

Proteins in urine scent marks of male house mice extend the longevity of olfactory signals

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Abstract. The binding of volatile semiochemicals to lipocalin proteins in many mammalian scent marks may provide a gradual release of volatile ligands, extending the life of airborne odour signals. We tested this by using menadione to displace semiochemical ligands from major urinary proteins (MUPs) in urine streaks obtained from adult male house mice, *Mus domesticus*, and assessed the responses of other males to these and to intact urine marks as they aged. Dominant male mice scent-mark their territories extensively with urine streaks; MUPs in these marks bind at least two semiochemically active molecules, 2-*sec*-butyl-4,5-dihydrothiazole (thiazole) and 2,3-dehydro-*exo*-brevicomine (brevicomine), associated with the males' aggressive status. Wild-caught males ($N=24$), housed in individual enclosures, were presented with two glass slides, behind mesh to prevent contact, on which 10 μ l of both unfamiliar urine and 0.5 mg/ml menadione in ethanol had been streaked. On one slide the urine and menadione solution were mixed to displace ligands; on the other they were separate (intact urine). We carried out tests 0, 0.5, 1 or 24 h after deposition, and matched them to changes in the concentration of thiazole and brevicomine within the intact and displaced marks. Males were hesitant to approach intact urine up to 1 h old but, when ligands were displaced, or were reduced to low levels by natural evaporation from intact urine streaks aged 24 h, their approach was similar to that to water and to menadione controls. Ligands did not appear to cause any longer term avoidance and, after the first approach, investigation increased with the freshness of urine regardless of when the ligands were displaced. This is the first direct demonstration that proteins evince a slow release of olfactory signals from mammalian scent marks. The nature of their response suggests that, from a distance, mice may be unable to tell whether airborne signals emanate from scent marks or from the donor himself and we suggest that this may provide territory owners with a major advantage in defending their territories.

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While we now understand much about the behavioural mechanisms underlying the use of scent marks in territory defence and dominance signalling (e.g. Gosling 1982, 1990; Hurst 1993; Hurst & Rich, in press), we know comparatively little about the chemical design of such signals and, particularly, how this interacts with behaviour. One particular feature of scent marks deposited in the environment is that they can provide chemical signals over a comparatively long period, in the

absence of the signaller. Mammalian scent marks typically comprise a complex mixture of volatile and non-volatile components, although not all of these may be involved in signalling, particularly in the many cases where animals use excretory products (urine or faeces) for scent marking (reviewed by Brown & Macdonald 1985). Territory scent marks may be deposited at high density around territory borders, along trails, or over the entire territory (Macdonald 1980; Gosling 1982; Gorman 1990), and are usually investigated at close quarters by competitors and potential mates. This would allow animals to use long-lasting signals of relatively low volatility to advertise their identity and dominance or defence of a territory.

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The majority of the chemicals in scents that have been associated with social dominance appear to be highly volatile, however (e.g. Harvey et al. 1989; Novotny et al. 1990). Furthermore, associations between volatile and non-volatile components may also play an important role in chemical signalling. In particular, non-covalent binding of ligands to proteins may modulate the expression of the smaller molecule. The most important of these are the members of the lipocalin family, found in urine, saliva and scent-gland secretions in a range of mammals including humans and at least some of these are known to bind volatile semiochemicals (Singer & Macrides 1992; Zeng et al. 1996). These proteins bind hydrophobic ligands within a central calyx or pocket formed by their unique tertiary structure (Flower 1996), but the precise role(s) of these protein–ligand complexes in chemical signalling has yet to be established. The proteins may be involved in the transport of semiochemicals and may protect them from oxidative and other changes (see review by Flower 1996), but it has also been hypothesized that lipocalins in scent marks may provide a gradual release of their volatile ligands into the environment, extending the longevity of airborne odour signals (Robertson et al. 1993).

The behavioural and biochemical basis of odour communication and territorial scent marking has been studied most thoroughly in house mice, *Mus domesticus*. Like many other rodents, house mice scent-mark their territories extensively with urine (Hurst 1987). Male mouse urine contains a high concentration of lipocalins termed major urinary proteins (MUPs) which are manufactured in the liver and are efficiently filtered from the circulation by the kidneys (Finlayson et al. 1963; Lehman-McKeeman & Caudill 1992). In adult male mice, these MUPs bind at least two semiochemically active molecules (Bacchini et al. 1992; Robertson et al. 1993), 2-*sec*-butyl-4,5-dihydrothiazole (thiazole) and 2,3-dehydro-*exo*-brevicomine (brevicomine), which are associated with the males' aggressive status (Apps et al. 1988; Harvey et al. 1989). Brevicomine and thiazole act synergistically in provoking aggressive competition between males (Novotny et al. 1985) and in attracting females and stimulating oestrous cycling (Jemiolo et al. 1985). Male mice use their urinary scent marks to advertise dominance over their defended territories (Hurst 1993), and both urine marking (Maruniak et al. 1977) and

the production of MUPs (Clissold et al. 1984; Johnson et al. 1995) and associated ligands (Schwende et al. 1986; Harvey et al. 1989) are under social and hormonal control. The urine of isolated or dominant males is partially avoided by subordinate intruders (Jones & Nowell 1973, 1989) and evokes this response for up to 48 h, although aversive potency is lost by 72 h (Jones & Nowell 1977).

Menadione can rapidly displace semiochemical ligands from MUPs (Robertson et al., in press). In this study, we used menadione as a novel chemical tool to displace the ligands from MUPs in the urinary scent marks of male house mice to examine (1) whether these lipocalin proteins do provide a slow release of volatile odorants from urinary scent marks, (2) whether the release of these volatiles from scent marks appears to provide an olfactory signal to other males, and (3) the nature of any response by competitors. These studies are a first step towards establishing the functional significance, if any, of the protein–ligand complex in competitive communication between male mice. We compared the responses of wild-caught male house mice, with natural social experience, to urine scent marks in which volatile semiochemicals were associated or dissociated with MUPs, and matched the time course of changes in responses to changes in the concentration of the semiochemicals within intact and displaced marks as these age. We expected that other males would be cautious in approaching scent marks containing naturally high concentrations of these ligands. If MUPs prolong their release from scent marks, displacement by menadione should curtail any cautious response. Mice should thus show little hesitation in approaching ageing scent marks from which ligands have been dissociated from MUPs allowing their rapid evaporation.

METHODS

Behavioural Response

We presented 24 wild-caught adult male house mice (>13 g) with a series of paired odour choices in their home enclosures (0.6 × 1.2 m and 0.8 m high, made of melamine and containing a nestbox and a food and water station). Males (18 caught from a poultry farm and maintained on poultry food, six from a variety of other livestock and arable farms maintained on laboratory mouse pellets TRM9607, Harlan Teklad, Hull, U.K.)

were housed individually after capture (for 3–12 months before the tests). They were tested with urine from unfamiliar wild-caught donor males ($N=32$), also housed individually and kept on the same diet as the subjects. It was very unlikely that subjects were previously familiar with donors caught from the same farm as they were from large infestations split between several separate buildings. After the experiment, subjects and urine donors were kept in the wild rodent unit at Nottingham for further behavioural studies.

Tests were carried out under dim red light during the dark phase of the 12:12 h light:dark cycle (white lights on at 2000 hours). We collected urine by holding individual donors briefly by the scruff and tail base over an Ependorff tube; such direct handling usually stimulates immediate urination in wild mice (J. L. Hurst, personal observation). The individual urine samples were frozen immediately at -18°C until use. Each male was given a choice between two mixed streaks of urine and menadione in ethanol (displaced ligands) versus two separate parallel streaks (intact urine), presented immediately after application to a test slide, or after the streaks had been left to air dry for 0.5, 1 or 24 h. Pilot tests and biochemical analyses of changes in ligand concentration (see below) had indicated that this would be a suitable range of time over which to test responses. Males experienced urine from a different unfamiliar donor in each trial. They were also given two control tests: water versus water, and menadione solution (0.5 mg/ml menadione in ethanol) versus water; these were presented immediately after application to the test slides. We conducted all tests in a balanced design to avoid any order effects or left:right bias, after first testing each male with fresh urine (no menadione) versus water. Mice experienced a maximum of one trial per day after being established in their test enclosures for at least 4 days (the six males maintained on laboratory diet had been established in their test enclosures for several weeks before the tests).

We applied odour cues (urine, water or 0.5 mg/ml menadione in ethanol) to microscope slides as 10 μl streaks, using the edge of another slide, in a neighbouring laboratory, where they were also left to air dry for the 0.5, 1 or 24-h urine tests. We created mixed urine and menadione streaks by depositing the urine on top of the menadione solution before streaking them. We then presented the slides in pairs in two mesh

holders fixed 15 cm apart at floor level on a male's enclosure wall, sited on either side of the subject's nestbox. The holders prevented contact with the test odours, ensuring that subjects could respond only to airborne volatiles, and bore the same scent marks as the surrounding enclosure; we placed clean test slides inside these mesh holders for 18–24 h before applying the test odours so that the slides would not themselves induce a novel odour or object response. Immediately before a test we placed the odour slides in the holders and removed the nestbox and food and water station from the subject's enclosure while the subject ran into a far corner of the enclosure. A test area (15 \times 50 cm) surrounding the paired test holders was video-recorded remotely for 5 min from the subject's first entry into the test area to measure the latency to the first approach to each holder (close enough to make contact) once the subject had been in the vicinity (i.e. within 15–20 cm), and the total frequency of visits to, and the duration of sniffing closely at, each odour holder. Trials were abandoned if the subject failed to enter the test area within 5 min, to be repeated on another day, although as their nestbox was normally in this area mice usually entered within 1–2 min. Test sites were almost always visited within 30 s, but occasionally a subject ran through the test area at the start and failed to return for more than 60 s. This appeared to be due to the subject's inactivity rather than a response to the odours (occurring in 4.8% of control trials and 3.5% of urine trials), so latencies to approach that were greater than 60 s were excluded from the data.

We transformed data logarithmically to meet the assumptions of parametric analysis, although they are shown in the figures as raw values ($\bar{X} \pm \text{SE}$). Repeated measures analyses of variance assessed the effects of ligand displacement and time since deposition on behaviour between tests. Matched-pair *t*-tests checked for bias in response between paired sites within each test.

Biochemical Analysis

To check the effect of menadione displacement on the concentration of thiazole and brevicomin in urine samples at each time point, we applied urine from adult male Balb/c laboratory mice (housed in caged groups) to microscope slides with menadione solution (0.5 mg/ml in ethanol) as two separate or two mixed streaks, each on

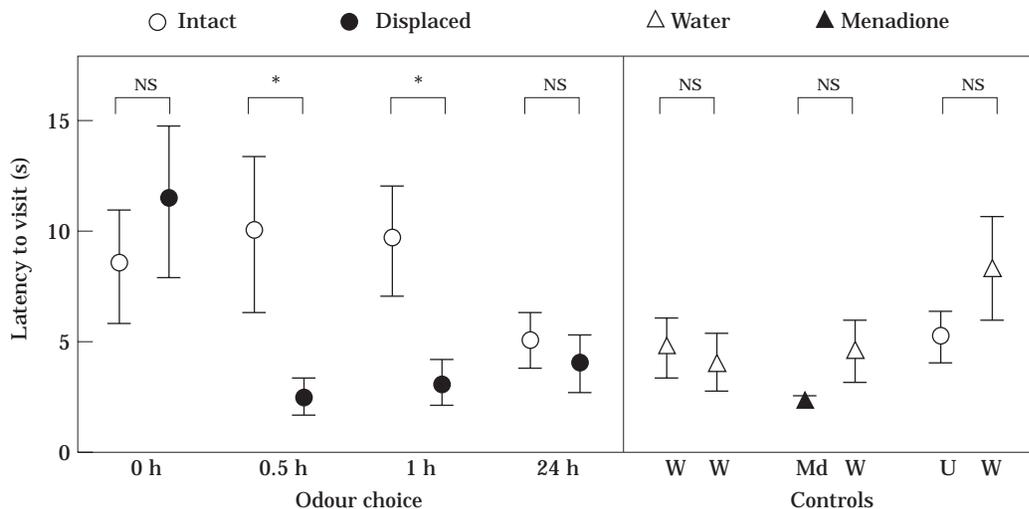


Figure 1. Effect of displacing ligands from major urinary proteins on the latency to visit urinary scent marks introduced into a male's territory. Each male was given a choice between intact urine with a separate menadione streak (Intact) versus urine mixed with menadione to displace ligands (Displaced), presented 0, 0.5, 1 or 24 h after deposition; they were also given water (W) versus water, menadione solution (Md) versus water, and intact urine (U) versus water control tests presented 0 h after deposition. Data are shown as raw values ($\bar{X} \pm \text{SE}$) but were transformed by logarithms for parametric analyses. Matched-pair *t*-tests show the bias between paired-sites within each test (* $P < 0.05$). Over all four time periods, there was a significant interaction between ligand displacement and the time since deposition on the males' latency to approach urine scent marks (repeated measures ANOVA: $F_{3,13} = 4.45$, $P < 0.025$). See text for further analysis.

four slides. Samples were recovered from the slides after they had stood at room temperature for 3 min (0 h), 33 min (0.5 h), 63 min (1 h) and 24 h (the average times elapsing before males first encountered odours in behaviour tests, allowing for the average latency to enter the test area) by serially overlaying with three 75 μl aliquots of distilled water. We extracted thiazole and brevicomin from the recovered urine by adding 75 μl of chloroform, containing an internal standard of 1 mg/ml ethyl undecanoate, before vortexing for 10 s. These extraction mixtures were allowed to stand for 1 h before we removed the chloroform, which was subsequently screened for the presence of thiazole and brevicomin using GC/MS in selected ion monitoring mode. The selected ions were m/z 60 (thiazole), 88 (ethyl undecanoate) and 95 (brevicomin).

RESULTS

Latency to the First Approach

As expected, mice were cautious in approaching samples emitting high levels of volatiles associated

with male dominance. They were significantly slower to approach both intact and displaced urine immediately after these had been applied to test slides than clean slides in a water-only control test (repeated measures comparison between urine and menadione after 0 h versus water-only tests: $F_{1,16} = 14.37$, $P < 0.002$; Fig. 1). Ligand displacement had no immediate effect on this hesitation to approach fresh urine, although freshly displaced ligands tended to evince the longest latencies to approach (Fig. 1), consistent with a sudden increase in the emission of volatiles from these urine samples.

When urine samples were allowed to stand for 0.5 or 1 h, mice were quicker to approach displaced urine than intact (Fig. 1). Comparison between tests using samples aged for 0, 0.5 and 1 h confirmed that this was because ligand displacement caused a significant reduction in the latency to approach these samples after 0.5 or 1 h (effect of time since deposition on latency to approach displaced samples: $F_{2,16} = 6.50$, $P < 0.01$). However, there was no significant change in latency to approach intact urine whether fresh or up to 1 h old ($F_{2,15} = 1.16$, NS). Biochemical analysis

confirmed that mixing urine with the menadione solution caused rapid displacement of both thiazole and brevicomin (Fig. 2), with almost total loss from the slides into the environment by the time the majority of mice encountered the freshly mixed samples (approximately 3 min after application). By contrast, intact urine samples released their ligands much more slowly over the first hour. The mice appeared to detect this natural gradual release of volatile ligands from intact MUPs and evaporation into the air, responding with initial caution. Compared with fresh urine (0 h), within-subjects contrasts showed that changes in latency to approach aged urine depended significantly on volatile displacement by menadione, both after 0.5 h ($F_{1,15}=12.94$, $P<0.005$) and after 1 h ($F_{1,15}=4.60$, $P<0.05$) with no significant difference in approach behaviour between the tests at 0.5 or 1 h ($F_{1,17}=0.007$, NS). This provides a clear demonstration that binding of these volatile ligands to MUPs acts to prolong their release into the environment at a concentration sufficient to be detected by mice at a distance and delay their first approach to a urine streak.

After urine streaks had been aged for 24 h, there was little further evaporation of brevicomin and thiazole from intact urine (Fig. 2). Correspondingly, the latency to approach intact urine 24 h after deposition was no greater than for displaced urine, both being significantly reduced compared with fresh deposits ($F_{1,20}=10.81$, $P<0.005$) and very similar to that for water controls (Fig. 1). Thus, while mice were hesitant to approach urine up to 1 h old, this response disappeared when ligands were displaced from MUPs by menadione and allowed to disperse, or when ligands were reduced to low levels by natural long-term evaporation from intact urine streaks over 24 h. The same pattern of response was shown by males from the poultry farm maintained on poultry food and by males caught from other commensal populations maintained on standard laboratory diet (Table I).

In the absence of urinary ligands, menadione in ethanol appeared to attract mice to approach the test site immediately. Compared with their latency to approach clean slides in a water-only control test (averaged across the paired test sites), mice were quicker to approach a control slide treated with menadione solution only ($t_{16} = -3.59$, $P<0.005$). They were also quicker to visit displaced urine (mixed with menadione in ethanol)

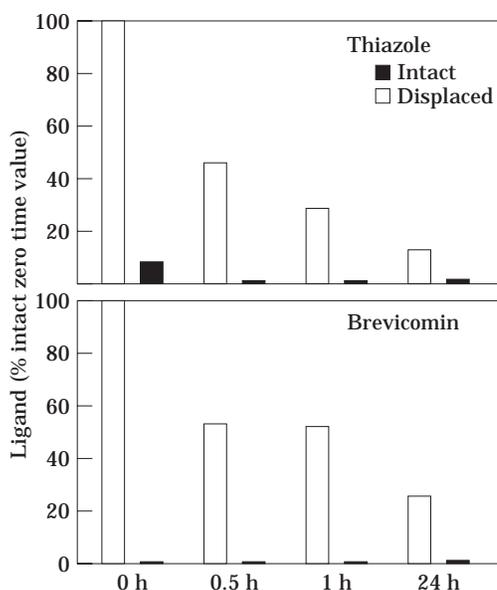


Figure 2. Concentration of thiazole and brevicomin in urine samples at each time point, screened using GC-MS with selected ion monitoring mode. Data are expressed as a percentage of their abundance in the 0 h intact urine sample.

after 0.5 h ($t_{15} = -2.64$, $P<0.05$), after 1 h ($t_{18} = -2.58$, $P<0.05$) and after 24 h ($t_{17} = -2.19$, $P<0.05$) than clean slides in the water-only control test. Within the menadione control test itself (choice between fresh menadione solution versus water), mice tended to visit the menadione solution more quickly than water but this was not statistically significant (Fig. 1), probably because in this case volatiles from the menadione in ethanol attracted mice to approach one test site immediately, where they were then likely to visit the nearby clean water site too. We noted that urine retained a distinctive 'mouse' odour to the human nose even after the MUP ligands had evaporated. However, we could not assess whether these urinary volatiles attracted mice to approach urine once the MUP ligands had been lost, since menadione was also present on all test slides where urinary ligands had been displaced and dispersed. When mice were presented with a choice between fresh intact urine only versus water (with no menadione to attract them to either site), there was no difference in their latency to approach the urine or clean site, both being significantly greater than towards two clean sites

Table I. Latency to approach unfamiliar urine shown by mice on different diets

Time (h)	Diet	Intact urine (s)		Displaced urine (s)	
		$\bar{X} \pm SE$	<i>N</i>	$\bar{X} \pm SE$	<i>N</i>
0	Poultry feed	7.4 ± 3.0	17	10.9 ± 3.4	18
	Laboratory diet	12.2 ± 3.7	4	12.9 ± 9.2	5
1	Poultry feed	7.8 ± 3.0	18	2.3 ± 0.6	18
	Laboratory diet	14.2 ± 4.0	6	5.3 ± 3.9	6
24	Poultry feed	4.0 ± 1.4	17	3.7 ± 1.4	17
	Laboratory diet	8.3 ± 2.8	5	4.5 ± 2.6	5

Data shown in Fig. 1 are here broken down by diet type (subjects and urine donors). Insufficient data were obtained for a similar comparison of response to urine 0.5 h after deposition because two of the six males on laboratory diet died before this test while two others failed to enter the test area within the maximum time period.

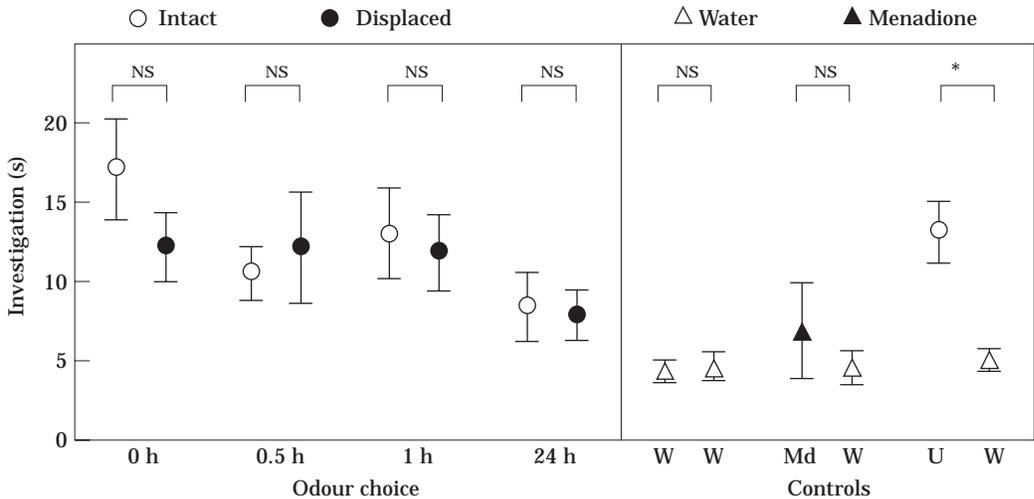


Figure 3. Effect of ligand displacement on duration of odour investigation in paired choice tests. Tests and key as outlined in Fig. 1. Data were transformed logarithmically to meet the assumptions of parametric analysis but are shown as raw values ($\bar{X} \pm SE$). Matched-pair *t*-test examined bias between paired sites within tests (* $P < 0.001$).

in the water-only test (repeated measures effect of test: $F_{1,15} = 5.10$, $P < 0.05$; Fig. 1), again indicating that fresh intact urine induced a hesitancy to approach the odour source.

Odour Investigation

While MUP ligands induced an initial hesitation to approach urine, they did not cause any longer term avoidance with respect to investigation or total frequency of visits to samples over the 5-min test. Indeed, after the first approach, mice were strongly attracted to investigate unfamiliar urine, the duration of investigation

increasing with the freshness of the urine regardless of displacement of MUP ligands. In the initial test of fresh urine versus water, urine stimulated considerably more investigation than the clean slide (Fig. 3). When presented with intact versus displaced urine, investigation decreased with time since deposition ($F_{3,17} = 6.08$, $P < 0.005$) but ligand displacement had no effect ($F_{1,19} = 2.49$, NS; interaction between displacement and urine age: $F_{3,17} = 1.30$, NS; Fig. 3). Mice showed a similar reduction in investigation as urine aged regardless of their diet (repeated measures ANOVA, interaction between diet and urine age: $F_{3,16} = 1.12$, NS), although poultry farm mice spent significantly less

time than the other males investigating all of the urine samples ($F_{1,18}=5.54$, $P<0.05$). Menadione in ethanol initially drew mice to approach the test site (see above), but they then showed no further interest in this, investigation being stimulated only by urinary signals (Fig. 3). Odours did not alter how frequently mice visited test sites within or between tests except that, given a choice between fresh intact urine and water, mice visited the urine slightly more frequently (7.7 ± 0.9 versus 6.3 ± 0.7 visits; $t_{23}=2.19$, $P<0.05$).

DISCUSSION

The sudden loss of thiazole and brevicomin (and possibly other unidentified ligands) from urine streaks after displacement by menadione, compared with the much more gradual loss from intact urine, is the first direct confirmation of the hypothesis that MUPs in urine scent marks provide a gradual release of their ligands into the environment (Robertson et al. 1993). More importantly, the sharp change in behaviour towards urine samples after MUP ligands had been lost shows that the normal slow release of ligands from MUPs provides a functionally significant olfactory signal. These responses were shown by all of the males (note the small standard error bars in Fig. 1) even though they were wild-caught from a variety of populations and would have had quite different prior social experiences. Bacchini et al. (1992) suggested that brevicomin and thiazole would not be sufficiently volatile to provide an airborne olfactory signal and would instead act through contact with the vomeronasal system. However, we have shown here that these ligands, once released from MUPs, could provide a highly volatile signal that was detected several centimetres away from a scent mark. When ligands were dissociated from MUPs by rapid displacement using menadione, they evaporated completely within a few minutes and the hesitant response towards the mark was lost. It has been suspected for some time that peptides in mouse urine might bind and slowly release volatile odorants involved in reproductive priming effects (Albone 1984), but this appears to be the first demonstration that proteins evince a slow release of olfactory signals from mammalian scent marks, confirmed by a predicted behavioural response.

Within the context of male urine marks introduced into the territory of another male, MUP ligands had a very specific effect on investigatory behaviour, inducing a hesitation to approach as expected but no avoidance in the longer term. If the males had responded to the volatiles as an indication of a competitor's fresh scent mark, and thus an area to be avoided in case a potentially dangerous competitor was still nearby, their hesitancy to approach the area should have continued even after their first close investigation of the mark. However, their initial hesitation was very similar to the caution that male mice usually show when approaching conspecifics that might attack them, when they generally pause to investigate their odour from a 'safe' distance (Hurst 1993). Cox (1984, 1989) also found that captured males show strong and sustained avoidance of a continuous airflow carrying volatiles emanating from unfamiliar males or those from neighbouring territories. Scent marks introduced into another male's territory are usually investigated and counter-marked rather than avoided, however (Hurst 1990, 1993). The initial caution shown when our males approached a source of the same volatiles that are emitted continually from the bodies of potential aggressors (Apps et al. 1988) suggests that the mice may have been unable to detect from a distance whether the odour source was a fresh urine deposit or the donor himself. Once visited, it would be apparent that the source of volatiles was not the donor male and that caution in approaching the site was no longer necessary.

The nature of the males' response has thus given an indication of at least one likely function of this signal in competitive interactions between males. MUPs prolonged the release of these highly volatile signals from deposited urine marks for 1 h at least, raising the intriguing possibility that male mice use MUPs in the scent marks they deposit all over their territories to prolong signals that other mice may mistake for the male himself when detected at a distance. This is likely to provide a resident with a distinct advantage in defending his territory, since intruders would be unsure of his precise location, especially since mice interact mostly in the dark (at night or within covered sites).

This interpretation of the functional significance of the signal and response seems to be consistent with the pattern of scent marking by

territory holders and responses of other males. Exposure to competitors greatly increases the rate and distribution of urinary scent marking by dominant males (Desjardins et al., 1973) which cover their entire territories with urine marks, not just borders or limited sites (Hurst 1987, 1990), depositing up to 400 marks/h (Maruniak et al. 1974). While males that have been attacked may become cautious in approaching marked areas (e.g. Jones & Nowell 1973; Hurst 1990), scent marks do not normally prevent territory invasion (Hurst 1990, 1993), and even attract subordinates that reside within the male's territory (Hurst 1990) or unfamiliar males of relatively high body weight (Gosling et al. 1996a, b) which closely investigate such substrate signals. Thus males do not appear to invest in this slow release of airborne volatiles just to advertise defence of their territory from a distance and to prevent intrusions. Gosling & McKay (1990) and Hurst (1993) have shown that other males investigate and use the scent marks to assess the competitive ability of a resident and determine whether to flee or challenge should they meet the territory owner or another male. If scents were deposited simply to mark out the extent of a defended territory and warn intruders of the identity and dominance of the owner over the area (Gosling 1982), selection should favour a long-lasting signal of low volatility that would remain in the environment, to the advantage of both dominant residents and competitors wishing to avoid attack (see Hurst 1993). However, male mice invest heavily in a mechanism that provides a sustained release of highly volatile odours from the substrate (the same as those emanating from the males themselves), through the expensive production of MUPs and ligands and continuous effort in depositing marks around their territories. Their scent marks may thus act as 'scarecrows' which, while not preventing other mice from invading the territory, may provoke extreme caution and avoidance from males that have previously been attacked (Hurst 1990; Hurst et al. 1997).

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