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The ownership signature in mouse scent marks is involatile

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Male house mice advertise their territory ownership through urinary scent marks and use individual-specific patterns of major urinary proteins (MUPs) to discriminate between their own scent and that of other males. It is not clear whether recognition occurs through discrimination of the non-volatile proteins or protein–ligand complexes (direct model), or by the detection of volatile ligands that are released from MUPs (indirect model). To examine the mechanism underlying individual scent mark signatures, we compared investigatory and countermarking responses of male laboratory mice presented with male scent marks from a strain with a different MUP pattern, when they could contact the scent or when contact was prevented by a porous nitrocellulose sheet to which proteins bind. Mice investigated scent marks from other males whether these were covered or not, and biochemical analysis confirmed that the porous cover did not prevent the release of volatiles from scent marks. Having gained information through investigation, mice increased their own scent marking only if they had direct contact with another male's urine, failing to do this when contact was prevented. Individual signatures in scent marks thus appear to be carried by non-volatile proteins or by non-volatile protein–ligand complexes, rather than by volatiles emanating from the scent.

Keywords: olfactory communication; scent marking; individual recognition; major urinary proteins; rodents

1. INTRODUCTION

Scent marking is an important mode of communication among many mammals, particularly for communication of individual territory ownership or competitive ability among males (Ralls 1971; Gosling 1982; Brown & MacDonald 1985). Because deposited scent marks provide information in the absence of the donor, they need to provide stable and persistent information about the donor's identity. Attention has focused largely on the volatile components of scents as sources of individuality signals, particularly those associated with major histocompatibility complex (MHC) odour-types (reviewed by Singh *et al.* 1987; Singer *et al.* 1997; Singh 2001). However, recently we have shown that the pattern of major urinary proteins (MUPs) in the urine scent marks of house mice seems to be essential in allowing mice to distinguish another male's scent mark from their own (Hurst *et al.* 2001). The only known functions of MUPs are in chemical communication. Urinary MUPs are expressed at high concentration by adult mice of both sexes (Beynon *et al.* 2001; Payne *et al.* 2001; Beynon & Hurst 2003), although males invest more than females in both scent marking (Hurst 1990*a*) and MUP production (Beynon *et al.* 2001). These proteins are highly polymorphic, coded by a multigene family on chromosome four (Bishop *et al.* 1982). Individual mice express a combination of MUPs (typically at least 7–12), and many different MUP patterns are found even among mice captured from the same population (Payne *et al.* 2001; Beynon *et al.* 2002). This genetically coded diversity of MUPs thus has considerable potential for signalling the identity of the owner of scent marks, as the patterns expressed by individuals are fixed and, unlike the many factors that influence volatile metabolites contributing to an individual's scent (Brown 1995), do not appear to be influenced by environmental effects such as changes in status or food source (R. J. Beynon and J. L. Hurst, unpublished data). Once deposited in a scent mark, they are highly persistent and resistant to enzymic or chemical degradation.

It is not clear whether the recognition of different MUP patterns occurs through discrimination of the non-volatile proteins themselves (direct model) or by detection of volatile ligands that are released from MUPs (indirect model). MUPs are members of the lipocalin family of proteins, and are small (18–20 kDa) barrel-shaped structures that bind volatile semiochemicals in the central calyx (Bacchini *et al.* 1992; Robertson *et al.* 1993; Timm *et al.* 2001). The urinary MUPs of male house mice bind several species and sex-specific volatile pheromones, including 2-*sec* butyl 4,5 dihydrothiazole and 3-4 dehydro-*exo*-brevicomin, and release these slowly from urinary scent marks (Hurst *et al.* 1998; Robertson *et al.* 2001). These volatile ligands are attractive to both female (Jemiolo *et al.* 1985) and male mice (Humphries *et al.* 1999; Mucignat-Caretta & Caretta 1999), stimulate aggression between males (Novotny *et al.* 1985) and have reproductive priming effects on females (Jemiolo *et al.* 1986; Novotny *et al.* 1999; Marchlewska-Koj *et al.* 2000). The specificity of these volatile ligands to male house mice suggests that they must signal the species and sex of the

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scent owner, and may also play a part in signalling social status (Harvey *et al.* 1989). MUPs may also carry other ligands (Robertson *et al.* 1998) and it is possible that different MUP profiles bind and release specific patterns of volatiles that are used to identify the owner of a scent mark. For example, Singer *et al.* (1993) showed that the complex pattern of volatiles that allows mice to discriminate between MHC odourtypes is bound to and released by the protein fraction of mouse urine. Although it has been assumed that these volatiles are bound and released by fragments of MHC proteins in the urine (e.g. Pearse-Pratt *et al.* 1999; Singh 2001), it is also possible that individual-specific volatiles are bound and released by MUPs that are present at concentrations up to a million times higher and that have evolved, specifically, to bind low-molecular-weight hydrophobic molecules. However, non-volatile components that are more persistent and 'hard-wired' in the genome, such as the urinary proteins themselves, might be more suitable for signalling the ownership of scent marks that are deposited to provide information over an extended period.

In this study, we assess whether mice can identify another male's scent mark from only the volatiles emanating from the mark, or whether contact with non-volatile components is essential for recognition, by comparing the response to scent marks with or without direct contact. We prevented contact by covering scent marks with a sheet of nitrocellulose to which proteins bind, to ensure that the small proteins would not be inhaled by a mouse sniffing closely at the scent even when they could not directly contact the source. We measured investigation (to see whether mice detected the presence of the scent) and scent marking responses. The latter is a specific test of whether mice recognize scent that is not their own because dominant (competitive) male mice increase their scent marking in the vicinity of scent marks from other males to countermark the scent, but show no such response to their own scent marks or to those from males genetically identical to themselves (Hurst 1990*b*; Nevison *et al.* 2000; Hurst *et al.* 2001). This countermarking response is an important part of male competitive advertisement to other males and to females (Hurst 1993; Hurst & Rich 1999) and thus provides a strong functional test of the recognition of scent ownership.

2. METHODS

(a) *Subjects and urine donors*

Thirty-two adult male inbred BALB/c mice (Harlan UK, Bicester, Oxfordshire, UK) aged 7–16 weeks acted as subjects while 32 adult C57BL/6 males (Harlan UK, Bicester, Oxfordshire, UK) provided the unfamiliar male urine used in tests. All males were housed in same-sex and same-strain pairs from five weeks of age in polypropylene cages (M3, 490 $cm^2 \times 12$ cm, North Kent Plastics, Rochester, Kent, UK) on sawdust substrate (Lignocel three-quarters, RS Biotech, Finedon, Northamptonshire, UK) with shredded paper nesting material (Datesand, Brooklands, Cheshire, UK). Food (TRM9607 rat and mouse pellets, Harlan Teklad, Hull, UK) and water were provided *ad libitum*. Mice were maintained at 19–23 °C, relative humidity 50–60%, under a reverse 12 L : 12 D light schedule with white lighting on at 22.00. All tests were conducted during the first 6 h of the active dark phase under dim red lights.

Stimulus urine was obtained from adult C57BL/6 donors aged 7–16 weeks. This inbred strain is derived from a separate genetic lineage to the BALB/c strain and the two strains differ in MUP patterns (Robertson *et al.* 1996) and MHC haplotype. In the week prior to the start of experimental tests, urine was collected by picking donors up gently by the scruff of the neck and, if urine was not voided voluntarily, the bladder region was gently massaged. Voided urine was collected directly into Eppendorf tubes, labelled and frozen at −20 °C within 15 min of collection. To standardize the stimulus used in different trials, equal volumes of urine were pooled across 16 individual donors, divided into 20 µl aliquots for each trial and re-frozen until immediately prior to use.

(b) *Assessment of social status*

Because only dominant males respond to the scent marks of another male by increasing their rate of scent marking to countermark (Desjardins *et al.* 1973; Hurst 1990*b*; Nevison *et al.* 2000), social status was assessed by observing aggressive behaviour within the home cage of each pair of subject mice during the week prior to tests. Observations took place during the first half of the dark period for three 4 h periods on separate days. Aggression was not deliberately stimulated, was relatively rare and mice were checked daily for injuries (only occasional minor bite marks were seen). Social status was assigned to mice only when the sum total of aggressive acts scored within the pair was at least 10 (range of 10–34), and when more than 75% of acts were directed by one individual (the dominant) towards the other (the subordinate), giving 10 pairs. It was not possible to assign social status within the remaining pairs owing to very low aggression, and these animals were excluded from analyses.

(c) *Urine recognition tests*

Each subject was used in two 10 min urine recognition tests, one in which subjects were allowed to contact unfamiliar male urine and one in which contact was prevented by a porous sheet of nitrocellulose, conducted one week apart in balanced order. Tests were conducted in clean bottomless varnished wood arenas (60 cm × 30 cm × 30 cm) placed over absorbent paper (Benchkote, Whatman, Over, Cambridgeshire, UK). A 10 µl streak of male C57BL/6 urine was pipetted centrally onto the Benchkote at one end of the arena and an equivalent streak of deionized water at the opposite end (location balanced between trials). A 3 cm × 3 cm square of nitrocellulose (pore size 0.2 µm, Schleicher & Schuell, London, UK) was placed over the urine mark in the 'no contact' test, or over the water mark in the 'contact' test. Open mesh grids (3.5 cm × 3.5 cm) were then placed over the top of both the urine and water marks, each held down by two staples. The grids were sufficiently open (3 mm × 2 mm apertures) to allow nasal contact with the surface below but to prevent mice from chewing at the nitrocellulose membrane. Two days prior to each urine test, subjects were also given a control test using two 10 µl water streaks, one of which was covered with nitrocellulose membrane as above (location balanced between trials), to assess any investigation response to the nitrocellulose membrane and to assess the baseline scent marking of each subject in the absence of any urine stimulus (nitrocellulose was present in the arena in all tests). Mice were introduced into the centre of the arena and their behaviour was recorded remotely using an overhead video camera. Stimulus streaks had dried prior to introduction of the subject. Mice from the same subject pair were tested simultaneously in separate arenas and were replaced together in their home cage at the end

of each test. To familiarize the mice with the arenas prior to tests, mice aged six to seven weeks were placed individually into a clean arena for two 10 min periods, spaced 2 days apart.

We recorded the total amount of time spent investigating each stimulus (urine or water) when the subject's nose was touching or over the mesh grid. After the 10 min test, Benchkote sheets were scanned using a FluorS imager (BioRad) to visualize deposited urine scent marks under ultraviolet light and the number of scent marks deposited was counted electronically using ScionImage software (Beta v. 4.02 for Windows, www.scioncorp.com). To avoid counting footprints, we counted only urine marks that were at least 50 pixels in area, equivalent to greater than 6 mm^2.

(d) *Biochemical analysis*

To check that the nitrocellulose membrane was effective in stopping contact with non-volatile proteins in the urine stimuli, a random sample of 26 of the nitrocellulose sheets that had been placed over urine samples during behavioural tests were probed with MUP antisera after blocking with 0.5% (w/v) Tween 20 in Tris buffered saline (TBS) pH 7.5 for 1 h. Nitrocellulose membranes were washed with 0.05% (w/v) Tween 20 in TBS then incubated with goat anti rabbit IgG alkaline phosphatase conjugate (Biorad) in TBS for 1 h. The nitrocellulose membranes were then washed as above and incubated with 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue Tetrazolium (Sigma-Aldrich) substrate for *ca*. 20 min. The reaction was stopped with running water and the nitrocellulose membranes air-dried. This highly sensitive immunoassay confirmed that there was no sign of any protein on the upper side of the nitrocellulose sheet in most tests (21 out of 26) but, in a few cases (5 out of 26), a small amount of protein was visible on the upper surface. However, even in these cases, the protein was bound firmly to the nitrocellulose membrane, as evidenced by the retention during extensive washing of the immunoblot process, and could not be inhaled.

To assess whether the nitrocellulose membrane reduced the release of volatiles from covered scent marks, we replicated the procedure for the behavioural tests for six contact and six no contact samples. After 10 min, volatiles were recovered from each urine streak (plus nitrocellulose sheet if samples were covered) and from 10 µl samples of fresh, non-deposited urine by multiple extractions with hexane. Volatiles were analysed using a ZB wax (Phenomenex) capillary column (30 m × 0.25 mm i.d.) fitted to a Thermo Finnegan Trace 2000 GC. Detection of resolved molecules was achieved using a Thermo Finnegan PolarisQ ion trap mass spectrometer. Data were obtained in total ion current mode. Detection of 2-sec butyl 4,5-dihydrothiazole (thiazole) was enhanced by using selected ion monitoring mode for the m/z 60 ion. We examined differences in the amount of thiazole remaining in covered and uncovered samples because this volatile is consistently found at high intensity in male urine samples.

(e) *Data analysis*

Investigation of each stimulus mark fitted a normal distribution (Kolmogorov–Smirnov tests, n.s.). Separate repeated-measures ANOVAs examined: (i) the effect of nitrocellulose on investigation of water marks within the control tests; (ii) the difference in investigation of urine and water marks within each of the two urine stimulus tests; and (iii) investigation of urine marks according to whether mice could contact the scent. In each case, subject status was included as a between-subjects factor. Scent marking was not normally distributed as there was a wide range in the number of marks deposited by different subjects. Mann–Whitney *U*-tests first confirmed that dominant males deposited more marks than subordinates in control tests (total number of marks deposited in the two tests) and in each urine stimulus test. Wilcoxon matched-pair specific tests assessed the hypothesis that dominant males increase the number of scent marks deposited in the presence of unfamiliar male urine compared with the preceding water control test. A non-specific Wilcoxon matched-pair test finally compared the number of scent marks deposited in urine tests when the male could or could not contact the urine.

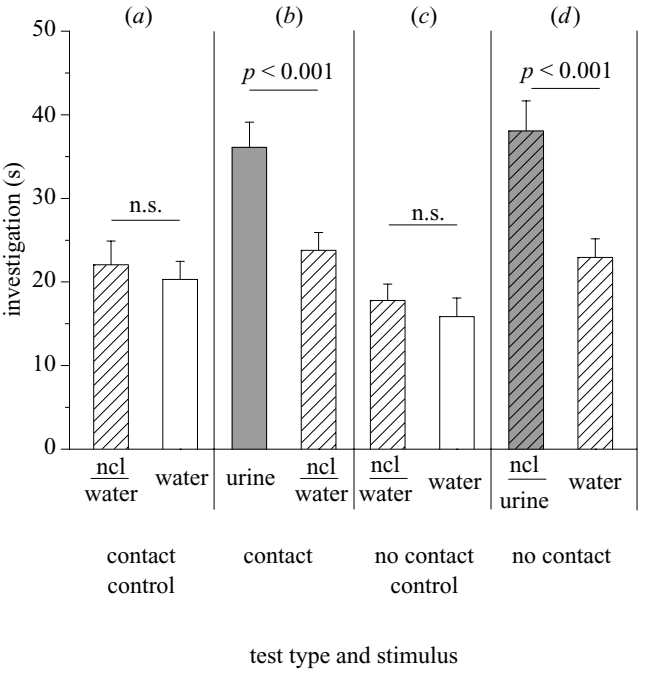

Figure 1. Duration of stimulus investigation by BALB/c male mice during each of four 10 min tests (mean ± s.e.). In each test, a nitrocellulose membrane (ncl, hatched bars) was placed over either a water stimulus (unshaded bars) or a urine stimulus from a C57BL/6 male (shaded bars) while the water or urine stimulus at the opposite end of the arena was not covered. (*a*) Control test prior to urine contact test; (*b*) urine contact test; (*c*) control test prior to no contact test; (*d*) no contact test.

3. RESULTS

(a) *Investigation*

First, we confirmed that the nitrocellulose membrane used to prevent contact with a stimulus mark did not stimulate any significant investigation or avoidance in control trials when no urine was present (effect of nitrocellulose on investigation of water stimuli, repeated-measures ANOVA, $F_{1,18} = 3.24$, n.s.; figure 1*a*,*c*). We also confirmed that there was no difference in levels of investigation between the two control trials that preceded urine trials with and without contact ($F_{1,18} = 0.10$, n.s.).

When presented with a streak of unfamiliar male urine, mice clearly detected it and spent much longer investigating the urine than a nearby streak of water, whether mice were able fully to contact the urine ($F_{1,18} = 26.72$, $p <$

0.001; figure 1*b*) or contact was prevented by a sheet of nitrocellulose ($F_{1,18} = 17.37$, $p < 0.001$; figure 1*d*). Indeed, ability to contact the source had no significant effect on the amount of time that mice spent investigating the unfamiliar scent ($F_{1,18} = 0.66$, n.s.) and in both cases mice investigated by applying their noses closely to the covering mesh grid.

Both dominant and subordinate males showed very similar investigation responses, with no effects of social status on stimulus investigation either in the two urine tests ($F_{1,18} = 0.86$, n.s.) or in the two control tests ($F_{1,18} = 1.75$, n.s.). The proportion of total thiazole remaining in the covered scent marks or on the nitrocellulose after 10 min (95 ± 1%) was similar to that remaining in the uncovered marks (87 ± 5%; Mann–Whitney *U*-test, $U = 13.0$, $N_1 = N_2 = 6$, n.s.), confirming that the nitrocellulose did not significantly reduce the emission of volatiles from the marks.

(b) *Scent marking*

In contrast to scent investigation, scent marking responses are not very closely localized around a stimulus as mice do not attempt to deposit their scent directly on top of another mouse's scent as an overmark (Hurst 1989; Humphries *et al.* 1999). Because scent marks were deposited throughout the test arena, we examined the total number of scent marks deposited in each test (see also Hurst *et al.* 2001). As expected, dominant males deposited many more urine marks than subordinate males in all tests (total marking in two control tests: $U = 23.5$, $p < 0.025$; contact with urine: $U = 0$, $N_1 = N_2 = 10$, $p < 0.001$; no contact with urine: $U = 19.5$, $p < 0.025$). Because only dominant males respond to the scent marks of a potential competitor by elevating their rate of scent marking to countermark, we focused on the scent marking responses of the dominant male within each subject pair.

Dominant males clearly recognized an unfamiliar male's scent mark when allowed full contact and significantly increased the number of scent marks that they deposited compared with their rate of marking when exposed to a water control test (Wilcoxon matched-pairs specific test: $z = -2.50$, $p < 0.01$; figure 2). However, when males could not contact the unfamiliar male urine, they did not elevate their rate of scent marking compared with the water control test (Wilcoxon matched-pairs specific test: $z = -0.97$, n.s.; figure 2). Direct comparison of the two urine tests confirmed that males deposited more scent marks when they could contact the urine stimulus than when they could not (Wilcoxon non-specific test: $z = -1.99$, $p < 0.05$), whereas there was no difference in marking between the two control tests ($z = -0.56$, n.s.).

Subordinate males deposited very few scent marks in each test (number of scent marks in contact control: 20.9 ± 9.9; contact: 8.7 ± 2.7; no contact control: 28.2 ± 12.7; no contact: 19.2 ± 9.3). Interestingly, they deposited fewest marks when they had full contact with unfamiliar male urine, but this was not significantly lower than either the control test (Wilcoxon non-specific test: $z = -1.36$, n.s.) or when they could not contact the urine ($z = -1.36$, n.s.).

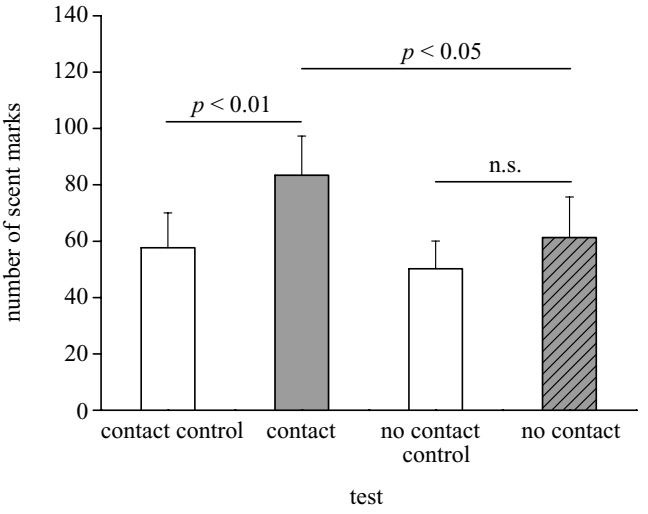

Figure 2. Total number of urine scent marks deposited by dominant males during each of four 10 min tests (mean ± s.e.). In the contact test, mice could contact the urine stimulus (shaded bar) but a sheet of nitrocellulose prevented contact with the urine stimulus in the no contact test (hatched and shaded bar). No urine was present in control tests (open bars). Note that nitrocellulose covered a water stimulus in both control tests and in the contact test, and thus was present in all tests.

4. DISCUSSION

Mice clearly detected the presence of unfamiliar male urine and investigated the scent mark closely, even when a sheet of nitrocellulose prevented direct contact. The very strong investigation response demonstrated that nitrocellulose did not create a strong barrier to the release and detection of volatiles. However, although dominant males responded appropriately to the urine marks of another male by increasing their own scent marking when they could contact the scent mark, they failed to do this when contact was prevented. The failure to countermark suggests that mice did not recognize urine from another male in the absence of information from non-volatile components of the scent mark. This is consistent with the finding that countermarking is induced by the high-molecular-weight fraction of urine that contains urinary proteins and larger peptides, even when most of the volatile ligands are artificially displaced or lost through ageing (Humphries *et al.* 1999).

Volatile components that are species- and sex-specific (Schwende *et al.* 1986) should signal that the urine was from an adult male mouse. Other volatile components are thought to provide information about the individual identity of the scent owner (e.g. Singer *et al.* 1993, 1997; Singh 2001). Why would males fail to respond appropriately when access was restricted to these volatile components of a scent mark? One possibility might be that non-volatile MUPs simply stimulate increased scent marking but do not provide information about the owner's individual identity. In this 'indirect' model, a volatile ownership signal would modulate a non-specific scent marking response induced by contact with non-volatile scent components. Mice recognizing volatiles from their own urine, or those from mice genetically identical to themselves, would suppress their scent marking response. However, this

'indirect' model fails to explain why wild mice elevate their scent marking when they contact urine from a brother with a different MUP pattern from their own, and spend much time in the vicinity of the scent, but do not do so when they contact urine from a brother sharing their own MUP pattern (Hurst *et al.* 2001). Genetically heterogeneous wild mice will each express different patterns of volatiles that are associated with multiple loci across the genome, inherited independently from their MUP pattern (e.g. volatiles associated with MHC and with sex chromosomes (Yamazaki *et al.* 1986; Singer *et al.* 1997)). Urine from a brother stimulates much more initial close investigation than their own urine, whether males share the same MUP-type or not (J. L. Hurst, unpublished data), suggesting that differences in volatile profile are detected. None the less, males show no functional recognition of scent marks that share their own MUP pattern (Hurst *et al.* 2001). Thus, a model in which volatiles communicate owner identity and modulate the scent marking response to non-volatile components is untenable.

In the alternative 'direct' model, contact with non-volatile components is essential for recognition of the scent owner's identity. MUPs would communicate owner identity through the pattern of proteins themselves, or through non-volatile protein–ligand complexes, rather than through volatiles bound and released by urinary proteins. Although mice detect volatiles emanating from a scent mark, and are sensitive to changes in these volatiles, their initial response is to approach and attempt to contact the odour source. Mice appear to need the additional non-volatile information to recognize ownership of the scent mark. This is consistent with the recent finding that neurons in the accessory olfactory bulb (part of the vomeronasal system) are only activated when mice investigate and their snout makes physical contact with the face or anogenital region of another individual. Further, individual neurons are only activated or inhibited when animals of specific combinations of sex and strain are investigated. This suggests that the vomeronasal organ accesses non-volatile information concerning sex and genetic identity through active pumping during close contact investigation of the scent source (Luo *et al.* 2003).

The use of non-volatile components to detect scent ownership may overcome the problem of plasticity of volatile components of scents, which are influenced by many environmental factors. MUPs, by contrast, are 'hard-wired' into the genome and are stable throughout the life of the animal. Although rodents can be trained to recognize volatiles associated with genotype, this recognition appears to be disrupted if animals are fed on different food types or if there are changes in their bacterial gut flora that also affect volatile metabolites (Brown 1995). Such apparent variability in volatile signatures of ownership would not be sustainable in natural populations where mice are exposed to many different food sources and other varying environmental conditions. Volatiles also vary according to social status and induce differences in investigation, but again such status differences do not appear to be involved in the recognition of individual scent ownership (Nevison *et al.* 2000). Although mice easily discriminate between scents based on volatile cues such as those associated with MHC type, discrimination has largely been based on trained responses (e.g. Singer *et al.* 1993) or investigatory responses, such as habituation–dishabituation tests (e.g. Carroll *et al.* 2002). Although such tests show that animals clearly detect a difference between two scents, they can provide no information about the functional significance of scent differences. Investigation is likely simply to reflect the need to gain further information and is influenced by other factors such as scent location, as well as by owner familiarity (e.g. Mayeaux & Johnston 2002). Functional tests such as mate choice (Potts *et al.* 1991), pregnancy block (Yamazaki *et al.* 1983; Brennan & Peele 2003) or nest sharing (Manning *et al.* 1992) indicate that MHC-associated scents are important in the selection of mates or recognition of kin, but only tests of pregnancy block have distinguished between volatile and non-volatile components (Brennan & Peele 2003) and it is not yet clear what part volatiles play in the individual recognition of scent owners in other contexts.

Changes in volatile components of scents are none the less likely to be very important in alerting mice to potential changes in their social environment, inducing them to closer investigation. Mice recognize their own scent and that of familiar group members and do not need constant contact with the scent to confirm individual ownership. It is likely that mice learn to associate familiar patterns of volatiles with hard-wired information concerning individual identity. For example, only non-volatile components of male-soiled bedding exert an innate attraction to naive female mice. However, male-derived volatiles become attractive to females after repeated exposure to male-soiled bedding, presumably owing to a learnt association with the innately attractive non-volatile components (Moncho-Bogani *et al.* 2002). If changes are detected in the volatile profiles of scents, mice respond by contacting the scent source where MUPs, or non-volatile MUP–ligand complexes, provide reliable information for individual recognition. Only then do mice countermark scents that have been confirmed not to be their own. The data thus support the 'direct' model. Although both volatile and non-volatile components play important roles in mouse scent marks, it is the non-volatile MUPs that are the critical components that signal individual ownership.

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