reinforcing sexual isolation[14], whereas negative assortative mating has been found in different strains within subspecies[15–17] reeinforcing outbreeding and thereby increasing heterozygosity.

In a twin-study[18], olfactory discrimination proved at least one of the mechanisms contributing to positive assortative mating in mole rats. Additional behavior such as vocal[6,19] and/or tactile[6] cues may complement olfaction, and they are currently being investigated in our laboratory.

In speciating mole rats[4,5] positive assortative mating together with other species-specific signals such as aggression patterns[20], may act as an important premating isolating mechanism. The latter presumably complement chromosomal incompatibility, thereby contributing to finalize the process of species formation.

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Olfactory Discrimination as an Isolating Mechanism in Speciating Mole Rats

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Summary. Olfactory discrimination was tested in two chromosome forms of the speciating fossorial rodent, *Spalax ehrenbergi*, in Israel. Females of the chromosome forms 2n = 52 and 2n = 58 were tested for male odour discrimination, the source of odour being either cage litter or urine. Estrous females of both forms preferred homochromosomal odours, whereas diestrous females showed no discrimination. These results suggest that olfactory discrimination may serve as a reproductive isolating mechanism in the speciation of mole rats.

Fossorial mole rats of the *Spalax ehrenbergi* superspecies complex in Israel involve four main chromosome forms (2n = 52, 54, 58, and 60) representing four closely related species at final stages of speciation[2–4]. The four karyotypes inhabit vast parapatric regions and are distributed clinally, from north to south Israel (see distribution map in [17]). Selective matings between the karyotypes serve as premating reproductive isolating machanisms providing species-specific recognition signals[5,6]. However, the nature and operation of the communication signals by which females discriminate between males have not hitherto been elucidated. The objective of the present study was to test olfactory discrimination as a potential mechanism in sexual preference and isolation between the karyotypes.

Materials and methods. Based on the results of previously conducted mating experiments[5] and female discrimination tests[6], this study involved both estrous and diestrous females of two karyotypes. Experimental animals were sampled at two northwestern Israeli populations: Ma'alot (2n = 52) and Kabri (2n = 58). These collecting sites lie, at the closest point, approximately 3 km apart, and just on opposite sides of the 300 m hybrid zone between the 2n = 52 and 2n = 58 karyotypes[4]. The sexually adult animals were live-trapped in the field during November–December 1974 and 1975, caged individually in the laboratory, and tested during the December 1975–January 1976 breeding season[7].

The testing apparatus was a two-choice olfactorium comprising a square perspex box (40 × 40 × 20 cm), with two short (11 cm) removable tunnels protruding on opposite sides. Six volt light bulbs and corresponding photocells were connected across the tunnels. The amount of time a test animal spent in each tunnel was automatically recorded. At the end of each tunnel there was a small odour stimulus receptacle, and odours diffused into the rest of the apparatus via a perforated perspex plate. When the test female was placed into the olfactorium, the odour stimuli were already in position, no stimulus-free period of adaptation being allowed prior to testing. Test length was standardized to 1 h. The initial orientation of homo- and heterochromosomal stimuli (i.e., of the same, or different chromosomal form as the tested female) was arbitrarily decided, but in order to eliminate possible bias due to a directional preference of the test females, runs were generally repeated in clean apparatus using fresh stimuli in the reversed positions. After each test the olfactorium was dismantled and thoroughly washed with hot water and detergent.

Estrus was artifically induced. About 44 h before testing, females were injected 0.1 mg estradiol benzoate (suspended in olive oil), followed by 0.8 mg progesterone 6–8 h before the run. Females normally came into estrus 42–48 h after the initial injection of estrogen. The high doses were found to be necessary for consistent induction of estrus. Estrous state was determined by taking samples of vaginal fluid prior to testing and assessing the relative amounts of leucocytes, epithelial cells and cornified epithelium present. Females were considered estrous when their vaginal smears included more than 80% cornified epithelial cells, and diestrous when the smears consisted primarily of leucocytes.

[1] Acknowledgments. Our thanks to M. Avrahami for field assistance, to I. Kornfield and S. Mendlinger for critically commenting on the manuscript, and to M. Haber for statistical advice. This project was supported in part by a Stiftung Volkswagenwerk, grant No. A 3: 11:1434, and an Israel National Academy of Science, grant No. 184 to E. Nevo.
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Olfactory discrimination by female mole rats belonging to two karyotypes of *Spalax ehrenbergi*[a]

Female's diploid No. and estrous state		No. of tested females	No. of tests	Time (min) per test, spent by female in odour receptacle				T[b]	P
				Homochromosomal		*Heterochromosomal*			
				Mean	S. E.	Mean	S. E.		
General odour									
58	E	10	20	17.95	2.43	6.42	2.35	53.5	< 0.03
52	E	6	12	15.95	3.13	1.93	0.65	13.0	< 0.025
58	D	8	11	7.90	3.53	13.25	3.95	41.5	n.s.
52	D	10	12	5.33	2.27	11.03	2.45	60.0	n.s.
Urine odour									
58	E	8	18	25.32	2.32	0.63	0.50	7.0	< 0.005
52	E	8	14	8.30	3.05	5.33	2.60	27.0	~ 0.06

[a] 2n = 58, Kabri population and 2 n = 52, Ma'alot population, tested either in the estrous (E) or diestrous (D) state. [b] T = The statistic obtained from a one-tailed Wilcoxon's matched-pairs signed-ranks test by comparing in each group the times that the tested female spent in homo as compared to heterochromosomal combination for every test.

Two sets of experiments were conducted. The first, *general odour* experiments, used wood shavings from males' cages as the stimulus. These were presumed to contain a complete cross-section of the male odour repertoire: urine, faeces, and possibly saliva and glandular exudates; for the second set, *urine odour* experiments, urine collected during handling males was absorbed into cotton. The analysis was based on the time spent by the tested female in homochromosomal and heterochromosomal tunnels for each experiment. As the primary interest was in the final discrimination by the female, only the last half hour of each test was analysed. Preference significance was examined by a one-tailed Wilcoxon's signed-ranks test[8].

Results and discussion. The results are given in the table. In the general odour set, estrous females from both populations show a similar strong homochromosomal preference, despite the rather small sample size from Ma'alot. By contrast, neither diestrous group showed such a preference, though there is a non-significant trend in the opposite direction. The urine odour tests were run only on estrous females and in the Kabri case the preference is strikingly significant, but it is only marginally so in Ma'alot.

Olfactory communication is of paramount importance in a variety of animals[9] including rodents[10]. In fossorial, blind mole-rats, chemical and auditory communication replace visual cues. The present experiments, in which auditory and tactile cues are excluded, indicate that odours serve in mole rats as sexual attractants. Furthermore, while this is certainly true in the general odour experiments (involving a mixture of odoriferous substances), the urine experiments suggest that the active compound or pheromone exists at least partly in the urine, and it appears to be species-specific. We therefore conclude that olfactory cues alone, without the operation of auditory or tactile cues, seem to provide effective guide for the sexual discrimination by females. Species-specific odours may differ due to both genetical and environmental factors[10]. However, since laboratory conditions and diet were identical for both karyotypes, genetic variation in the pheromones of the karyotypes is suggested. Chemical identification of the pheromones in the four karyotypes may elucidate some aspects related to the genetics of speciation and the origin of isolating mechanisms. Other stimuli besides olfaction may reinforce reproductive isolation among the karyotypes of *S. ehrenbergi*. Vocal signals are extensively used during the mating behaviour of mole rats[5], and both their physical patterns[11] and relative importance in female discrimination tests are currently being investigated in our laboratory.

Olfactory cues have been shown to act as reproductive isolating mechanisms in a variety of mammalian species including rodents such as *Clethrionomys*[12], *Peromyscus*[13,16], *Mus*[14] and *Gerbillus*[15]. Female discrimination tests conducted on karyotypically different males of *Spalax ehrenbergi*[6], suggested that both 2n = 52 and 2n = 58 females significantly ($p < 0.001$) selected homochromosomal males. The present experiments indicate that females can discriminate conspecific (or homochromosomal) males on the basis of odour, possibly urine pheromones. Since this olfactory discrimination ability is present only in estrous females, it probably plays a role in premating sexual isolation among the karyotypes.

Aggression patterns have also been suggested as a factor in speciation among three *Spalax ehrenbergi* chromosomal forms in Israel[17]. Levels of aggression are higher in heterogametic encounters than in homogametic ones, and they are also higher between contiguous (2n = 52–58) than between noncontiguous (2n = 52–60) karyotypes. Since all mole rat matings are initially aggressive[5], it is conceivable that olfaction may also function as a recognition trigger in aggressive interactions. Hence, olfaction may provide a major mechanism mediating both selective matings and aggression patterns, thereby reinforcing reproductive isolation at the final stages of speciation.

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