Short-Term Contact Elicits Heterospecific Behavioral Discrimination of Individual Odors in Mound-Building Mice (Mus spicilegus)

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The authors used a habituation–dishabituation procedure to test the ability of male mound-building mice (Mus spicilegus) to discriminate individual odors from males of another species of mouse. Male mound-building mice failed to spontaneously discriminate individual odors from Mus musculus males, a natural competitor. After 24-hr contact with a male of one of the M. musculus subspecies (M. m. musculus or M. m. domesticus), experienced M. spicilegus males discriminated the individual odors of unfamiliar males of the same subspecies. These results confirm that discrimination of individual chemosignals is not confined to olfactory cues of a single species and provide new information about the effect of short-term contact on discrimination of individual odors across species.

Many studies on communication in rodents have demonstrated the primordial role played by olfactory cues (for reviews see Brown, 1979; Doty, 1986; Halpin, 1986). The use of olfactory cues in communication often implies the discrimination of individual conspecific odors. Such ability has been demonstrated in several rodent genera (e.g., Mus: Bowers & Alexander, 1967; Meriones: Halpin, 1974; Tamias: Keevin, Halpin, & McCurdy, 1981; Microtus: Newman & Halpin, 1988; Stoddart, 1982; Mesocricetus: Johnston, Derzie, Chiang, Jernigan, & Lee, 1993; Phodopus: Johnston & Robinson, 1993; Spalax: Todrank & Heth, 1996; Tamiasciurus: Vaché, Ferron, & Gouat, 2001). Discrimination of individual odors from another species has been demonstrated in various species such as rats and mice (Beauchamp et al., 1985, 1995; Heth, Beauchamp, Nevo, & Yamazaki, 1996). Most studies, however, used learning tasks that did not reveal information about the spontaneous responses of animals. In fact, in an operant conditioning procedure, animals are highly motivated to respond to odors to obtain water or food reinforcement, whereas in a habituation–dishabituation procedure, subjects respond to stimuli that are important to them (Johnston et al., 1993; Schellinck & Brown, 1994). The habituation–dishabituation method requiring no external motivation or reward is, therefore, more ecologically valid and provides information on the importance of odors presented (Schellinck & Brown, 1994). Using a habituation–dishabituation procedure, Todrank and Heth (1996) demonstrated that two chromosomal and allopatric species of subterranean mole-rats, Spalax ehrenbergi, perform cross-species discrimination of individual odors. A similar result was found by Johnston and Robinson (1993) in two allopatric species of hamsters, Mesocricetus auratus and Phodopus campbelli. According to these authors, the ability to discriminate between individual scents is not confined to a single species’ chemosignals. They suggested that similar abilities probably exist in most species of mammals.

Feral mice have an accurate sense of smell as demonstrated by their ability to discriminate individuals of the same congenic strain (Penn & Potts, 1998). Moreover, chemical cues are clearly important in intraspecific communication (e.g., Hurst, 1989, 1990a, 1990b, 1990c). Given this background, we expected other Mus species to discriminate between odors of heterospecific individuals. A previous study showed, nevertheless, that male mound-building mice (Mus spicilegus) spontaneously discriminated individual odors from conspecifics but failed to discriminate individual odors from male house mice (Mus musculus domesticus; Gouat, Patris, & Lalande, 1998). Male M. spicilegus may be unable to perceive the differences between the chemosignals of M. m. domesticus because of a functional specificity of chemical cues (Tubbiola & Wysocki, 1997). This explanation seems rather improbable because species specificity is not absolute, at least in the peripheral olfactory pathway (Fiber, Adames, & Swann, 1993; Hildebrand & Shepherd, 1997), and the two species are genetically close (Bonhomme, 1992; Boursot, Aufray, Britton-Davidian, & Bonhomme, 1993). Gouat et al. (1998) suggested, therefore, that mound-building mice may be able to distinguish between the odors of two M. m. domesticus individuals but that the attractiveness of these heterospecific odors was too low to induce a behavioral discrimination. Our aim was thus to test whether behavioral discrimination between odors of heterospecific individuals might be elicited by using a more ecologically relevant heterospecific odor (i.e., the odor of a natural competitor) or by enhancing the reactivity of mound-building mice to a given heterospecific odor.

Method
To elicit behavioral discrimination of individual odorant signatures, we conducted two different experiments. In the first experiment, we acted on...
the donor species to arouse the interest of mound-building mice in the heterospecific odor. Some rodent species (voles: De Jonge, 1980; spiny mice: Friedman, Haim, & Zisapel, 1997; Haim & Rozenfeld, 1993) respond spontaneously to odors of their heterospecific competitors. Animals generally avoid places with the odor of a heterospecific competitor. According to these results, we used a natural competitor (Duryadi, 1993), Mus musculus musculus, as the donor species. M. m. domesticus is sympatric with M. spicilegus (Bonhomme, 1992; Orsini et al., 1983; Sage, Atchley, & Capanna, 1993) whereas M. m. domesticus is mainly allopatric with mound-building mice (Boursot et al., 1993; Krystufek & Macholan, 1998).

In the second experiment we tried to enhance the reactivity of mound-building mice to a heterospecific odor. Krasnov and Khokhlova (1996) demonstrated that Mus musculus mice avoid odors from their natural competitors, Meriones meridianus, only after contact with individuals of this species. We therefore allowed a heterospecific short-term contact to increase the reactivity of mound-building mice to the odors of the encountered species to promote a behavioral discrimination of their individual olfactory signatures.

**Strains and Breeding Conditions**

*M. spicilegus* males, strain ZYP, originated from a population in Yugooslavia (Pancevo) and were raised in captivity for at least 19 generations. The original population is sympatric with *M. m. musculus*. *M. m. domesticus* males, strain MDH, used as scent donors originated from a population in Denmark (Hov) and were raised in captivity for 15 generations. *M. m. domesticus* males, strain DDO, used as scent donors in Experiment 2 originated from a population in Denmark (Oddis) and were raised in captivity for 23 generations. At the beginning of the experiments animals were 3 to 8 months old.

Animals were maintained at 21 ± 3°C on a 14:10-hr reversed light-dark cycle. Mice were placed in individual polycarbonate cages (26 × 16 × 14 cm) 2 weeks before the beginning of the experiments to homogenize their social status (De Catanzaro & Gorzalca, 1979; Kimura & Hagiwara, 1984; Koyama, 1995). Prior to isolation, animals were housed in groups of brothers from weaning at 21 days of age (two to five mice per group). Food (mouse pellets, type A04, Usine d’Alimentation Rationelle, Epinay sur Orge, France) and water were provided ad libitum, and cotton was provided for nesting material.

**Habituation–Dishabituation Procedure**

A habituation–dishabituation procedure was used in both experiments according to a protocol derived from Johnston et al. (1993). An experimental mouse was presented with the scent from the same stimulus animal for five trials. On the sixth trial, the scent from a second mouse was presented. Trials lasted 5 min each and were separated by 2 min intervals. Tests were performed in the home cage of the experimental mouse during the first half of the dark phase. Two plastic dishes (3.5 cm diameter, 4 cm between the two dishes), fixed to a plastic plate (12 × 4 × .5 cm) so that the mice could not displace or overturn them, were placed in the cage of the experimental mouse opposite the nest. One dish was filled with soiled shavings (shavings with urine and droppings) collected from the cage of a scent donor, prior to each trial; the second dish was filled with clean shavings. The two dishes were removed and cleaned after each trial. The position of each type of stimulus (i.e., left or right) alternated between each trial, and the initial position of the dishes was designated at random for each test. The two donors were assigned to an experimental mouse at random following the rule that the three animals were unfamiliar and unrelated.

The time spent investigating the scent stimulus by the experimental mouse was measured during each trial. The decrease of investigation time between the first and the fifth trial indicated that a habituation process occurred. On the sixth trial, a significant increase in investigation time indicated that the experimental mouse perceived the change of the presented stimulus (i.e., scent of another donor).

**Experiment 1**

We tested the ability of male *M. spicilegus* to discriminate individual odors of male *M. m. musculus*. The experimental mound-building mice had never encountered any *M. m. musculus* males before testing (naive mice). Sixteen naive *M. spicilegus* males were tested with *M. m. musculus* odors (experimental group, *N* = 16). Eight *M. m. musculus* males were used as scent donors for the experimental group. Ten other naive mound-building mice, tested with homospecific odors, served as controls (control group, *N* = 10). The 10 mound-building mice of the control group were used as scent donors.

**Experiment 2**

We tested whether a short-term contact with a heterospecific male promotes the discrimination of individual odors by male *M. spicilegus*. Two groups of experienced animals were built up. In one group (*N* = 9), male mound-building mice had a short-term contact with a *M. m. musculus* male before being tested with male *M. m. musculus* odors. The other group (*N* = 7) had a short-term contact with a *M. m. domesticus* male before being tested with male *M. m. domesticus* odors. Eleven different *M. m. musculus* males and seven *M. m. domesticus* males were used as scent donors and animal stimuli. A control group (*N* = 8) had a short-term contact with a conspecific male before being tested with odors from male *M. m. musculus*.

Short-term contact consisted of a 24-hr limited encounter between an *M. spicilegus* male and an unfamiliar *M. m. musculus* subspecies male (experienced animals) or a conspecific male (control group). A transparent plastic box (16 × 18 × 8 cm) was fixed on the home cage of each mouse, creating a new zone with the home cage wired lid serving as its floor. The half-open lid allowed the mice to climb into this zone. Two circular windows (5 cm diameter) were cut on the side of the transparent box connected to the home cage of each mouse. Each window was closed by a wired netting (mesh: 5 × 5 mm). The two home cages were coupled and fixed to each other, permitting the two mice to interact through the wired windows (see Figure 1).

This system, compared with a direct dyadic encounter, is advantageous because it allows the mice to interact while it prevents highly agonistic interactions that may lead to injuries. Agonistic interactions, moreover, might increase aversive responses toward heterospecific odors (De Jonge, 1980). In this case, *M. spicilegus* might not investigate shavings soiled by the heterospecific mice.

Each mouse was first accustomed to the apparatus for a 24-hr period (cages were not coupled and were placed so that mice could not have any contact). After this period, home cages were coupled and contact was

Figure 1. Apparatus used for short-term contact.
possible. The setting in contact began at the onset of the nocturnal phase and lasted 24 hr.

Dyads of the experimental group were videorecorded during the nocturnal phase to check that mice did interact. Interactions essentially consisted of nose-to-nose sniffing, with the wire netting permitting no further investigations. The total time of nose-to-nose sniffing between the mice of the dyads was ($M \pm SE$) 115.1 ± 60.4 s for the $M. m. musculus$ group and 273 ± 143 s for the $M. m. domesticus$ group.

The discrimination test was carried out during the dark phase following the contact, using the habituation-dishabitation procedure. Donor males were different and unrelated to the mouse used for the short-term contact.

**Data Analysis**

We measured the time spent by an experimental mouse investigating each dish. The data collected were not normally distributed, and because of the small number of animals in each group, we used nonparametric statistics. For the same reasons, we used the median and the first and third quartiles in the figures and in the text. Tests were performed using exact statistics. For the same reasons, we used nonparametric procedures on StatXact for Windows (Mundry & Fischer, 1998).

Comparisons of the duration of investigation were made using the Wilcoxon matched-pairs signed-ranks test ($T$ statistic is given with the two-sided exact probability). To check the occurrence of a habituation process, we compared time spent investigating soiled shavings between the first and the fifth trial. To determine the effect of donor change, we compared the duration of investigation of soiled shavings between the fifth and the sixth trial. The attractiveness of the social odor was evaluated by comparing the duration of investigation between soiled and clean shavings on the first trial.

We used the Wilcoxon–Mann–Whitney test to compare results between experimental and control groups ($U$ statistic is given with the two-sided exact probability). The attractiveness of the odor stimulus was evaluated through the difference between the time spent investigating soiled and clean shavings during the first trial. The effect of donor change was compared through the difference in time spent investigating the soiled shavings between the sixth and the fifth trials.

**Results**

**Experiment 1: Naive Mice**

When presented with a conspecific odor (see Figure 2a, control group) on the first trial, $M. spicilegus$ males spent more time investigating soiled shavings than clean shavings ($T = 1, N = 10, p = .02$). Males habituated to the scent of the first donor, as was shown by the decrease in investigating time of soiled shavings between the first and the fifth trial ($T = 5, N = 10, p = .02$). On the sixth trial, $M. spicilegus$ mice detected the change of donor, with the increase in investigating time of soiled shavings being significant ($T = 3, N = 10, p = .01$).

When presented with a heterospecific odor on the first trial (see Figure 2b, experimental group), $M. spicilegus$ males did not spend more time investigating soiled shavings by a male $M. m. musculus$ than clean shavings ($T = 57, N = 16, p = .57$). The difference in duration of investigation between clean and soiled shavings on the first trial differed significantly between the experimental ($Mdn = 0.40$ s, first quartile = $−0.57$ s, third quartile = $1.23$ s) and the control ($Mdn = 2.87$ s, first quartile = $0.15$ s, third quartile = $6.13$ s) groups ($U = 37, N_1 = 16, N_2 = 10, p = .02$). A habituation process occurred between the first and the fifth trial ($T = 0, N = 16, p < .01$), but no increase was detected on the sixth trial ($T = 46, N = 16, p = .45$). The slight increase in the investigation time observed on the sixth trial (see Figure 2b) was mainly due to a single male who spent 17.00 s investigating soiled shavings. The median time spent investigating the novel scent by the 15 other males was 0.97 s (first–third quartiles = 0.25–2.04 s). The difference of duration of investigation of soiled shavings between the fifth and sixth trial differed significantly between the experimental ($Mdn = 0.02$ s, first quartile = $−0.40$ s, third quartile = $1.16$ s) and the control ($Mdn = 3.94$ s, first quartile = $1.72$ s, third quartile = $19.99$ s) groups ($U = 32, N_1 = 16, N_2 = 10, p = .01$).

**Experiment 2: Heterospecific Tests After a Short-Term Contact**

After a short-term contact with an $M. m. musculus$ male, experienced $M. spicilegus$ males (see Figure 3a) spent more time investigating soiled shavings from $M. m. musculus$ males than clean shavings on the first trial ($T = 1, N = 9, p = .01$). A habituation process occurred as shown by the decrease in duration of investigation of soiled shavings between the first and the fifth trial ($T = 0, N = 9, p < .01$). Experienced mice clearly detected the change of donor on the sixth trial and increased their investigating time of soiled shavings ($T = 0, N = 9, p < .01$).

$M. spicilegus$ males from the control group (short-term contact with a conspecific male) did not spend more time on the first trial investigating shavings soiled by a $M. m. musculus$ than clean shavings ($T = 7, N = 8, p = .15$; see Figure 3b). A habituation process occurred between the first and the fifth trial ($T = 0, N = 8, p = .01$), but no increase was detected on the sixth trial ($T = 8, N = 8, p = .20$). The difference of duration of investigation
between clean and soiled shavings on the first trial differed significantly between experienced (Mdn = 2.32 s, first quartile = 1.09 s, third quartile = 3.24 s) and control (Mdn = 0.85 s, first quartile = −0.14 s, third quartile = 1.72 s) animals (U = 14, N₁ = 9, N₂ = 8, p = .04). The difference of duration of investigation of soiled shavings between the fifth and sixth trial differed significantly between experienced (Mdn = 4.92 s, first quartile = 2.13 s, third quartile = 7.41 s) and control (Mdn = −0.18 s, first quartile = −0.32 s, third quartile = −0.08 s) animals (U = 9, N₁ = 9, N₂ = 8, p = .01).

After a short-term contact with an M. m. domesticus male, experienced M. spicilegus males (see Figure 4) did not spend more time investigating shavings soiled by a male M. m. domesticus than clean shavings on the first trial (T = 14, N = 7, p = 1.00). Nevertheless, experienced M. spicilegus males reacted to the odor of a male M. m. domesticus, but individuals differed in the way they responded. The time spent investigating soiled shavings differed, plus or minus, from the time spent investigating clean shavings by 50% (median) and 45–80% (first–third quartiles). A habituation process occurred, as shown by the decrease in investigating time of soiled shavings between the first and the fifth trial (T = 0, N = 7, p = .02). Experienced mice clearly detected the change of donor on the sixth trial and increased their investigating time of soiled shavings (T = 1, N = 7, p = .03).

Discussion

Male M. spicilegus clearly discriminated individual odors of two M. spicilegus males, as already shown by Gouat et al. (1998). On the first trial, males spent more time investigating soiled than clean shavings. Homospecific olfactory cues are relevant enough to induce a recognition process and a behavioral discrimination.

M. spicilegus males that had no experience with M. m. musculus, however, did not discriminate between individual odors from males of this subspecies. On the first trial, male M. spicilegus did not investigate soiled shavings more than clean shavings. Information conveyed by heterospecific scent does not seem important in this context to M. spicilegus males. A similar result was previously obtained with odors from M. m. domesticus males (Gouat et al., 1998). Using odors from the sympatric subspecies M. m. musculus, a natural competitor (Duryadi, 1993), did not improve behavioral cross-species discrimination of individual odors.

After heterospecific short-term contact with an M. m. musculus male, M. spicilegus clearly discriminated between individual odors from males of this subspecies. A short-term contact with an M. m. domesticus male elicited, in a similar way, the discrimination between individual odors from males of this subspecies. The behavioral discrimination of individual odors was coupled with an increase of attractiveness to the heterospecific social odor because M. spicilegus males that had a short-term contact with an M. m. musculus male investigated soiled shavings more than clean shavings on the first trial. M. spicilegus males that had a short-term contact with an M. m. domesticus male reacted to the first presentation of the heterospecific odor, but they avoided as well as preferred the soiled shavings. On the other hand, control mice, which had a conspecific contact, neither discriminated heterospecific individual odors nor showed any preference for or avoidance of soiled shavings on the first trial. Heterospecific short-term contact was responsible, therefore, for the interest manifested by male M. spicilegus toward heterospecific odors on the first trial (i.e., heterospecific odors became relevant for M. spicilegus and induced behavioral discrimination). Our results demonstrated that, after a short-term contact with a heterospecific male, male mound-building mice became able to discriminate between individual odors from males of the other species. This result confirms that individual discrimination of olfactory cues is not confined to a single species’ chemosignals but might occur even between allo-
The attachment of significance to a social odor because this structure (amygdala) after a highly agonistic social encounter (Martinez et al., 1998). In rats, the preoptic area regulating sexual behavior (Fiber et al., 1993; Stark et al., 1993). Although conspecific and heterospecific chemosignals involved in olfactory discrimination.

Social experience is also required to improve discrimination of olfactory signatures of close kin (Todrank, Heth, & Johnston, 1998, 1999). Adult hamsters (Mesocricetus auratus) discriminated only the individual scents of familiar siblings they were reared with until weaning at 30 days of age. Direct interactions were necessary, and long-lasting memories for social odors corresponded to a long-term social encounter. Social experience allowed hamsters to learn the subtle nuances between their littermates’ scents. In comparison with these studies on hamsters, male M. spicilegus did not have the opportunity to learn the individual characteristics of unknown donors. A relatively short-term contact was sufficient, therefore, to promote a behavioral discrimination, without the possibility of learning precise characteristics of familiar donors. By this, we do not imply that no learning phenomenon occurred during the short-term contact, but our results suggest that the mechanisms implicated in the two processes are different.

Neural structures involved in differential responsiveness according to species specificity have been studied in two species of hamsters, Mesocricetus auratus and Phodopus sungorus (Fiber et al., 1993). Although conspecific and heterospecific chemosignals stimulated a similar response in the main olfactory bulb and in the accessory olfactory bulb, only conspecific chemosignals evoked a response in three central areas (i.e., the medial nucleus of the amygdala, the bed nucleus of the stria terminalis, and the medial preoptic area) regulating sexual behavior (Fiber et al., 1993; Stark et al., 1998; Tubbilola & Wysocki, 1997; Wood & Coolen, 1997) and social behavior (Martinez, Phillips, & Herbert, 1998). In rats, c-fos expression increased in two of those central areas (the bed nucleus of the stria terminalis and the medial nucleus of the amygdala) after a highly agonistic social encounter (Martinez et al., 1998). Orbital cortex has been hypothesized to be involved in the attachment of significance to a social odor because this structure has been implicated in assigning or modulating valence to particular events, relationships, or odors (see review in Petruis, DeSouza, Schiller, & Johnston, 1998). Our results showed that short-term contact allowed M. spicilegus males to express their discriminative abilities. M. spicilegus may prove to be an invaluable biological model for the understanding of neural processes involved in olfactory discrimination.

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