis. If a male interest towards a female was observed, a swab was taken from the female to define the phase of the sexual cycle according to the method described by Zorenko and Skinderskaya (1996). Once the proestrus phase was noted, the behaviour of the animal was observed until mating. This helped to determine which female was the first to mate with the male. The following variables were checked: period between group formation and first parturition and litter size straight after parturition. The offspring was considered 'survived' after reaching 60 days of age. The motherhood was defined by maternal behaviour and lactation; offspring mortality rates were

checked daily in the first three days, then every third day. After the experiment, in the groups where pregnancy did not occur, voles were placed with unrelated individuals in order to evaluate reproductive activity. Females were marked by cutting off fur. Newborn pups were marked by nontoxic pigment with coloured markers ("Vivarii" Ltd "Evitas"). The mark lasted for five days until pups developed the coat. Peak offspring mortality occurred during the first days after birth, and this method made it able to detect accurately which litter exhibited higher mortality rates.

Experiment 3: Three-choice behavioural olfactory kin recognition test.

Three-choice behavioural test was used in the study. Male and female siblings were separated from their parents and kept apart from each other for 5–7 days before the test. A glass aquarium (700×360×400 mm) was used with all walls covered except the front. Three Petri plates were placed along the back wall: one filled with clean sawdust (control) and placed in the centre, and the other two filled with substrate from the inhabited cage, soaked in the urine of its inhabitant. Sawdust was taken from the cage of a kin and the cage of a nonkin individual of the opposite sex. The Petri plates were marked at the bottom with a sticker stating 'kin' or 'nonkin', which helped avoid accidental falsification of results. Each vole was placed in the aquarium for 5 min, with time recorded using a stopwatch. The quantity of approaches (direct olfactory contact with sawdust) to each plate and the duration of each approach were noted. For RP and CAP, 15 experiments were conducted with males and females at the age of 35–40 days, and 15 experiments with animals at the age of 53–58 days, totaling 120 specimens. Preference for related or unrelated individual's smell during living on parents' territory and during the onset of natal migration might differ. The first period (35–40 days; Y) is chosen due to its connection with age of sexual maturation, while the second (53–58 days; O) is when the first migrations occur.

Statistical data analysis

In R, a two-tailed Fisher's exact test was used to compare the proportions of pairs producing litters and successfully reared offspring over 60 days between SIBS and NSIBS, DF and NDF, and SM and NSM treatments as well as between datasets on aggression. Test value was marked as F. The arithmetic mean, the standard deviation, standard error and range were evaluated for litter size, offspring survival, and duration between the test onset and the first parturition in days. Continuous data on olfactory recognition were calculated for each specimen separately, including the total time spent at each Petri plate. To find out whether the selection of animal odour samples tested in the study differs from

the theoretically expected selection, a nonparametric χ^2 test (p = 0.05, df = 2) was performed in MS Excel. The graphical representation of the data was created in Python (matplotlib, numpy) and Adobe Photoshop.

3. Results

3.1. E1: Reproduction of related individuals in experimental pairs

Decrease in reproduction probability was observed in every type of CAP pairs in proportion of 2 (SIBS; F = 4.3; p < 0.001), 2.3 (SM; F = 3.33; p < 0.001), and 2.5 (DF; F = 3.24; p < 0.001) times to control pairs. On the contrary, in RP, reproduction rates were similar in inbred and control pairs (Table). In CAP and RP, parturition would happen later for SIBS compared to NSIBS. No differences were observed in DF and SM pairs compared to NDF and NSM pairs (Table). Over a span of 60 days, voles from both populations typically produced 1–2 litters. Two litters were gained in 5/20 NSIBS CAP pairs and 2/20 SIBS pairs (F = 1.28; p > 0.05; ND). In CAP, reproduction probability between relatives was significantly reduced in comparison to control pairs: NSIBS and SIBS (F = 4.30; p < 0.001); NDF and DF (F = 3.24; p < 0.001); NSM and SM (F = 3.33; p < 0.001), while RP did not exhibit any differences. Second litter appeared in RP more frequently than in CAP in every group type: NSIBS in 10/20 (F = 1.66; p-value < 0.05), SIBS in 3/20) (F = 0.48; p-value > 0.05; ND), SM in 2/13 (F = 1.92; p-value < 0.01) and DF in 4/13 (F = 2.80; p < 0.01) (Table). Litter size variation both in CAP and RP (Table) was significant only in RP SIBS/NSIBS (t = 3.24; p < 0.001) and RP DF/NDF (t = 2.24; p < 0.05). Mortality rates in inbred pairs of CAP were high; 47% of pups (61/129) did not survive, while in outbred pairs, the mortality of pups was only 11% (11/100) (F = 6.31; p < 0.001). On the contrary, offspring mortality in RP in both related and unrelated pairs was low: 8% (20/245) and 5% (9/191), respectively (F = 1.47; p > 0.05; ND). Results reflected substantial differences between RP and CAP reproduction probability in every experimental group (Table). CAP SM groups exhibited the sharpest decline. Offspring mortality appeared to be significantly lower in every CAP group except for SM.

E2: Polygynous reproduction and inbreeding: Female mortality.

Female antagonistic behaviour was observed in CAP groups at the very start of trio formation and expressed in the form of bites and chasing. Aggression levels rose during pregnancy or before parturition (Figure 1). We tried to prevent deaths by removing the weakened female, but in most cases, it died afterwards. Results show significant differences between aggression levels occurring in the first and second half of the experiment. In CAP, aggression led to the death of the female at the

Table. Parameters of reproduction of related individuals of two forms of *M. hartingi* in experimental pairs: *comparison of siblings SIBS and unrelated NSIBS; daughter-father DF/ nrelated with older males NDF, and son-mother SM/ unrelated with older females NSM; ** comparison of populations.

| Population	CAP	RP	CAP: RP
	SIBS		
Proportion of reproducing females in 60 days, %	10/20 (50)	20/20 (100)	*RP: ND *CAP: F = 4.3; p < 0.001 **CAP: RP: F = 4.97; p < 0.001
Number of pups in the first litter, $x \pm se$/range	3.9 ± 0.35 3–6	4.8 ± 0.47 2–9	*CAP: ND *RP: df = 20; t = 3.24; p < 0.001
Number of successfully reared pups in the first litter, $x \pm se$/ range	3.0 ± 0.39 1–5	4.5 ± 0.47 2–8	*CAP: ND *RP: df = 20; t = 3.35; p < 0.001
Period from pair formation to first birth, days, x ± se/range	51.4 ± 4.77 33–60	37.4 ± 2.78 23–50	*CAP: df = 20; t = 2.61; p < 0.05 *RP: df = 28; t =2.88; p < 0.05
Number of litters per female giving birth in 60 days, x ± se	1.2 ± 0.13	1.2 ± 0.08	*ND
Proportion of successfully reared pups in 60 days, %	30/39 (77)	100/106 (94)	*CAP:F = 4.3; p < 0.001 *RP: ND
	NSIBS		
Proportion of reproducing females in 60 days, %	12/12 (100)	10/10 (100)	** RP vs CAP F = 4.97; p < 0.01
Number of pups in the first litter, $x \pm se$/range	3.6 ± 0.38 2–5	5.5 ± 0.45 4–7	
Number of successfully reared pups in the first litter, $x \pm se$/range	3.1 ± 0.48 1–5	5.4 ± 0.48 3–7	
Period from pair formation to first birth, days, $x \pm se$/range	37.3 ± 2.51 25–53	25.9 ± 0.72 22–30	
Number of litters per female giving birth in 60 days, x ± se	1.2 ± 0.11	1.4 ± 0.16	
Proportion of successfully reared pups in 60 days, %	42/47 (89)	70/71 (97)	**F = 2.27; p < 0.01
	DF		
Proportion of reproducing females in 60 days , %	4/10 (40%)	13/13 (100)	*CAP: F = 3.24; p < 0.001 RP: ND
Number of pups in the first litter, $x \pm se$/range	4.2 ± 0.63 3–6	3.9 ± 0.50* 2–6	CAP: ND RP: df = 12; t = 2.24; p < 0.05
Number of successfully reared pups in the first litter, $x \pm se$/range	2.8 ± 0.95 0–4	3.6 ± 0.60* 1–6	CAP: ND RP: df = 12; t = 1.62; p > 0.05
Period from pair formation to first birth, days, $x \pm se$/range	39.2 ± 5.28 27–53	29.2 ± 1.24 22–35	CAP: ND RP: ND
Number of litters per female giving birth in 60 days, x ± se	1.0	1.4 ± 0.15	ND
Proportion of successfully reared pups in 60 days, %	11/17 (65)	58/59 (98)	*CAP: F = 2.72; p < 0.001 *RP: F = 1.73; p < 0.05
	NDF		
Proportion of reproducing females in 60 days, %	5/5 (100)	10/10 (100)	**F = 4.21; p < 0.01
Number of pups in the first litter, $x \pm se$/range	4.3 ± 0.51 3–6	5.4 ± 0.45 4–9	

Table (Continued).

Number of successfully reared pups in the first litter, $x \pm se$ /range	4.1 ± 0.37 3–5	4.9 ± 0.53 3–9	
Period from pair formation to first birth, days, $x \pm se$/range	29.2 ± 2.00 26–37	25.5 ± 2.13 22–34	
Number of litters per female giving birth in 60 days, x ± se	1.2 ± 0.20	1.6 ± 0.22	
Proportion of successfully reared pups in 60 days, %	23/24 (96)	73/79 (92)	**F = 3.67; p < 0.001
	SM		
Proportion of reproducing females in 60 days, %	8/18 (44)	13/13 (100)	*CAP: F = 3.33 p < 0.001
Number of pups in the first litter, $x \pm se$/range	3.8 ± 0.59 2–6	4.6 ± 0.51 2–7	*RP: df = 12; t = 0.86; p > 0.05
Number of successfully reared pups in the first litter, $x \pm se$/range	2.8 ± 0.41* 1–4	3.7 ± 0.57 0–7	*RP: df = 12; t = 1.67; p > 0.05
Period from pair formation to first birth, days, $x \pm se$/range	35.1 ± 2.36 26–42	35.5 ± 3,06 22–50	
Number of litters per female giving birth in 60 days, x ± se	1.0	1.2 ± 0.10	
Proportion of successfully reared pups in 60 days, %	22/30 (73)	67/80 (84)	*CAP: ND *RP: F = 2.00; p < 0.01 **ND
	NSM		
Proportion of reproducing females in 60 days, %	5/5 (100)	6/6 (100)	** F = 4.62; p < 0.01
Number of pups in the first litter, x ± se/range	4.4 ± 0.75 2–6	5.2 ± 0.48 4–7	
Number of successfully reared pups in the first litter, $x \pm se$/range	3.2 ± 0.37 2–4	5.0 ± 0.54 3–7	
Period from pair formation to first birth, days, $x \pm se$/range	28.8 ± 4.87 22–48	28.3 ± 2.54 24–40	
Number of litters per female giving birth in 60 days, x ± se	1.4 ± 0.24	1.3 ± 0.21	
Proportion of successfully reared pups in 60 days, %	24/29 (83)	39/41 (95)	** F = 1.69; p < 0.05

very beginning: 7/20 (35%) CAP-O and 4/16 (25%) CAP-Y were lost on 1.1 ± 0.14 and 1.2 ± 0.25 days after trio formation, respectively. Nonkin and kin were at equal risk of being killed: 4/10 (40%) nonkin and 3/10 (30%) kin females died in CAP-O and 2/8 (12.5%) kin and 2/8 (12.5%) nonkin females died in CAP-Y. Age did not influence the aggression. No females died in the second half of the experiment (CAP-O: F = 4.0; p < 0.001 and CAP-Y: F = 2.74; p < 0.01). In RP, the aggression levels at the beginning of the experiment were lower than in CAP: no specimens died in RP-Y (F = 2.96; p < 0.01), and only one RP-O nonkin female was killed on the second day after trio formation (F = 2.58; p < 0.01). In the second month of the experiment, RP-Y aggression was significantly higher than in CAP-Y, as in 2/5 trios, both females were lost on the 49th and 50th day, at the time the attacked females were about to give birth (4/10; 40% overall. F = 3.40; p < 0.001). In RP-O, one kin female was lost on the 50th day (2/20; 10% overall in RP-O). Age influenced aggression in RP (F = 3.06; p < 0.001).

E2. Polygynous reproduction and inbreeding: Mating order. Both females survived only in 3 CAP-O trios and in 3 CAP-Y trios, so the mating order was recorded in 6 cases. Overall, in both CAP age groups, the first mating with a nonkin female was 67% (4 out of 6) and with a sister was 33% (2 out of 6). Of the 9/10 RP groups in which both females survived, the mating order was recorded only in 7 cases, since mating did not occur in one group, and the copulation sequence was not recorded in the other group. The male first copulated with his sister in a 6/7 trio, and in a 1/7 trio with an unrelated female (F = 2.98; p < 0.001). In RP-Y groups, a male preferred mating first with a sister
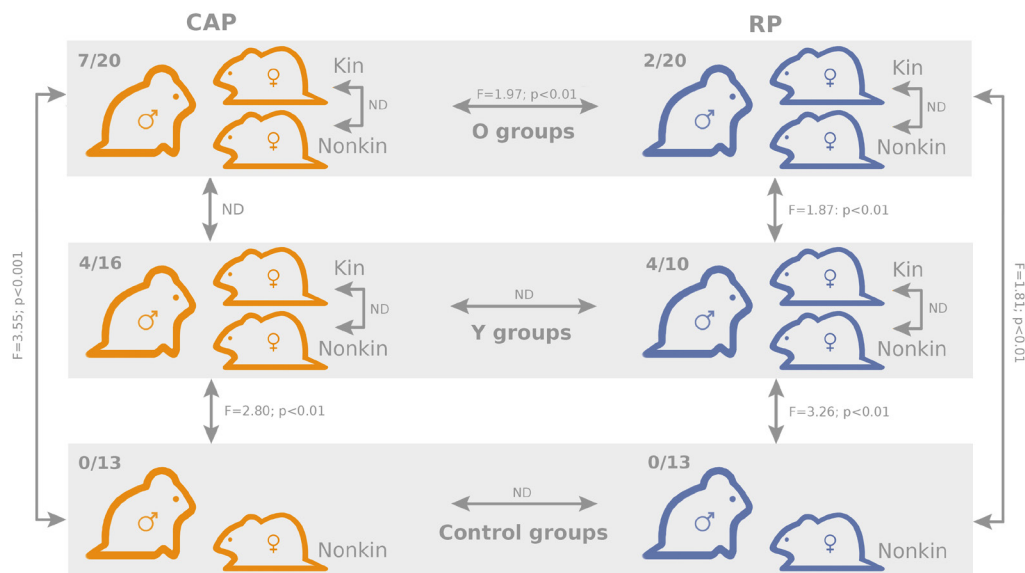
Figure 1. Differences in female aggressive behaviour with deadly outcome in polygynous groups in two populations of *M. hartingi* voles: O-groups (60–70 days of age), Y-groups (49–52 days of age) and control pairs. CAP analogous groups compared to RP; O-groups and Y-groups compared to each other and to control within populations; sisters compared to nonkin in all groups, by Fisher's exact test

in 3/5 cases, or with an unrelated female in 2/5 cases (F = 0.64; p > 0.05; ND). In general, in both age groups (7 and 5 cases), sisters mated first in 75% (9/12), and unrelated females in 25% (3/12) of cases (F = 1.66; p < 0.05). Thus, siblings preferred each other statistically three times more often than nonrelatives.

E2. Polygynous reproduction and inbreeding: Communal reproduction. The reproduction probability in polygynous trios reached 90% for CAP (9/10) and 90% (9/10) for RP in O-groups, while in Y-groups rates were 87.5% (7/8) and 100% (5/5), respectively. Communal breeding was observed in 2/10 CAP-O (20 %) and 6/10 RP-O (60%) trios, as well as in 3/8 CAP-Y (37.5%) and in 3/5 RP-Y (60%) trios. Offspring mortality was total in all CAP trios, except for a single pup of nonkin Y-group female. On the contrary, RP-O sisters successfully reared 24 out of 31 (77%) pups; it was 2.1 times as much as nonkin females (8 out of 22; 36%). Age positively affected reproductive success only in RP-O sisters (F = 5.68, p < 0.001). In RP-Y, no sister pups (0/9) and 17% (2/12) nonkin pups survived. Thus, offspring survival appeared to vary in O-groups by female type: sister (F = 5.42, p < 0.001); nonkin (F = 3.14, p < 0.001). Communally reproducing RP females delivered 1.3 litters in O-groups and 1 litter in Y-groups in 60 days, while all females of CAP had only one litter.

E2. Polygynous reproduction and inbreeding: Reproduction after the death of one female. In both CAP

age groups, female reproductive success increased after the removal of the competitor. The proportion of survived pups increased substantially compared to communally reproducing groups: Kin-O (F = 3.81); Nonkin-O (F = 4.86); Kin-Y (F = 4.51) and Nonkin-Y (F = 3.06) (all p < 0.001) (communally breeding Y/O kin/nonkin were compared accordingly to single females breeding after the removal of the competitor). In addition, offspring mortality was 30%–40%, and no difference in offspring survival was observed for kin and nonkin females. However, all sisters in CAP-O groups delivered only one litter in 60 days, while many unrelated females had two litters. In the Rhodopean population, there were few groups with only one reproducing female (both survived, but one was suppressed): 2 (O) and 2 (Y). In O-groups, the nonkin female weaned 4/7 pups, while sister managed to rear 5/5 pups. In Y-groups, kin and nonkin females gave birth to 2 offspring, who died in the very first days.

E3. Olfactory kin recognition. Specimens from both populations preferred the odours of conspecifics exclusively compared to clean sawdust (p < 0.001). Males and females sniffed clean sawdust for 0–4 s. Older voles at 55 days of age were more likely to approach the sawdust of nonkin individuals: CAP males and females (χ^2 = 11.2 and 6.40, p < 0.05 accordingly) and RP (males and females χ^2 = 19.6 and 6.6, p < 0.05) (Figure 2). Differences in approach duration are statistically significant for both sexes in CAP and in RP. Younger voles at the age of 35–40
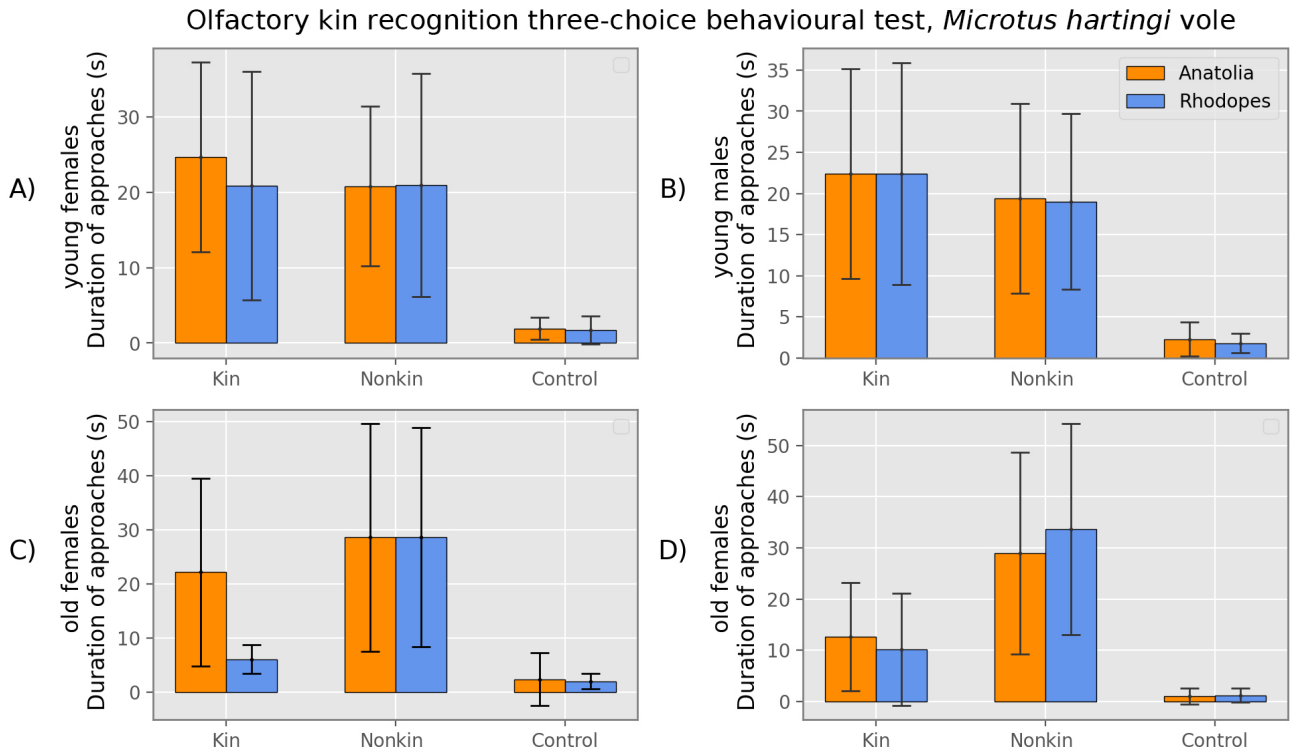
Figure 2. Odour choice between kin vs. nonkin individuals of the opposite sex in *Microtus hartingi* populations (%), regarding duration of approaches to odour samples in seconds. Females A) and males B) at the age of 35–40 days; females C) and males D) — at the age of 53–58 days.

days did not display any olfactory preferences for kin or nonkin: CAP (males χ^2 = 5.20 and females χ^2 = 2.80, p > 0.05) and RP (males χ^2 = 1.60 and females χ^2 = 0.42; p > 0.05) (Figure 2). However, young voles reacted to the odour differently. For example, 8/15 CAP males sniffed the odour of kin females substantially longer, and 6/15 CAP males examined the odour of nonkin females longer.

4. Discussion
Data analysis on inbreeding among voles from the subfamily Arvicolinae shows that most of the species tend to mate with close relatives in the laboratory. As a whole, inbreeding avoidance mechanisms are most pronounced in monogamous species (McGuire and Getz, 1981; Schadler, 1983; Bashenina et al., 1994; Zorenko and Kaprale, 2003), while high inbreeding tolerance is a feature of polygynous species (Pugh and Tamarin, 1988; Bashenina et al., 1994; Berger et al., 1997; Zorenko and Kaprale, 2003). *M. hartingi* from the Rhodopes exhibited high inbreeding tolerance. In this aspect, it is the only studied taxon among Arvicolinae with 100% effectively reproducing relatives of any kinship degree, including pairs of parents and offspring. It is the common characteristic in RP to form communal groups with successfully reproducing sisters (Zorenko, 2022). On

the contrary, in Asian *M. h. ankaraensis* (CAP) population, social monogamy seems to prevail in nature (Çolak et al., 2002). Representatives of this taxon react negatively to polygynous group formation in laboratory conditions (Zorenko, 2022). Despite the monogamous reproductive strategy of *M. h. ankaraensis*, the inbreeding avoidance mechanisms are weakened and successful reproduction is observed in sibling pairs, as well as in parent-offspring pairs. As a whole, CAP voles reproduce in inbred pairs in 45.8% of cases. In monogamous social vole *M. s. socialis*, inbreeding is recorded only in 9.4% cases (Zorenko and Kaprale, 2003; Zorenko, 2013). In this subspecies, pairs of voles tend to stay together for a while and have their own permanent burrows (Kasatkin et al., 1998). Territories of constant pairs do not overlap, and nests are actively protected. The territory might be temporarily inhabited by juveniles of one or two litters. In other social vole subspecies *M. s. goriensis*, consanguineous reproduction was observed 2.3 times more often in 21.3% of cases (Zorenko and Kaprale, 2003). In monogamous species, inbreeding between siblings is the most common, while mother-son reproduction is not observed at all in *M. s. socialis* and is rare in *M. s. goriensis*. However, in *M. h. ankaraensis*, it reaches 40%. In polygynous species,

the inbreeding between mothers and sons is rare in *L. brandtii* (18%) (Zorenko and Kaprale, 2003) and is not recorded in *M. rossiaemeridionalis* (Ognev, 1924) but often appears in both *M. hartingi* populations, especially in RP. Harting's voles in both populations are equally prone to breed both in mother-son and father-daughter pairs (CAP 40% and RP 100%). The probability of getting offspring from father and daughter is higher, for example, 42% in *M. rossiaemeridionalis* (Bashenina et al., 1994) and 46% in *L. brandtii* (Zorenko and Kaprale, 2003). The more pronounced the polygyny and promiscuity, the higher the possibility of consanguineous reproduction, and the less developed are the inbreeding avoidance mechanisms.

Inbreeding tolerance can depend on the possibility of natal dispersal. In Central Anatolia, where a monogamous population lives, the steppe is the most common biome, offering a wide range of opportunities for natal and reproductive dispersal (Çolak et al., 1998; Tabur et al., 2015). On the contrary, in the eastern Rhodopes, mountains make the habitats highly fragmented (Markov and Dimitrov, 2010; Kryštufek et al., 2018; observations of one of the authors). Restricted natal dispersal could be the trigger of changes in reproductive strategy (transition to communal reproduction) of RP voles. During the settlement in Europe, animals might have fallen into a mountain 'trap'. The heterogeneity of the habitats and isolation of RP resulted in the 'bottleneck effect' (Golenischev et al., 2022). The population managed to develop a new reproductive strategy—polygyny, together with group structure change and communal reproduction (Zorenko, 2022). On the contrary, the Asian ancestral CAP population kept being monogamous as it had unlimited dispersal possibilities. However, the negative effects, such as calcium metabolism disorders, thinning of the skull (Golenischev et al., 2022), and incisor (unpublished data) growth abnormalities are observed in RP voles more often. Philopatry is related not only to the availability and quality of the suitable habitat but also to the structure of the group: *My. townsendii*'s choice between migrating and helping on population density and levels of intrasexual aggression (Lambin, 1994). It is known that monogamous *My. ochrogaster* voles can form communal families of sisters (Hodges et al., 2002). The same situation was observed in Harting's voles in the study featuring experimental groups that consisted of similar trios (sister, brother, nonkin female), and of trios with two sisters or two unrelated females (Zorenko, 2022). In the RP, reproduction rates appeared to be in line with control groups, while in CAP, two unrelated females reproduced communally more likely than two sisters. Philopatry is most often the result of female altruism in polygynous species, as cobreeding of young females increase reproductive success, and thus can partially

correct the effects of inbreeding depression via inclusive fitness (Puurtinen, 2011). As this strategy aims to spread a genotype similar to one's kin, altruistic relationships are more likely to be formed between close relatives. It is known that in mandarin voles communal groups are not always formed through the philopatry of daughters alone, as nonkin females can successfully reproduce in the group too (Smorkatcheva, 1999; Gromov, 2001).

It is worth mentioning that the probability of reproduction in inbred pairs in monogamous CAP population did not drop to zero, indicating the plasticity of the species. Current research has shown that monogamous voles have different attitudes to mating with different types of relatives. The lowest reproduction rates are in mother-son groups, which points to inbreeding avoidance mechanisms. At the same time, the polygynous subspecies reproduced with any relatives of any type in 100% of cases. It is known that inbreeding mortality may depend on MHC genes: the smaller the distance in this variable in parents, the higher the embryonic death and mortality of the juveniles on the first-third) day after birth (Erofeeva et al., 2022). Selective reaction to olfactory signals is one of the inbreeding avoidance strategies. Kin recognition problem and the role of kinship degree receive much attention, which is reflected in many publications (McGuire and Getz, 1991; Potapov and Evsikov, 1994; Lambin and Mathers, 1997; Penn and Potts, 1999; Zorenko, 2013). Kin selection can be determined by genetic mechanisms, imprinting (Bekoff, 1981) and spatial relationship type. Imprinting in social voles occurs in the early postnatal ontogenesis. In *M. socialis*, distinction and preference for parental odour happens during eye opening, which correlates with cephalization index (Zorenko, 2013). At 20 days of age, voles start to manifest an interest in the odour of genetically related (nonparents) and unrelated individuals, but by 60 days of age, they prefer the latter. Olfactory experiments on Harting's voles show that in both populations, juvenile animals under 40 days are equally attracted to the odour of both siblings and unrelated partners (Figure 2). However, the strict preference for genetically nonrelated odour is formed after reaching 2 months of age. Experiments on the pairing of *M. socialis* siblings after 60 days of isolation displayed that consanguineous reproduction in *M. s. socialis* subspecies reduced by 40%, and in *M. s. goriensis* by 32% compared to nonkin (Zorenko, 2013), which implies the influence of genetic factors. It is proven that MHC plays a central role in mice behaviour (Penn and Potts, 1998). It might be presumed that Harting's voles choose the partner with an odour dissimilar from kin. The role of stress on group formation features was revealed in the current research. In CAP, pair formation and reproduction in trios consisting of a brother, sister,

and nonkin female was not successful. Communal reproduction was observed in one-third (28%) of all CAP groups, and almost all pups died before reaching maturity regardless of kinship degree (although unrelated females delivered thrice as many offspring as sisters). Communal reproduction in RP is significantly more frequent (60% of trios) and RP sisters have successfully weaned pups twice more than nonkin females (60% and 29% accordingly). This reverse dynamics points to inbreeding avoidance mechanisms in CAP and inbreeding preference in RP, as the same situation was reflected in male choice. In both CAP age groups, the first mating occurred twice as often (67%) with an unrelated female compared to a sister (33%). It is assumed that in nature, CAP sisters avoid mating with brothers, as they can choose another partner after dispersal and finding new territory. In RP, limited natal dispersion lowers the chances of meeting unknown mates. Males mate with equal frequency in groups composed of both related and unrelated females. Moreover, males mated with the sister first, and only after that approached the nonkin female, which indicates the benefits of consanguineous reproduction, as inbreeding may alternate with outbreeding, resulting in increased population fitness (Kokko and Ots, 2006). It is possible that for RP, it is beneficial to mate predominantly with relatives, as inbreeding in this isolated population became an advantage. Natural selection benefited the survival and reproduction of individuals with new adaptive traits (Joly, 2010), such as tendency to reproduce communally. Prolonged isolation of the population, coupled with the reinforcement of inbreeding tolerance, facilitated the formation of new coadapted gene complexes, which can be destroyed by outbreeding. When faced with a choice between mating with an unrelated and related partner, Rhodopean voles prioritize kinship over unrelated partners. During the early group formation, CAP females exhibit competitive behaviour marked by open aggression. The level of social stress increases together with aggression, a common trait observed in monogamous animals (Hayes et al., 2004).

Almost total offspring mortality was another result of social stress experienced by females in polygynous CAP trios. Aggression developed early in age, and was equally high in Y and O groups, reaching 25% and 35%, respectively. After the death of the competitor, the reproductive success of the remaining female increased substantially, and unrelated females had more litters than sisters. The reason for this might be that the consequences of social stress have a greater impact on sisters due to inbreeding avoidance mechanisms. In the other experiment on *M. hartingi* polygyny, aggression was rare in CAP trios with two nonkin females and did not occur in the trios with two sisters (Zorenko, 2022). The aggression of sisters may

possibly be explained as a reaction of kin group members to the arrival of a stranger, as in rodents, females tend to be territorial (Boonstra et al., 2002). Establishing a clear hierarchy in a group reduces stress (LeClair et al., 2021); therefore, open aggression indicates the inability of *M. h. ankaraensis* to adapt to artificial polygyny. Stress in females is known to cause behavioural abnormalities in their offspring due to hormone hypersecretion and changes in the hypothalamic-pituitary-adrenal system (Carter et al., 1995). This suggests that the Anatolian form of Harting's vole is not prone to communal reproduction due to social stress. A different situation was observed in the RP population. Female voles in RP show almost no aggression towards each other at the beginning of group formation; however, their competition intensifies during the reproductive phase. At the beginning of the experiment, one vole (3%) was killed, while during the reproduction phase rates increased to 17%. Initially low aggression levels made it possible for RP females to start communal or cooperative reproduction and offspring survival in communal trios remained high. In O-trios, sisters were twice as successful (77%) in rearing offspring as unrelated females (36%), which is another sign of inbreeding preference. The aggression during the reproductive phase was significantly more pronounced in younger RP animals, and, in both cases, resulted in the death of both females. The dynamics of female relationships in artificially created polygynous trios clearly illustrate how differences in the initial reproductive strategy in two populations affect the levels of aggression, stress, and reproductive success. Both populations of *M. hartingi* are genetically close and can interbreed, although a significant proportion of first- and second-generation males, as well as backcrosses, are sterile (Golenischev et al., 2022). Gene drift and inbreeding in RP might have contributed to the reproductive isolation (Matute, 2013). In the current research, kinship is proven to influence reproductive performance in the monogamous Anatolian population (CAP). In contrast, it has less effect on the Rhodopean population, which might be the evidence of a long inbreeding history due to isolation in nature. It is proven that inbreeding 'purges' harmful alleles, making the species stable even at a low level of genetic diversity (Crnokrak et al., 2002). When a certain genetic uniformity is achieved, the level of aggression directed at 'outsiders' decreases and communal reproduction becomes more frequent (Lacey and Sherman, 1997). Individuals of Harting's vole polygynous population may be genetically less diverse than monogamous voles due to the long history of inbreeding, which increases the chance of communal care of offspring regardless of the kinship degree. The results stated that in polygynous groups, the weaning success depended on whether females reproduce

separately or communally with all rates being lower for the latter case. The reason may be lactation loss due to stress. Many sources confirm that older females are often inclined to suppress younger females and that related females are more likely to suppress unrelated females (Dunbar, 1980). In our case, aggression in CAP affected all females equally, although unrelated females produced more pups. Genetic diversity in the natural Rhodopean population may be maintained by the migration of females instead of males due to increased population density in their natal deme. This is the second possible explanation for the reduced aggression level in the polygynous trios of RP, which, however, does not explain the mechanism for controlling social stress. The type of reproductive strategy is related to the number of dopamine receptors in *nucleus accumbens*, being higher in monogamous voles (Gingrich et al., 2000). It is known that inbreeding and social stress are connected with DNA methylation (Harony-Nicolas et al., 2014), so it is not unreasonable for this process to be directly related to social (maternal) behavioural disorders in inbred animals. We assume that inbreeding causes genetic and epigenetic changes in the dopamine system, which could theoretically turn the evolution of the species towards sociality. Social stress is directly related to the reward system of the brain, so its regulation can occur through changes in the system's functioning (Blanchard et al., 2001). However, our assumption has not yet been proven and is currently still a hypothesis. Thus, the level of sociality (pair living and communal breeding), the breeding strategy (polygyny and monogamy), and the natal dispersal (unlimited and limited) potentially are directly related to the level of inbreeding tolerance in *M. hartingi* vole species populations. The monogamous Anatolian population is less tolerant to inbreeding and communal reproduction, indicating the impossibility of forming such groups in nature. On the contrary, Rhodopean voles successfully reproduce communally and exhibit low levels of aggression and higher reproductive success in communal reproduction, which indicates the evolution of this population towards higher levels of sociality.

In Europe, Harting's vole range is composed of several fragments (Kryštufek et al., 2018), and one of them is in Eastern Rhodopes. The collected data allow to create the preliminary reconstruction of the early formation of the Rhodopean population (Golenischev et al., 2022). This population is isolated and lives in small patches separated from each other by mountain forests. Harting's vole entered southeastern Europe from Anatolia via a land corridor, beginning in the Middle Pleistocene (McHugh et al., 2008), but the separation of European forms of Thracia *M. hartingi* from Asian forms of Anatolia *M. hartingi* happened approximately ~0.73 Mya.

Available data suggest a reduction in steppes due to the formation of the Mediterranean mountain-forest complex in the Balkans (Okay and Okay, 2002), periods of increased genetic drift of the ancestral forms of Harting's voles and a population bottleneck (Golenischev et al., 2023). As a consequence of these processes, stress caused by the lack of vacant territories for the dispersal and inbreeding formed the specific population. This population not only avoided extinction under the influence of unfavourable conditions in Eastern Rhodopes but also developed a number of behavioural adaptations. In fragmented biotopes, the species developed the ability to reproduce communally with maintaining high reproductive skew (Zorenko, 2022), together with lowered aggressiveness and tolerance towards inbreeding with any type of relatives (current research). Consanguineous mating between siblings and other relatives in RP has occurred over many centuries and could have lowered the inbreeding depression by genetic purging of malevolent alleles (Lacy and Ballou, 1998). In insect species, inbreeding tolerance can be a preadapted trait for invasion success (Eyer et al., 2018). Isolation and enhanced level of inbreeding may have led to the formation of coadaptive gene complexes in the population, which can be disrupted by outbreeding with outsiders (recombination load), while inbreeding serves as the only strategy of maintaining interlocus allele associations (Joly, 2011).

It might be assumed that the invasion of voles in the European steppes happened in the area expansion process. The ancient vole form, just as CAP, was prone to limited inbreeding, which could serve as a preadaptation to new environments in Europe. Possibly, it is typical for other small rodent species with weakened inbreeding avoidance, such as *Ellobius tancrei* (Smorkatcheva, 2021).

Ethical implications
All applicable international, national, and/or institutional guidelines regarding the care and use of animals for investigations were adhered to. All the procedures performed in this study followed the European directive no. 2010/63/EU and Latvian regulations (Latvian Council of Science, 2018) resulting from this directive.

Conflicts of interest
The authors declare no conflicts of interest.

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