The genus *Mus* as a model for evolutionary studies
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Male–female associations and female olfactory neurogenesis with pair bonding in *Mus spicilegus*

CLAUDE BAUDOIN\(^1\*,\) NICOLAS BUSQUET\(^1\), F. STEPHEN DOBSON\(^2\), GILLES GHEUSI\(^1\), CHRISTOPHE FERON\(^3\), JEAN-LUC DURAND\(^1\), GIORA HETH\(^3\), BRUNO PATRIS\(^1\) and JOSEPHINE TODRANK\(^3\)

\(^1\)Université Paris 13, CNRS UMR 7153 Laboratoire d’Ethologie Expérimentale et Comparée, 99 avenue J-B. Clément, F-93430 Villetaneuse, France
\(^2\)Auburn University, Department of Biological Sciences, Auburn, AL 36849, USA
\(^3\)University of Haifa, Institute of Evolution, Haifa 31905, Israel

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Mound-building mice *Mus spicilegus* exhibit life-history traits that are unique among the *Mus* species complex, such as the cooperative mound-building behaviour that gives the species its common name. In this and other socially coordinated activities, such as those associated with reproduction, these mice should be able to recognize individuals (via discrimination based on kinship, population and species) to mediate their interactions. Our previous studies have provided evidence of population and species recognition in *M. spicilegus*. The aims of the present study were: (i) to study associations of mice during their reproductive period (in outdoor enclosures), (ii) to investigate whether there is an influence of relatedness of females in these associations, (iii) to determine whether female *M. spicilegus* are able to make kin vs. non-kin discriminations, and (iv) to study certain neurobiological correlates of male–female bonds. Stable male–female associations were found in almost all the experimental groups, both those with unrelated and unfamiliar females and those with unfamiliar sisters. Kinship between females did not affect female associations in our enclosure experiment, but in our kin discrimination experiment females did distinguish between unfamiliar sisters and unfamiliar unrelated females. Shared kinship may not encourage cooperative rearing of pups but could enhance cooperation in building mounds where mice over-winter. Male–female associations could be based on a social bond, as hypothesized from previous laboratory experiments. This was substantiated in this study by increased olfactory bulbar neurogenesis in females that preferred (in two choice tests) their sexual partner 3 weeks after their first mating. Based on these results there is clear evidence to suggest that the mating system of *M. spicilegus* is social monogamy. © 2005 The Linnean Society of London, Biological Journal of the Linnean Society, 2005, 84, 323–334.


INTRODUCTION

Monogamous species are generally considered relatively rare (between 5% and 10% of mammalian species). They have been reported in a number of families in several orders: primates (Dixon, 1998), carnivores (Kleiman, 1997), ungulates (Komers, 1996), insectivores (Favre *et al.*, 1997; Rychlick, 1998) and rodents (Getz & Carter, 1980; Oliveras & Novak, 1986; Shapiro & Dewsbury, 1986; Shapiro *et al.*, 1986; Ribble, 1992; Carter & Getz, 1993; Sommer, 1996, 1997; Getz & McGuire, 1997). The *Mus* species group is a good biological model for comparative behavioural studies of species that exhibit different mating systems, for several differ-
ent reasons: (1) the phylogeny of this group is well known (Bonhomme et al., 1984; Boursot et al., 1993; Sage, Atchley & Capanna, 1993; Chevret, Veyrunes & Britton-Davidian, 2005, this issue; Tucker, Sandstedt & Lundrigan, 2005, this issue), (2) different species of Mus have been studied in various habitats, and (3) one species, the house mouse M. musculus, has been studied extensively and can be used as a reference (see Ganem et al., 2005, this issue).

The mating system of M. m. domesticus is generally described as polygynous (Crowcroft & Rowe, 1963; Bronson, 1979; Gerlach, 1990, 1996). Dominant males defend a breeding territory that includes females that form intrasexual associations during reproduction and mutual caring for the young in a communal nest (König, 1994; Dobson, Jacquot & Baudoin, 2000; Dobson & Baudoin, 2002). Cooperative breeding females are often close relatives (Wilkinson & Baker, 1988; Manning, Wakeland & Potts, 1992; Dobson et al., 2000). In M. m. musculus, a polygynous mating system also seems to be typical, but with important variations in space use depending on available resources (Krasnov & Khokhlova, 1986; Krasnov, 1988; Sokolov, Kotenkov & Michailenko, 1998).

Wild, non-commensal species of Mus have not been studied as extensively as has M. musculus. One of these species, the mound-building mouse M. spicilegus, exhibits remarkable adaptations to cold winters with a trait unique among Mus species: they construct over-wintering mounds from soil, plant parts, and food reserves (Naumov, 1940; Pisareva, 1948; Murariu, 1981; Orsini et al., 1983; Sokolov et al., 1998; Unterholzner, Willenig & Bauer, 2000). Yearling mice inside the mounds may originate from different litters, with different parents but with kin-related mothers (Garza et al., 1997). In natural populations, the sex ratio is female biased (Milishnikov, Rafiev & Muntianu, 1998), but it has been reported to be male biased during the spring–summer period (Simeonovska-Nikolova & Gerasimov, 2000). The mating system and spatial organization of this species are not clearly known. Several recent results from the laboratory suggest a socially monogamous mating system: the existence of a male–female pair bond (Patris & Baudoin, 1998), paternal behaviour and cooperation of parents in caring for their young (Patris & Baudoin, 2000), and a high level of aggression during encounters between unfamiliar mice, whether males or females (Patris et al., 2002; Simeonovska-Nikolova, 2003). The latter could indicate an exclusive use of space by bonded pairs in natural populations. Simeonovska-Nikolova & Gerasimov (2000) observed during summer in a field study in North Bulgaria that female home ranges were separated from each other, and Russian researchers found that pairs reproduced in enclosures, but they did not otherwise define group structure or spatial organization (Sokolov et al., 1998).

The purpose of the first part of our study (experiment 1) was to examine whether there were stable associations among M. spicilegus. Specifically we: (a) tested for the presence and temporal stability of associations, especially between males and females, in outdoor enclosures and (b) assessed whether kinship between females influenced the structure of such associations. According to our above-mentioned studies and to others on monogamous species (Kleiman, 1977; Wittenberger & Tilson, 1980; Dewsbury, 1981, 1987; Mock & Fujioka, 1990; Carter & Getz, 1993; Getz & McGuire, 1997), our expectations were to observe stable male–female associations, with no associations among male or among female M. spicilegus. If relatedness among females did influence the patterns of female social behaviour, as has been demonstrated in M. musculus, we would expect more associations among kin females than occur among unrelated females.

To be sure that female kin discrimination abilities would permit female social behaviour to be influenced by their relatedness, we examined the ability of M. spicilegus females to discriminate between sisters and unrelated females (experiment 2). Kin discrimination and kin recognition have been documented in various rodent species (ground squirrels: Holmes, 1986; hamsters: Heth, Todrank & Johnston, 1998, 1999; Todrank, Heth & Johnston, 1998; beavers: Sun & Muller-Schwarz, 1997), and the most salient signals of kinship in nocturnally active species like mice might be odours (Manning et al., 1992). Even though the biochemistry of such signals has not been yet analysed in M. spicilegus, we studied female discrimination abilities using unfamiliar odours of sisters and unrelated females. Our expectation was that M. spicilegus females would discriminate between these odours.

As recent studies in mice demonstrated a correlation between olfactory neurogenesis and olfactory learning (Rochefort et al., 2002), and as pair bonding in M. spicilegus involves a learning process (Patris & Baudoin, 1998), the purpose of the third part of our study (experiment 3) was to find neurobiological correlates to male–female pair bonding in M. spicilegus. Our expectation was to observe a correspondence between partner preference of females and olfactory neurogenesis. The existence of male–female associations and of an increase in olfactory neurogenesis linked to pair bonding would be important and complementary results in favour of social monogamy in M. spicilegus.
EXPERIMENT 1: STABLE ASSOCIATIONS DURING THE SEASON OF REPRODUCTION

MATERIAL AND METHODS

Animals and treatments
Seventy-two *M. spicilegus* (36 males, 36 females) were used in this study. All of them were of generation 15–20 (total breeding), bred in the Laboratoire d’Ethologie Expérimentale et Comparée (Villetaneuse), and originated from wild mice trapped in Yugoslavia by F. Bonhomme (CNRS UMR5171, Montpellier). Mice were paired in a manner that ensured avoidance of close inbreeding and pairs were always more distantly related than cousins. At the beginning of the study, mice were 5–10 months old and their body mass was 13.03 ± 1.37 g for females and 16.51 ± 1.79 g for males. None had any sexual experience. Each individual was identified with numbered metallic ear tags that were painted with coloured spots. The colours were assigned in a code that allowed us to recognize individual mice without handling. Mice were distributed among 14 outdoor enclosures in groups of two or three males and two or three females. Seven groups (enclosures H to N, Table 1) were comprised of unfamiliar and distantly related mice, termed ‘unrelated mice’ for convenience (the average kinship was second cousins), and seven groups (enclosures A to G, Table 1) had the same composition except that females were unfamiliar sisters from different litters. Treatments were assigned to enclosures in pairs, in a randomized block design (Dobson et al., 2000; Dobson & Baudoin, 2002). Before the experiment, mice were grouped with individuals of the same sex (two to five mice per cage) in standard polycarbonate cages (26 × 16 × 14 cm). In each enclosure, females originated from different cages and were unfamiliar to each other. During the experiment, some mice died from unknown causes during the first week (see enclosures C, E, I, L and N, Table 1).

Enclosures
Field enclosures were located in a deciduous forest at the Centre d’Etudes Biologiques de Chizé (CNRS), near Niort (Villiers-en-Bois, France). Each 8.8-m² cement-walled enclosure was divided into two equal-sized unroofed compartments, each 1.9 × 2.3 m. There was an opening (20 cm high and 22 cm wide) in the middle of the wall between the compartments. The cement walls were 1.2 m high, with a 20-cm band of smooth gypsum plaster around the top to prevent escape. Avian predators were excluded by a protective net of synthetic fibre that covered all the enclosures. The enclosures were built in two parallel rows of seven each, separated by about 6 m. Each compartment had a central metal box with food (standard lab chow) and a water bottle. Each compartment contained wooden nest boxes (24 × 12 × 10 cm), one for each mouse spaced roughly evenly along the walls. Thus, across the two compartments each enclosure had two food and water boxes (36 × 36 × 40 cm) and twice as many nest boxes as mice.

Observations
Mice were placed in the enclosures in June 1998. Enclosures were usually inspected twice daily, in the morning (between 09.00 h and 10.30 h) and afternoon (between 19.00 h and 20.30 h) in June and July. Because mice were active during daylight hours, the two inspections could be considered independent observations (Swihart & Slade, 1985). All the nest and food boxes were thoroughly inspected, and the locations of all the mice were recorded. Observations were also performed in June and July during the first 2 hours of the night, but because of the difficulty of recording representative social interactions, this part of the study was discontinued. During observation periods, mice were usually in the boxes. On the rare occasions when the experimenters entering the enclosures disturbed a mouse and it left a box, no observation was recorded for the mouse on that occasion. Occurrences when two or more mice were found together in a box (the same or different successive boxes) at least once per day during two successive days, we termed ‘associations’. When the same two or more individuals were found together during at least six successive days in the same or in different boxes, we termed this a ‘stable association’. The total number of days of observation per enclosure varied between 41 and 64 (Table 1). At the beginning of September the group composition was recorded and all the mice were removed from the enclosures.

RESULTS

In 13 of the 14 enclosures we observed stable associations between two or three mice, called ‘pairs’ and ‘trios’ (Table 1, column 3). In one enclosure (B) no stable association was observed. A total of 15 pairs was observed; 14 of them were male–female pairs and one was a male–male pair. Initial stable associations formed within 3–40 days (Table 1, column 5) with an average of approximately 20 days for male–female pairs. New male–female pairs appeared regularly during the first 41 days of observation (Fig. 1).

Trios were observed in four enclosures: in three cases one male and two females associated (enclosures A, C, N, Table 1) and in one case a female joined an male–male pair (enclosure I). Three of the four trios remained stable until the end of the observation period and one split into an male–female pair and an isolated female (enclosure N).
Table 1. Characteristics of associations in outdoor enclosures in *Mus spicilegus*

<table>
<thead>
<tr>
<th>Enclosure</th>
<th>No. mice at Day 0 (and at Day 7)</th>
<th>Types of stable associations</th>
<th>Isolated mice</th>
<th>Latency (days) of 1st stable association</th>
<th>Latency (days) to 1st litter born from 1st stable association</th>
<th>Duration (days) of stable association</th>
<th>Total days of observation</th>
<th>Group composition after the period of observation (Day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3M + 3F (3M + 3F)</td>
<td>1 MF pair (a), then 1 trio (a + 1F)</td>
<td>1 M</td>
<td>4 (a)</td>
<td>&gt; 60 (pregnant F) at D80</td>
<td>10 (a)</td>
<td>41</td>
<td>(D80) 1 MF pair (F pregnant) + 1M + 1F</td>
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<tr>
<td></td>
<td></td>
<td>+ 1 MF pair (b)</td>
<td></td>
<td>18 (trio)</td>
<td>22 (trio)*</td>
<td>18 (b)*</td>
<td></td>
<td></td>
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<tr>
<td>B</td>
<td>(3M + 3F)</td>
<td></td>
<td>3 M + 3F</td>
<td></td>
<td>No stable association (pups at D27)</td>
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<tr>
<td>C</td>
<td>3M + 3F (2 M + 3F)</td>
<td>1 MF pair</td>
<td>0</td>
<td>21 (pair)</td>
<td>&gt; 40</td>
<td>13 (pair)</td>
<td>64</td>
<td>(D103) 1 MF pair + 7 juveniles</td>
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<tr>
<td></td>
<td></td>
<td>+ 1 trio</td>
<td></td>
<td>23 (trio)</td>
<td></td>
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<tr>
<td>D</td>
<td>2 M + 2F (2 M + 2F)</td>
<td>1 MF pair</td>
<td>1 M</td>
<td>21</td>
<td>39</td>
<td>43*</td>
<td>64</td>
<td>(D102) 1 MF pair + 4 young</td>
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<td>E</td>
<td>3M + 3F (3M + 1F)</td>
<td>1MF pair</td>
<td>2 M</td>
<td>22</td>
<td>35</td>
<td>45*</td>
<td>64</td>
<td>(D103) 1 MF pair + 10 juveniles</td>
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<tr>
<td>F</td>
<td>2 M + 2F (2 M + 2F)</td>
<td>1 MF pair</td>
<td>1 M + 1F</td>
<td>3</td>
<td>24</td>
<td>24*</td>
<td>41</td>
<td>(D80) 1 M</td>
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<tr>
<td>G</td>
<td>2 M + 2F (2 M + 2F)</td>
<td>2 MF pairs (a, b)</td>
<td>0</td>
<td>1 (a)</td>
<td>&gt; 60</td>
<td>39 (a)*</td>
<td>42</td>
<td>(D80) 1 MF pair + 6 young + 1F</td>
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<td>30 (b)</td>
<td></td>
<td>10 (b)*</td>
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<td>H</td>
<td>(2 M + 2F)</td>
<td></td>
<td>2 M + 2F</td>
<td>22</td>
<td>15</td>
<td>26*</td>
<td>41</td>
<td>(D80) 1 MF pair (Pregnant F) + 3 juveniles</td>
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<tr>
<td>I</td>
<td>3M + 3F (3M + 3F)</td>
<td>1 MF pair</td>
<td>1 F</td>
<td>3 (pair)</td>
<td>&gt; 60</td>
<td>12 (pair)</td>
<td>64</td>
<td>(D103) 1 trio (2 M 1 F) + 6 young</td>
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<td></td>
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<td></td>
<td>23 (trio)</td>
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<td>40(trio)*</td>
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<tr>
<td>J</td>
<td>2 M + 2F (2 M + 2F)</td>
<td>1 MF pair</td>
<td>1 M + 1F</td>
<td>17</td>
<td>23</td>
<td>33*</td>
<td>56</td>
<td>(D93) 1 MF pair + 6 juveniles</td>
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<tr>
<td>K</td>
<td>2 M + 2F (2 M + 2F)</td>
<td>2 MF pairs (a, b)</td>
<td>0</td>
<td>11 (a)</td>
<td>&gt; 55</td>
<td>12</td>
<td>51</td>
<td>(D90) 1 MF pair + 7 juveniles</td>
</tr>
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<td></td>
<td></td>
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<td></td>
<td>12 (b)</td>
<td></td>
<td>15 (b)</td>
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<tr>
<td>L</td>
<td>(2 M + 2F)</td>
<td></td>
<td>1 M + 1F</td>
<td>40</td>
<td>10</td>
<td>28*</td>
<td>41</td>
<td>(D80) 1 MF pair (Pregnant F)</td>
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<tr>
<td>M</td>
<td>2 M + 2F (2 M + 2F)</td>
<td>1 MF pair</td>
<td>1 M + 1F</td>
<td>14</td>
<td>10</td>
<td>28*</td>
<td>41</td>
<td>(D80) 1 MF pair (Pregnant F)</td>
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<tr>
<td>N</td>
<td>3M + 3F (1 M + 2 F)</td>
<td>1 trio, then 1 MF pair</td>
<td>1 F</td>
<td>11</td>
<td>No pups</td>
<td>21 (trio)</td>
<td>64</td>
<td>(D101) 1 MF pair</td>
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</table>

Stable association (column 3): two (pair) or three (trio) mice were observed at least once per day during at least 6 successive days.

Duration of stable association (column 7): total number of days with mice sharing a box for pairs and trios (* = end of the period of observation).

Enclosures A to G: all the females in a particular enclosure were unfamiliar sisters on Day 0 (first day in enclosure); all the males were unfamiliar and unrelated.

Enclosures H to N: all the mice were unrelated and unfamiliar on Day 0.
The duration of male–female pairing varied from 10 to 45 days during the period of observation (Table 1, column 7) and 12 male–female pairs were still observed in September at the end of the study, in several cases around 80 days after pair formation. Only one trio with two males and one female was observed at that time (Table 1, column 9).

When two pairs or one pair and a trio were observed in the same enclosure at the same time (enclosures A, C, G, K), there was a clear separation between the groups, with each group occupying half of the enclosure. At the beginning of September only one male–female pair remained in each enclosure, in two cases (A, G) with isolated adults.

Reproduction occurred in 13 enclosures. In one enclosure (B) young mice were observed in a nest on Day 27 without any stable association. In another enclosure with a trio transformed into an male–female pair (enclosure N), no young were observed. The latency between the first stable association and the first litter produced (Table 1, column 6) was shorter in the enclosures with only one male–female pair (10–39 days in five out of six cases) than it was in enclosures with two male–female pairs or one male–female pair and one trio (> 40 days in all five cases). There was only one case where a trio (with two males) was found with young at the end of the study.

Close relatedness among female mice did not influence the number of pairs and trios in association, or reproduction (occurrence and latency) in the enclosures. Females did not cooperate in caring for young, but direct observations of male behaviour showed that males cared for young with the paired female, inside the communal nest and outside the nest when chasing intruders.

**DISCUSSION**

Laboratory studies on *M. spicilegus* have revealed behaviour that suggests a monogamous mating sys-

**EXPERIMENT 2: TEST OF FEMALE KIN DISCRIMINATION**

**INTRODUCTION**

The aim of our second experiment was to assess whether *M. spicilegus* females discriminate between unfamiliar kin and non-kin on the basis of individual odours. Working on whole body odours of laboratory strains of wild mice is especially relevant as rodents have to deal with such signals in the wild, and it seems reasonable to investigate the animals’ perception and categorization of complex individual odours.
We measured differences in the time females spent investigating an unfamiliar sister’s odour and a simultaneously presented odour of an unfamiliar and unrelated female. Differential treatment of an unfamiliar female’s odours based on kinship would demonstrate kin discrimination through chemical information. Our prediction was that females would investigate their sister’s odour less compared with the other odour, as observed in previous studies with odours of close and distant species and populations (Heth & Todrank, 2000; Heth et al., 2001).

MATERIAL AND METHODS

Animals
We tested 30 virgin 3–6-month-old female mice. Prior to testing, these mice lived in cages with same-sex littermates and they had not been used in other experiments. Ten females served as subjects, and 20 others served as odour donors. The two test odours were: (1) the odour of a sister from a subsequent litter (therefore unfamiliar to the subject), and (2) the odour of a same-age (± 1 week) unrelated and unfamiliar female. Tests were conducted when subjects and odour donors were in diestrus, as determined by vaginal closure (Féron & Gheusi, 2003).

Methods
Because environmental factors (diet: Brown & Schellinck, 1996; Ferkin et al., 1997; social status: Drickamer, 1992; health: Penn et al., 1998; Willis & Poulin, 2000) can affect odour qualities, these factors were controlled in this experiment. All animals were raised in the same standardized laboratory conditions. Subjects were tested in a clean polycarbonate cage covered with a clean glass plate. They were transferred to the test cage 5 min prior to the test to allow familiarization with the new area. Two stimuli were prepared during this time by gently rubbing a small Petri dish (3 cm diameter) on the ano-genital area of the female donors for a few seconds (Heth et al., 2001), and these were introduced simultaneously into the test cage. During the 5-min test, the time spent investigating each of the two odours was recorded with stopwatches by an observer who was unaware of the identity of the odour donors.

RESULTS
During the 5-min test, subjects spent significantly more time investigating the unfamiliar unrelated female odour (mean ± standard error, 5.31 ± 0.99 s) than they did the unfamiliar sister’s odour (2.59 ± 0.56 s) (Fig. 2, permutation test for paired samples, N = 10, exact P-value P < 0.001).

EXPERIMENT 3: OLFATORY NEUROGENESIS ASSOCIATED WITH PARTNER PREFERENCE OF FEMALES

INTRODUCTION
Both male–female spatial association and behavioural characteristics in the enclosures (experiment 1) and in
laboratory conditions (Patris & Baudoin, 1998, 2000; Patris et al., 2002) suggest a male–female pair bond. Experiment 3 was conducted in order to explore neurobiological correlates of male–female pair bonding as observed in studies on monogamous voles (Insel & Shapiro, 1992; Winslow et al., 1993; Wang, Ferris & De Vries, 1994; Carter, DeVries & Getz, 1995; Young, Wang & Insel, 1998; Wang et al., 1999).

Recent developments in neurobiology have led to the general acceptance that adult neurogenesis (i.e. the ability of the adult brain to produce and incorporate new neurones) occurs in the mammalian brain. Neuronal birth in adult mammals has been demonstrated in two restricted regions: the subventricular zone (SVZ) of the lateral ventricle and the subgranular layer of the hippocampal dentate gyrus (for reviews see Gage, 2000 and Gross, 2000). The SVZ harbours steadily dividing stem cells and progenitors that produce new neurones (Peretto et al., 1999; Temple & Alvarez-Buylla, 1999). These neuronal progenitors then migrate by way of the rostral migratory stream to populate the main olfactory bulb, where they differentiate into local inhibitory interneurones (mainly granule cells) and establish functional connections with their neuronal targets (Luskin, 1993; Lois & Alvarez-Buylla, 1994; Wichterle et al., 1999; Carleton et al., 2003). It was demonstrated recently that odour stimulation increases the survival of newly generated neurones in an activity-dependent manner and that such change improves olfactory memory in mice (Rochefort et al., 2002).

The aim of our experiment was to analyse whether pair-bond formation in female M. spicilegus might be associated with neural changes involving bulbar neurogenesis. This was hypothesized for several reasons. First, the main events occurring during olfactory learning seem to be at the mitral cell–granule cell synapses and indicate the critical contribution of inhibition provided by granule cells (Brennan & Keverne, 1997). This demonstrates the plausible contribution of the permanent supply of interneurones into the olfactory bulb to memory during adulthood. Second, in M. spicilegus, pair-bond formation between partners involves olfactory learning (mate recognition: Patris & Baudoin, 1998). Consequently, we examined whether an increase in the permanent supply of newly generated neurones within the olfactory bulb was associated with social attachment in this species. Using a combination of immunohistological and behavioural approaches, we examined the effects of the social environment, specifically pair-bond formation, on adult neurogenesis in female M. spicilegus. It was hypothesized that partner preference could affect the extent of olfactory neurogenesis in females and that newly generated neurones may be associated with individual recognition and odour memory.

### Material and Methods

#### Animals

We tested sexually naive adult male and female M. spicilegus from our laboratory colony. Ovariectomized females (N = 19) were primed with a subcutaneous estradiol benzoate injection on 3 consecutive days and received progesterone on the Day 4 just before pairing with a male partner. On the day of mating, females were given four injections of the cell proliferation marker 5-bromo-2’-deoxyuridine (BrdU, 50 mg kg\(^{-1}\) dissolved in 0.4 M NaOH with 0.9% NaCl, one injection every 2 h). Detection of the labelled progeny cells in the female’s olfactory bulb was done immunohistologically after a 20-day period of cohabitation with her male partner.

#### Partner preference test

The testing apparatus consisted of a central cage (43 × 22 × 26 cm) joined by hollow tubes with two parallel identical cages, each housing a male subject. Females were free to move throughout the three compartments whereas males were loosely tethered within their cage to eliminate contact between them. The familiar partner (housed with the female for 3 weeks) and an unfamiliar male were used as stimulus animals. The time spent by the females in each cage was recorded over 3 h using a time-lapse video recording system. For each female a partner preference was defined as spending 60% or more of the time in the cage of the familiar partner.

#### Perfusion and immunohistochemistry

The day following the partner preference test, females were deeply anaesthetized with sodium pentobarbital (100 mg kg\(^{-1}\)) and perfused transcardially with 0.9% saline followed by 200 mL 4% paraformaldehyde in 0.1 M PBS, pH 7.3 at 4 °C. The brain was excised and immersed overnight in the same fixative at 4 °C. Coronal sections were cut serially using a vibrating microtome and collected in 0.1 M PBS. BrdU staining was performed on free-floating 40-μm sections that had been pretreated by DNA denaturation (2 M HCl for 30 min at room temperature). The primary antibodies used were rat monoclonal anti-BrdU antibody (1 : 200). To determine the number of BrdU-positive cells, we stained for BrdU with the peroxidase method (ABC system) with biotinylated donkey anti-rat IgG antibodies (1 : 200) and diaminobenzidine (0.05%) as chromogene. Immunostained nuclei visualized through a 20× objective were counted in every third section, 120 μm apart.

#### Analysis

The number of BrdU-positive cells counted in the olfactory bulb 3 weeks after BrdU injection were compared between females preferring their familiar part-
ner and females showing no preference for either male. A Mann–Whitney U-test was used and the level of significance set at 0.05.

RESULTS

Twelve of the 19 females preferred their familiar partner and seven females showed no preference for either male. None of the females showed a preference for the unfamiliar male.

Three weeks after BrdU administration, a time by which newly generated neurones that survived had reached the olfactory bulb, most BrdU-labelled cells were found scattered throughout the granule cell layer. Differentiation of these newly generated cells was examined by using double labelling for BrdU and a neuronal marker, NeuN. In all cases, cell counts revealed that BrdU-labelled cells were mostly positive for NeuN (~85%, data not shown). Comparisons between females who preferred their familiar partner and those showing no preference for either male revealed that female *M. spicilegus* that spent more time in the cage of their mate also showed a greater number of newly generated neurones within the olfactory bulb (*P* < 0.05) (Fig. 3).

DISCUSSION

Pair bonding in *M. spicilegus* could involve olfactory learning (Patris & Baudoin, 1998). The results of experiment 3 showed that an increase in the permanent supply of newborn interneurones within the olfactory bulb was associated with social attachment in females of this species. This result supports the hypothesis that pair bonding involves olfactory learning. Further investigations are needed to determine whether the same phenomenon also occurs in males. This hypothesis could be more directly tested by blocking the increase in the number of newborn granule cells (Shors et al., 2001) and examining whether any impairment occurs at the time when female *M. spicilegus* form a preferential association with a male.

GENERAL DISCUSSION

The first objective of this study was to demonstrate in *M. spicilegus* the existence of male–female stable pairing during the reproductive period in outdoor enclosures. As expected from our previous studies under laboratory conditions (Patris & Baudoin, 1998, 2000; Patris et al., 2002), we observed male–female associations but no association among females (experiment 1), and no effect of kinship between females on the social organization in breeding groups. In a field study, Simeonovska-Nikolova & Gerasimov (2000) found no overlap between female home ranges during the breeding period. Our observations in enclosures revealed a high level of aggression between females, as described during dyadic encounters (Patris et al., 2002) and in cage groups of wild mice (Simeonovska-Nikolova, 2003). This lack of cooperation between females during the reproductive period may also exist between sisters (this study). The agreement among these studies supports the hypothesis of social monogamy in this species. Field studies under natural conditions are needed to verify the occurrence of the male–female associations that we observed, and other types of associations. In enclosures we also observed three trios with two females, which may indicate that associations between females could occur in the field, perhaps depending on resources. This latter suggestion has been made for other rodent species (Ostfeld, 1990). If dispersal depends on resources, females might form associations when food is abundant and disperse when food is scarce. A similar prediction can be made when *M. spicilegus* begin to disperse after communal wintering inside the mounds. If resources near the mound are sufficient, dispersal may be limited and familiar females could form associations. In our study, groups with young and associated females were never observed. Rather, we observed high levels of female–female aggression during the whole period of study, as has been described mainly during pregnancy and lactation in *M. musculus* (Floody, 1983). Thus, we predict that these hypothesized associations would not persist in

![Figure 3](image-url)
field conditions and that females will have become segregated by the time that they give birth to a litter.

In our experimental test of kin discrimination (experiment 2), we showed that females can discriminate between odours of unfamiliar sisters and unrelated females. Therefore, the lack of effect of kinship between females on male–female pair bonding (experiment 3), which are also involved in social memory. In future research, we need to know if M. spicilegus males exhibit the same neurogenesis capacities as do females. It would also be interesting to compare the olfactory neurogenesis patterns of non-choosy M. spicilegus females (experiment 3) and of M. musculus polygynous mice.

By considering the two behavioural mechanisms mentioned above (aggression and predisposition to pair bonding), it is possible to understand the general pattern of social monogamy in M. spicilegus in enclosures. Because we observed an important intergroup variability before the onset of stable male–female associations, and also because in a few cases trios were observed, we can infer that the social regulation of associations at the beginning of the breeding period is probably a complex process. Our results need to be validated in wild M. spicilegus, but they constitute a basis for testing new hypotheses in the field. When comparing M. spicilegus with M. musculus, breeding success may be obtained in very different ways. House mice are polygynous, with cooperation between kin females during nursing (e.g. sisters; Dobson et al., 2000; Dobson & Baudoin, 2002). We found that M. spicilegus are monogamous under particular conditions (this study), with cooperative reproductive behaviour between males and females, but without cooperation between females. It would also be of great interest to compare M. spretrus and M. macedonicus with M. spicilegus for all the behavioural traits examined in this study, because these three species are free-living in nature and are not commensal, unlike M. musculus. Two of these Mus species are closely related (M. spicilegus and M. macedonicus), and thus could help to indicate whether monogamy is ancestral or a more recent response to social and ecological conditions as has been described in other monogamous mammals (Komers & Brotherton, 1997).

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REFERENCES

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Wichterle H, Garcia-Verdugo JM, Herrera DG, Alvarez-


