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# Unravelling the chemical basis of competitive scent marking in house mice

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Major urinary proteins (MUPs) in the urine of male house mice, *Mus domesticus*, bind the male signalling volatiles 2-*sec*-butyl-4,5-dihydrothiazole (thiazole) and 3,4-dehydro-*exo*-brevicomin (brevicomin) and slowly release these volatiles from urinary scent marks. To examine the role of urinary proteins and volatiles, either attached or unattached to the proteins, in competitive scent marking, we fractionated urine from isolated male BALB/c laboratory mice, *Mus musculus*, by size-exclusion chromatography into three pools. Pool I contained all of the urinary proteins and their bound ligands while pools II and III contained lower molecular weight components including unbound signalling volatiles. In experiment 1, pools I–III were streaked out on to absorbent paper (Benchkote) and introduced into enclosures housing single wild-caught male mice, together with a clean control surface. Each male was tested with fresh stimuli and with aged stimuli deposited 24 h previously. Only pool I stimulated significantly more countermarking and investigation than the control, attracting mice to investigate from a distance even when the rate of ligand release was considerably reduced after 24 h. Experiment 2 examined responses to pool I when this was fresh, aged by 7 days, or had been mixed with menadione to displace ligands from the proteins. Although all three protein stimuli were investigated and countermarked more than a clean control, the aged and menadione-treated pool I stimulated the strongest responses, despite containing the lowest levels of thiazole and brevicomin. Thus competitive countermarking is stimulated by proteins or by nonvolatile protein-ligand complexes in male urine, while release of volatile ligands attracts attention to a competitor's scent marks.



Many male mammals advertise their competitive dominance and ability to defend territories by depositing scent marks and by countermarking those deposited by their competitors (reviewed by Ralls 1971; Johnson 1973; Gosling 1982, 1990; Hurst 1993; Hurst & Rich 1999). While traditionally such scent marks have been viewed simply as 'territorial markers', serving to keep intruders out while the territory owner is elsewhere, closer analyses have revealed that scent marks play a much more sophisticated role in dominance advertisement. Individuals can use the temporal and spatial deposition dynamics of scent marks to assess the dominance status and territory ownership of their competitors (Gosling 1982; Hurst 1993). Perhaps more importantly, these signals are used by the opposite sex to select high-quality mates that are able to countermark any challenging marks from competitors (Rich & Hurst 1998; Hurst & Rich 1999). Information concerning dominance status is held both in the chemical components of the scent mark (odour quality) and in the relative positioning and age of marks. Scent marks deposited more recently than any competitor's scents in the proximity signal a more dominant competitor, and thus a more attractive mate, since only animals successfully dominating a scent-marked area are able to ensure that their marks are the most recent (Hurst & Rich 1999); the relative age of competitors' scent marks can be signalled by overmarking directly on top of older scents (e.g. hamsters, *Mesocricetus auratus*: Johnston et al. 1995; meadow voles, *Microtus pennsylvanicus*: Johnston et al. 1997), or animals may be able to assess the age difference from chemical changes in the scent marks as they age (e.g. house mice, *Mus domesticus*: Rich & Hurst 1999). However, as yet, we know very little about the dynamics

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of competitive scent mark signalling or the chemical mechanisms involved.

Research into the chemical basis of competitive scent signals has focused largely on volatile odorants associated with individual social status. In laboratory mice, *Mus musculus*, for example, at least four volatiles in adult male urine have been associated with social dominance: 2-*sec*-butyl-4,5-dihydrothiazole (thiazole), 3,4-dehydro-*exo*-brevicomin (brevicomin) and α and β farnesenes (Apps et al. 1988; Harvey et al. 1989; Novotny et al. 1990). These volatiles are attractive to females (Jemiolo et al. 1985, 1991), stimulating early reproductive maturation and oestrus (Jemiolo et al. 1986; Novotny et al. 1999), while provoking aggressive competition between males (Novotny et al. 1985). The farnesenes are also reported to inhibit investigation and countermarking among subordinate male mice (Novotny et al. 1990; Jemiolo et al. 1992).

However, nonvolatile components of scents may also play an important part in chemical signals. Both inbred (Finlayson et al. 1963) and wild (Robertson et al. 1997; Pes et al. 1999) male mice produce a high concentration of proteins termed major urinary proteins (MUPs) in their urine. The thiazole and brevicomin volatiles associated with male dominance are largely attached to these proteins as ligands (Bacchini et al. 1992; Robertson et al. 1993), although farnesenes are released into urine from the preputial glands and little is known about their association with MUPs (Novotny et al. 1999). These protein–ligand complexes have been implicated in reproductive priming of females via the vomeronasal system (e.g. Guo et al. 1997; Keverne 1998; Krieger et al. 1999). MUPs also play a role in behavioural signalling by extending the release of volatile ligands from the numerous urinary scent marks that dominant male mice deposit around their territories (Hurst et al. 1998). The release of these volatile ligands into the surrounding airspace induces a cautious approach towards the scent mark from other males, probably because they are unable to distinguish in the dark whether the volatiles emanate from a scent mark or a dangerous competitor (Hurst et al. 1998). Although males are cautious in their first approach, the release of volatile ligands from urinary MUPs stimulates mice to explore a scent-marked area, even overcoming their normal reluctance to enter brightly lit areas after an initial investigation (Mucignat-Caretta & Caretta 1999).

Scents deposited in the environment as an advertisement of competitive dominance need to provide information on the species, sex and competitive status of the depositor together with its individual identity and the freshness of the deposit. While it might be predicted that animals would prefer to use relatively nonvolatile molecules that will remain in the environment for extended periods to advertise their dominance and identity, thus reducing the rate at which scent deposits need to be refreshed, nonvolatile signals may not be very effective. First, volatile airborne molecules may be required to draw attention to the presence of the scent mark (Beynon et al. 1999). Second, animals investigating a scent mark will need to establish its freshness if they are to use it as a reliable signal of the current dominance and recent presence of the depositor in the area. Signals that do not decay, or decay only very slowly, may thus not be deemed very reliable or useful since the depositor may not have been in the area for a long time. Assessing the freshness of a scent deposit from the loss or change in volatile molecules, on the other hand, may be difficult unless the amount or concentration in the original scent deposit is known. The concentration of a volatile molecule is the product of two variables: the amount deposited and the time since deposition. It is therefore impossible for a receiver to extract either variable from a single sampling. The attachment of highly volatile molecules to nonvolatile proteins in scents might fulfil both roles, however, by providing a volatile signal that will become airborne and attract attention while the ratio of volatile to nonvolatile components might provide information on signal age, regardless of the amount or concentration of the original deposit. Information on species, sex and individual identity could then be signalled by volatile and/or nonvolatile components of scents.

In this study, we examined the role of proteins and volatiles, either attached or unattached to the proteins, in stimulating the countermarking response of competitors to the urine scent marks of other male house mice.

## EXPERIMENT 1

To investigate whether protein–ligand complexes are implicated in competitive signalling among male house mice, we assessed competitive countermarking behaviour towards urinary components fractionated by size-exclusion chromatography, such that all urinary protein was contained in the highest molecular weight fraction. When active, male house mice frequently patrol their territories, continually depositing scent marks at a low rate to maintain fresh marks throughout the territory. If any new, clean (unmarked) surfaces are encountered, these are rapidly marked to ensure that all areas of the territory bear fresh marks (Maruniak et al. 1974; Hurst 1987, 1989, 1990). However, the challenge of a competitor's scent marks within the territory induces a much stronger countermarking response than the marks deposited on a novel (unmarked) patch of substrate (Hurst 1990). To assess competitive countermarking, we thus assessed which fractions of a competitor's urine stimulated significantly more countermarking when introduced into another male's territory than a clean surface marked only with buffer from the fractionation column.

We also examined the temporal dynamics of competitive signals by assessing the response of males to aged fractions of urine that had been deposited 24 h previously. In a previous study (Hurst et al. 1998), we showed that the volatile ligands that are associated with male social dominance and held in MUPs in adult male mouse urine are considerably reduced in whole urine deposits after 24 h. Such marks are no longer effective in inducing a hesitant approach from an unknown competitor. If males are signalling their competitive dominance through the slow release of these volatile molecules from their scent deposits, old scent marks that no longer

release ligands should provide no competitive challenge and should no longer stimulate significant countermarking. We thus compared the behavioural responses of mice towards each of the urine fractions with temporal changes in the concentration of volatile molecules in the deposited fractions and with their rate of release from the deposits.

## Methods

### Subjects

Since scent marking and responses to odours are influenced by a large number of environmental factors, particularly social experience, we used wild-caught, adult male house mice as subjects to ensure that the responses measured would be natural and not induced by highly artificial laboratory rearing conditions. Thirteen adult males were caught from two pig farms in Oxfordshire ($N$=9 feeding on a wheat-based pig diet, Livestock Modelling System, Cheltenham, U.K.) and Berkshire ($N$=4 feeding on a maize-based pig diet, Growell Feeds, Melksham, U.K.), and were held in individual cages (48 × 15 × 13 cm) on peat substrate with shredded paper nest material until the start of the experiment. Mice were provided with the same pig diet they had been feeding on prior to capture and water ad libitum throughout. At least 1 week before the start of countermarking trials, males were rehoused in individual enclosures (1.2 × 1.1 × 0.8 m) containing a single nestbox (15 cm diameter containing shredded paper nest material) and a food and water station (a clean cage top), both placed centrally in the enclosure. To stimulate competitive behaviour, a straight mesh-capped tunnel (9 cm long, 5 cm diameter) linking pairs of neighbouring male enclosures provided olfactory and some visual contact with their neighbour. Two days before the tests, shredded paper nest material from the cage of an isolated female caught from the same farm was scattered around each male's enclosure and one of the two mesh caps was removed from the connecting tunnel so that males could interact with their neighbour through the remaining grille for 30 min, providing a limited amount of direct contact without risk of fighting and injury. We removed the males' nestbox lids and bedding for this period to encourage activity and we observed interactions to ensure that the mice did not bite each other through the grille. If neighbouring males appeared to attempt to bite each other they were gently disturbed by tapping on the enclosure or pushing them away so that they withdrew from the grille. Female nest material was removed again the next day, 1 day before tests began, and mesh caps on the tunnels linking neighbours were replaced with solid caps to remove any neighbour influence during the experiment. Solid caps were replaced by mesh caps again in between successive tests to maintain the males' competitive behaviour, but tunnels were always blocked with solid caps 1 day prior to any tests. Pilot tests indicated that these stimuli induced a more reliable competitive countermarking response than isolation alone. Adult male house mice will normally attempt to exclude any other males from their territory by direct aggression, so housing adult males in separate territories was appropriate for this species. Provision of female odours and contact with a neighbouring male helped to reduce any sense of social isolation.

Males were kept under reverse day lighting with white lights on between 0000 and 1200 hours and darkness from 1200 to 0000 hours when all tests were carried out. At the end of the study, we kept the males in captivity for further behavioural tests.

### Stimulus urine

We tested the males using pooled urine collected from 10 singly housed male inbred BALB/c laboratory mice, allowing us to collect a large amount of standardized urine stimuli for use in both behavioural and biochemical analyses. Laboratory mice are derived from the house mouse and males of the BALB/c strain produce very similar levels of urinary proteins and associated volatiles to those of wild mice (Robertson et al. 1998; Beynon et al. 1999). Males were housed in cages (29 × 16 × 13 cm) on sawdust substrate with food (RDS RM3 diet, Special Diet Services, Norwich, U.K.) and water ad libitum. We obtained urine by holding each donor by the scruff over a clean Eppendorf tube and gently massaging the bladder. Collected urine was frozen immediately at −20°C until use. Immediately prior to use, test urine was defrosted and split into three fractions according to molecular weight by size-exclusion chromatography. A column, of dimensions 8 mm × 22 cm, was packed with 10 ml of Sephadex G-25 the day before and allowed to settle. This was then equilibrated with 10–50 ml of 0.1 M phosphate buffer/0.1 M NaCl, pH 7.4, before we applied 1 ml of urine to the top of the column. The urine sample was pooled from a minimum of five individual donors. The urine was considered to have fully penetrated the gel bed when the column eluent ceased to flow. We then developed the column by adding 10 1.5-ml aliquots of buffer to the column head. Each aliquot was allowed to penetrate the gel bed fully and, in each case, all the eluent associated with that aliquot was collected as a fraction. The first of the 10 1.5-ml fractions (assumed to be column buffer) was discarded, whilst the remaining fractions were pooled in the following manner based on prior biochemical analysis: fractions 2, 3 and 4 (pool I), fractions 5, 6 and 7 (pool II) and fractions 8, 9 and 10 (pool III). We used these pooled fractions for subsequent behavioural and biochemical analyses.

### Biochemical analyses

*Determination of protein concentration.* We determined the protein concentration of urine samples and gel filtration column eluent by using the Coomassie Plus assay (Pierce Chemicals, Chester, U.K.), in a microtitre plate format. The assay was calibrated with standard solutions containing 0, 10, 20, 30, 40 and 50 μg/ml of bovine serum albumin, prepared from a stock solution (2 mg/ml) supplied with the assay. We prepared samples by diluting whole urine or column fractions 1:100 (5 μl+495 μl). Aliquots (100 μl) of samples and standards thus prepared

were pipetted in duplicate into the wells of a 96-well microtitre plate. Coomassie Plus protein assay reagent (250 µl), previously equilibrated to room temperature, was added to all sample and standard wells. The plate was then inserted into a Labsystems iEMS-MF plate reader which, after mixing and incubation at room temperature for 1 min, measured the absorbance of light at 620 nm in each well. The absorbance of the standard solutions was plotted as a function of their concentration and we used the resulting graph to determine the protein concentration of the samples.

*Determination of creatinine concentration.* The muscle metabolite creatinine, used to determine the elution position of low molecular weight urinary components, was measured by the alkaline picrate assay (Sigma Chemicals, Poole, Dorset, U.K.) in a microtitre plate format. We calibrated the assay using standard solutions containing 0, 5, 10, 15, 20 and 30 µg/ml creatinine, prepared from a stock 30 µg/ml solution supplied by the manufacturer. Aliquots (100 µl) of standards and samples were pipetted in duplicate into the wells of the microtitre plate. All samples and standards were then treated with a 150-µl aliquot of alkaline picrate solution. The plate was shaken and incubated for 15 min at room temperature inside a Labsystems iEMS-MF plate reader after which the absorbance of light at 492 nm was measured. Creatinine concentration in samples was determined by comparison to absorbance values obtained from standard solutions.

*Static headspace sampling and GC/MS analysis of volatiles.* To determine the loss of MUP ligands from deposited samples of urine and fractionated urine, we deposited samples on to squares (2 × 3 cm) of Benchkote (polythene-backed absorbent paper; Whatman International Ltd, Maidstone, U.K.). These were incubated at room temperature for 0 and 1 h and 1, 2, 3, 4 and 7 days. We conducted incubation in a reverse manner, culminating at 0 h. After incubation, the Benchkote squares were cut into pieces of ca. 10 × 2 mm and placed in a headspace vial. A 300-µl aliquot of human 'carrier' urine was added to each vial prior to sealing with gas-tight crimp caps. The samples were then placed in a Hewlett-Packard HP7694E headspace sampler. Each vial was incubated at 100°C for 20 min, prior to withdrawal of 3 ml of the headspace gas. This was subsequently injected into the split/splitless injection port of a Hewlett-Packard 5890(II) gas chromatograph, maintained at 250°C and fitted with a Zebron wax column of 30 M × 0.55 mm (Phenomonex, Macclesfield, U.K.). The initial column temperature was 100°C, rising to 200°C at 20°C/min. Compounds emerging from the GC column were detected by a Hewlett-Packard 5971A mass selective detector, run in selected ion mode for ions of m/z 60 (2-*sec*-butyl-4,5-dihydrothiazole), 95 (3,4-dehydro-*exo*-brevicomin) and 93 (α and β farnesenes). Chromatographic peaks corresponding to thiazole, brevicomin and farnesenes were identified by the presence of these ions and on the basis of their retention times. The abundance of each ligand was determined from their integrated peak areas.

To examine the partitioning of volatiles in each of the three pools eluting from the column prior to deposition, we analysed the volatiles in each 1.5-ml fraction directly from the liquid eluent of the column. In this instance, headspace sampling and GC/MS were performed on 150 µl of the column eluent. Headspace sampling and GC/MS parameters were the same as described above.

### Countermarking trials

We presented males with stimuli in their own home enclosures at the beginning of the active dark period. We gave them a choice of four stimuli simultaneously and measured their countermarking responses towards the four stimuli after 30 min and after 10 h. From a neighbouring laboratory, we also videorecorded their visits and investigatory behaviour over the first 30 min, under infrared lighting. The four stimuli consisted of the three urine pools and an equivalent volume of buffer from the fractionation column as a 'novel' control stimulus. The control column buffer was obtained from the fractionation column after the column had been conditioned with buffer but before any urine was added. For each stimulus, two 50-µl streaks were deposited in the centre of a square of Benchkote wrapped around a Perspex tile measuring 15 × 15 cm. Surgical gloves were worn to avoid any human contamination of stimulus tiles. Each tile was placed against a different side wall of a male's enclosure, allocated by random number; none was close to the corners of the enclosure or to the tunnel separating neighbouring enclosures where marking was likely to be heavier (personal observations). The lid of the male's nestbox and the nest material were then removed and the experimenter left the room to encourage immediate exploratory activity. We presented stimuli either 3–5 min after deposition or after 24 h. Stimulus tiles were prepared in a clean laboratory where 24-h aged stimuli were stored on open shelves until use. Each mouse experienced two trials, one with fresh stimuli and one with 24-h aged stimuli, presented 7 days apart in a balanced design such that half the males experienced the fresh stimuli first and half the aged stimuli first. After 30 min, the male's nestbox lid and nest material were replaced and we removed the four test tiles briefly to count the number of urine marks deposited (marks were visualized under ultraviolet light and ringed lightly with pencil to distinguish them from any subsequent marking). The tiles were then replaced in the same positions in the male's enclosure for a further 9.5 h to measure his longer-term marking response.

### Analysis of behavioural data

*Urine marking.* Urine marks deposited on the Benchkote-covered tiles were visualized under ultraviolet light (Desjardins et al. 1973). We measured the total number of urine marks on each tile deposited over the first 30 min and 10 h, together with the percentage of the tile surface covered with urine after 10 h. We also scored the number of marks that directly overlaid the stimulus marks and the total area covered by the stimulus marks. We measured the surface area covered by placing a grid of points 1 cm

square over the top of the tile and counting the number of points that overlaid urine or stimulus marks. This was divided by the total number of points in the grid ($N$=256 points) and multiplied by 100 to give the percentage coverage. Since mice occasionally chewed parts of the Benchkote, making less surface area available for marking and thus providing an underestimate of their marking response, we measured how many grid points had been chewed away and corrected both the coverage and frequency of marks to give marks per 15 $cm^2$ of surface area. Although the number of marks deposited after 30 min was recorded, tests with whole urine stimuli showed that the number of countermarks deposited was not significantly greater than on a water control after 30 min but was highly significant after 10 h (data not presented). Tests with whole urine also showed that while there was a strong correlation between the number of marks deposited and total area covered, the number of marks was a more significant measure of countermarking in response to a normal whole urine stimulus. In this paper, we thus present data only for the number of urine marks deposited over the full 10 h period, although the percentage of area covered with urine also showed the same pattern of response. Countermarking responses to urine pools after 30 min followed the same pattern as longer-term responses over 10 h but were not generally statistically significant.

*Investigatory behaviour.* To analyse the videotapes of investigatory behaviour we used Ethovision (Noldus Information Technology, Wageningen, The Netherlands), an automated image analysis system. We measured the latency to visit each tile, the frequency of visits and the total time spent on each tile over the first 30 min.

*Data analysis.* Two males failed to mark the test tiles. These males also failed to mark a whole urine stimulus used in pilot tests prior to this experiment to assess the best measure of countermarking. We therefore excluded these males from the analysis as nonresponders. We used nonparametric tests to analyse marking data since there was considerable variation in the numbers of marks deposited by different individuals. We first examined the response to fresh stimuli to establish which urine pool(s) stimulated countermarking. After confirming with a Friedman test that there was a significant difference in the number of marks deposited on the four stimulus tiles, we used specific Wilcoxon (signed-ranks exact) tests (SPSS Inc. 1999), to examine which of the three fresh urine pools stimulated more marking than the column buffer control. Since we had no clear prediction for effects on investigatory behaviour, we compared latencies to the first visit, the total number of visits and total time spent on each urine pool tile with those for the control stimulus, using nonspecific (two-tailed) Wilcoxon signed-ranks exact tests. Since the three simultaneous comparisons to the control were nonorthogonal, we adjusted probability values using the Bonferroni inequality (Meddis 1984).

We then examined whether the same urine pool(s) still stimulated countermarking and investigation 24 h after deposition. We also compared the total number of marks deposited on test tiles in the fresh and aged trials, and whether countermarking was greater on each urine pool when this was fresh, by using Wilcoxon signed-ranks exact tests, adjusting probabilities for multiple comparisons as above. We used parametric repeated measures ANOVA to examine the effects of urine pool and stimulus age on latencies to the first visit, total number of visits and time spent on each tile, after transformating latencies and total time on tiles by logarithms so that all investigation data approximated normal distributions (Kolmogorov–Smirnov tests of normality: NS). All data are presented as $\bar{X} \pm 1$ SE ($N$=11).

## Results

### *Biochemical analysis of pooled urine fractions*

Protein was confined to the early fractions eluted from the chromatography column (Fig. 1). These fractions contained the MUPs, which constitute over 99% of the protein in normal, adult male mouse urine. The creatinine assay indicated the elution position of low molecular weight molecules. However, very high proportions of the thiazole and brevicomin were eluted with the protein peak (Table 1), consistent with their known behaviour of binding in the central cavity of the MUPs. Furthermore, the small amounts of farnesenes that could be detected in the elution trace were protein associated. The recovery of farnesenes was only ca. 1% of applied material, and it is likely that free farnesenes were retained nonspecifically by the column. Thus, the protein fraction is associated with a very small proportion of the farnesenes in urine samples.

The column fractions were combined into three pools. Pool I contained the proteins and most of the thiazole and brevicomin and had a relatively weak 'mousey' odour to the human nose. Pool II contained the urinary creatinine and the unbound portion of the thiazole and brevicomin and had an acrid 'mousey' smell. Pool III had no detectable proteins or ligands, but possessed a similar but weaker acrid 'mousey' smell and was included in the analysis.

### *Response to fresh urine fractions*

As expected (Hurst 1989, 1990), males deposited urine marks on all of the introduced tiles as these all represented novel substrate, but there was a significant difference in the number of marks deposited on the four stimulus tiles after 10 h (Fig. 2a; Friedman test: $\chi^2_3$=10.53, $P$<0.025). Only pool I (protein) stimulated significantly more countermarking than the column buffer control (pool I: 103 ± 29 marks; Wilcoxon signed-ranks exact test: $Z$= − 2.40, $P$<0.025; Fig. 2a). Neither of the two free-volatile fractions stimulated significantly more marking than the control tile and a post hoc test confirmed that countermarking of pool I was significantly different from that of the volatile pools II and III ($Z$= − 2.31, $P$<0.025). Urine marks were mostly deposited on the clean surface of the tile, particularly around the edges, rather than over

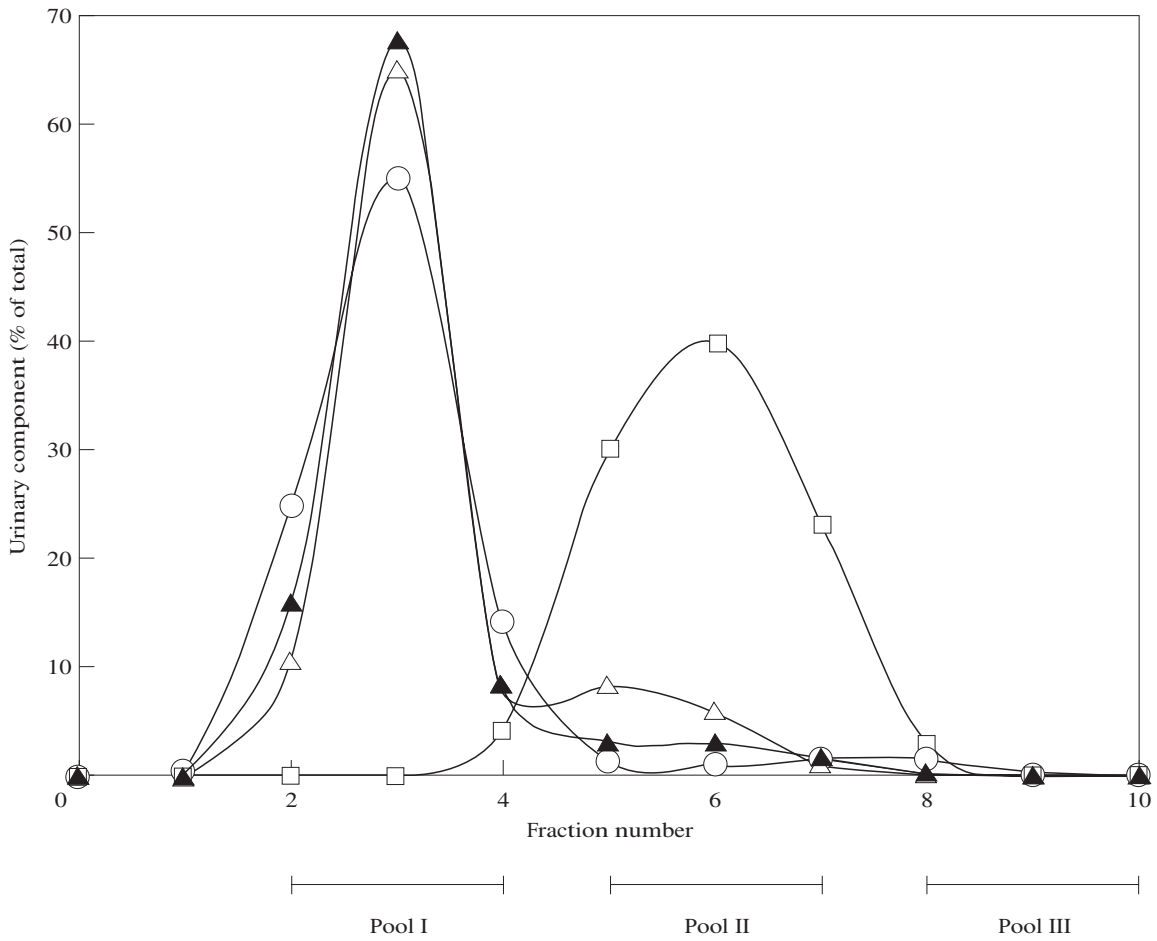


**Figure 1.** Separation of urinary components by gel-filtration chromatography. A sample (1 ml) of adult male BALB/c urine was applied to a 10-ml column of Sephadex G25 and 10 1.5-ml fractions were collected in succession as the column was developed. After analysis of each fraction for protein (○), creatinine (□), 2-*sec* butyl-4,5-dihydrothiazole (▲) and 3,4-dehydro-*exo*-brevicomin (△) the fractions were combined into three separate pools as indicated on the figure.

**Table 1.** Partition of specific urinary components among the three fractionated urine pools tested

| | Percentage partition of | | | |
|---|---|---|---|---|
| Pool | Protein | Creatinine | Brevicomin | Thiazole |
| I | 94 | 4 | 84 | 92 |
| II | 4 | 93 | 16 | 8 |
| III | 2 | 3 | 0 | 0 |

the top of the protein stimulus (4.1% of marks were deposited on top of the protein stimulus which covered 3.9% of the tile, and three out of 11 marked tiles showed no overlap at all; this was very similar to the degree of 'overmarking' on pools II and III). Even after 30 min, countermarking showed the same general pattern, but differences were not statistically significant (see Analysis of behavioural data).

Corresponding to this bias in urine-marking behaviour measured over a 10-h period, males showed a strong initial attraction to investigate the high molecular weight protein pool I over the first 30 min of exposure. There was normally a very high variance in the time it took for individuals to visit a stimulus tile in these tests if there was no stimulus to attract them to that particular location, since mice did not normally visit the edges of their enclosures very frequently; occasionally, mice failed to visit within the first 30 min of the recording period. However, the majority of mice visited the fresh protein pool I within the first 30 s of the test, with very little variance (Fig. 3), visiting this fraction significantly more quickly than the column buffer control ($Z = -2.54$, $P<0.025$). This suggests that they were attracted from a distance by volatiles emanating from the proteins in pool I. Although there was a tendency to visit pool II more quickly too (Fig. 3), this was not significantly different from the control ($Z = -0.99$, NS). Only pool I stimulated more visits in total over the first 30 min than the control tile (Fig. 2b), although this difference was not significant when probability values were adjusted for multiple comparisons with the control tile ($Z = -1.99$, $P=0.07$). Males spent no more time in contact with the protein pool than with the buffer control over the first 30 min, however (Fig. 2c).

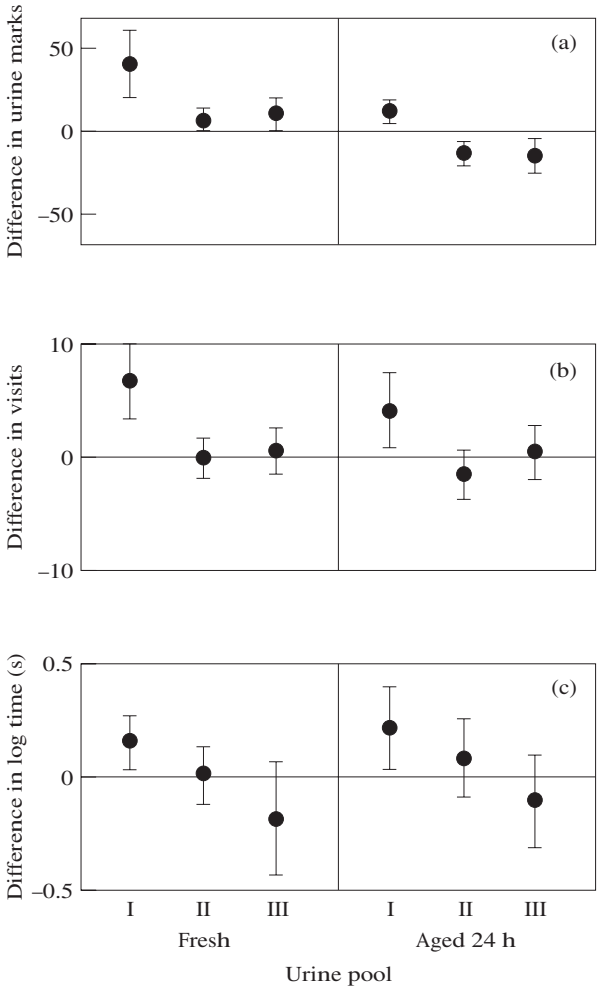


**Figure 2.** Response of male mice to fractionated urine pools I–III (see Fig. 1) when fresh or presented 24 h after deposition, relative to their response to a control tile marked only with column buffer. (a) Number of urine marks; (b) number of visits; (c) duration of visits. In each case the data show the response to the urine pool minus the response to the control presented simultaneously. Positive values represent a greater response to the urine pool than to an equivalent unmarked surface. Values are $\bar{X}$±SE ($N$=11).

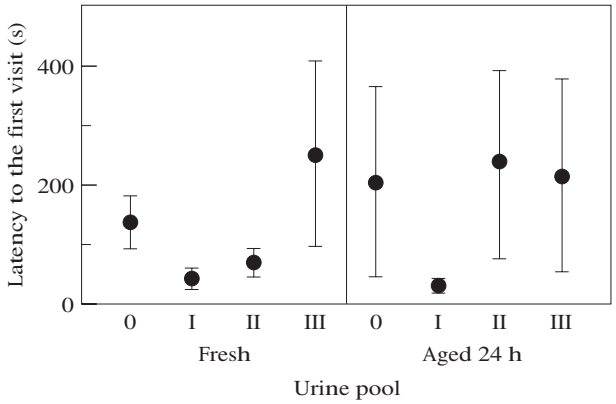


**Figure 3.** Latency to the first visit to fractionated urine pools I–III (see Fig. 1) and a control tile (0) when fresh or presented 24 h after deposition. A visit was scored when the mouse was close enough to touch the tile. Values are $\bar{X}$±SE ($N$=11).

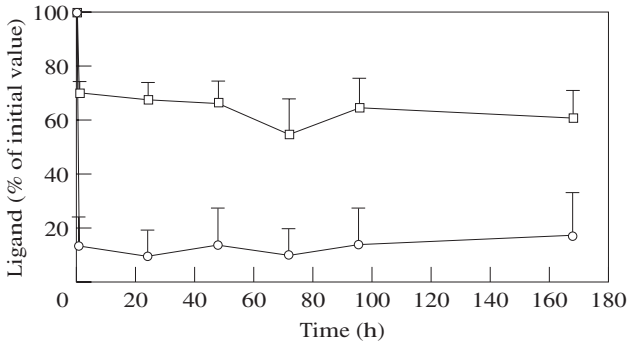


**Figure 4.** Loss of MUP-associated ligands from desalted MUPs dried on to Benchkote substrate. A portion (50 µl) of pool I material (see Fig. 1) was applied to Benchkote (absorbent side) and was allowed to dry. At different times after application of samples, the residual 2-*sec* butyl-4,5-dihydrothiazole (□) and 3,4-dehydro-*exo*-brevicomin (○) were measured by GC/MS. The residual level of these ligands is expressed relative to a sample of similarly treated pool I material that was spotted on to Benchkote and assayed immediately. Values are $\bar{X}$±SE ($N$=4).

It is notable that although some bias in investigation towards pool I was apparent over the first 30 min of exposure, stimulating more frequent visits, the bias in marking behaviour in favour of this pool was not apparent when tiles were removed after 30 min. This suggests that initially the males responded to the novelty of all introduced tiles by investigating and marking them all. The bias in marking developed only after a more prolonged period of exposure.

### *Response to aged urine fractions*

Mice deposited fewer marks in total when tested with urine fractions that had been deposited 24 h previously compared with their response to fresh test stimuli (306 ± 75 marks in fresh trials, 240 ± 70 marks in aged trials; Wilcoxon signed-ranks test: $Z = -2.0$, $P<0.025$), although there was considerable variation between individuals in the number of marks each male deposited (range 10–684 per 10-h test). One day after deposition, the levels of thiazole and brevicomin ligands associated with pool I had dropped appreciably compared with fresh deposits, thiazole levels having fallen to ca. 67% of their original concentration while brevicomin had dropped to ca. 10% (Fig. 4). The rate of release of these volatiles from the deposits had dropped to very low levels, from 30%/h over the first 1 h to 0.1% over the next 23 h for thiazole. For brevicomin, the corresponding figures were 86%/h and 0.2%, respectively. However, males still discriminated between the four aged stimuli in the number of urine marks deposited over 10 h (Fig. 2a; Friedman test: $\chi^2_3=8.67$, $P<0.05$). The aged protein pool I continued to stimulate significantly more marking than both the column buffer control ($Z = -1.69$, $P<0.05$) and the free volatile pools ($Z = -2.22$, $P<0.025$). Although the frequency of marking tended to be lower towards aged stimuli overall (see above), there was no significant difference in the number of marks deposited on fresh and

aged pool I in the two separate tests ($Z = -0.80$, NS). As expected from countermarking responses to fresh stimuli, there was no significant countermarking of the free volatile pools aged by 24 h and, indeed, these now tended to stimulate less marking than the column buffer control (see Fig. 2a).

The pattern of investigatory behaviour over the first 30 min was similar to that shown towards fresh stimuli. Again, all males consistently visited pool I within a few seconds of the start of the test in contrast to the very high variance in latency to visit the control tile (Fig. 3). A repeated measures analysis of response to control and protein pools confirmed that there was no significant effect of ageing on latency to the first visit ($F_{1,10}=1.60$, NS) but there was a significant effect of the presence of protein ($F_{1,10}=13.79$, $P<0.005$), with males attracted to visit the protein pool more quickly whether fresh or aged. The protein pool was also visited more frequently than the control ($F_{1,10}=12.88$, $P<0.005$) with no effect of stimulus age ($F_{1,10}=0.27$, NS) or interaction between stimulus age and type ($F_{1,10}=0.20$, NS; Fig. 2b). However, there was no difference in the total time spent on the protein pool I tile compared to the control (Fig. 2c), even when data for both aged and fresh tests were considered together ($F_{1,10}=3.26$, NS). There were no significant effects of the volatile pools II or III on investigatory behaviour.

## Discussion

While any novel object introduced into a mouse's home enclosure will induce some investigation and urine marking, the only fraction of urine that stimulated more investigation and more countermarking than this novelty response was that containing the urinary proteins and their associated volatile ligands. Surprisingly, the two free-volatile pools stimulated no more investigation than a column buffer control, even though they all had a very strong and characteristic 'mousey' odour to the human nose, particularly pool II which retained this very noticeable odour even after 24 h. The protein pool contained much higher levels of the known male signalling volatiles thiazole and brevicomin, however, and had a much 'sweeter' odour to the human nose than the acrid odour of the two lower molecular weight fractions. Although these signalling volatiles exist in unbound form in equilibrium with bound ligands in mouse urine (Robertson et al. 1993), free ligands evaporate very quickly when not bound to MUPs (see also Hurst et al. 1998). Attraction to investigate and countermark the protein components might therefore be stimulated by the continual slow release of these highly volatile male signalling molecules from the MUPs (Robertson et al. 1993; Hurst et al. 1998). However, although mice generally showed less countermarking overall when presented with stimuli deposited 24 h previously than with fresh stimuli, countermarking was not significantly reduced on the aged protein compared with the fresh protein pool, even though there was a substantial reduction in the level of volatile ligands in the aged deposit and the rate of release of airborne volatiles had dropped to very low levels after 24 h. This gradual loss of ligands from protein deposits over 24 h corresponds to our findings when urine was streaked out on to glass slides (Hurst et al. 1998), although a greater proportion of the thiazole and brevicomin was retained in the deposits on Benchkote. This absorbent substrate appears to retain some of the volatile signalling molecules even when they are not attached to MUPs, preventing their evaporation. Despite the great reduction in the rate of release of volatile ligands after 24 h, the consistent short latency to the first visit shows that mice were still attracted to investigate the aged protein pool I from a distance; thus they must still have been able to detect the release of volatile ligands.

The tendency to mark the aged volatile pools less than a clean control might reflect our observation that these tiles now bore just a faint 'mousey' odour which may have presented less of a contrast against the mouse's own marked territory than the introduction of a clean control tile completely devoid of mouse odours. Thus, there may have been less of a positive stimulus to mark a 'hole' in its scent-marked territory.

Based on these results, we suggest that either (1) countermarking is stimulated by detection of a volatile signal released from proteins even at the low levels that remained in the deposit after 24 h, or (2) competitive countermarking is stimulated by nonvolatile proteins or by nonvolatile protein–ligand complexes in urine deposits and the release of volatile male signalling molecules from the deposits acts only to attract mice to investigate the scent marks closely. When investigating urine marks, mice always sniff them very closely (Hurst 1990, 1993; present study), which would allow even nonvolatile materials to be taken up into the nose by direct contact, as an aerosol or in particulate form. Although there was no significant difference in the strength of their response to fresh and aged protein pools in these two separate tests, despite the difference in ligand concentration after 24 h, mice were not given a direct choice between these stimuli and they may have responded strongly to a very weak, aged protein–ligand stimulus because this was the only competitive stimulus present in their territory at the time.

# EXPERIMENT 2

To test further whether competitive countermarking is stimulated by the male signalling volatiles associated with MUPs or by the proteins themselves, we manipulated the concentration of ligands in the protein fraction of mouse urine either by natural evaporation over a more prolonged period or by displacement with menadione. Menadione (vitamin K) is a familiar and natural component of a mouse's diet but will displace the natural signalling ligands from the MUP calyx when mixed with urine in vitro (Robertson et al. 1998). Once displaced, ligands quickly evaporate from the scent deposit (Hurst et al. 1998). Menadione thus provides a natural chemical tool for ligand displacement that is familiar to mice and that does not appear to stimulate any response in itself (Hurst et al. 1998). In this experiment, we gave mice a direct choice between freshly deposited urinary proteins,

protein deposits that had been aged for 7 days to allow them to lose a very high proportion of their volatile ligands naturally, and fresh proteins from which ligands had been displaced by mixing with menadione, together with a column buffer control.

## Methods

We used 12 wild-caught males, seven of which had been used in experiment 1 and another five caught from the same pig farms, allowing at least 6 weeks between the two experiments to ensure that males did not habituate to the urine stimuli. The urine donors and experimental protocol were the same as in experiment 1. Stimulus urine was fractionated by size-exclusion chromatography as before, but in this experiment we used only pool I, containing virtually all of the urinary protein and most of the detectable thiazole, brevicomin and farnesenes attached as ligands to the MUPs (Fig. 1, Table 1).

Mice were presented with the following four stimuli simultaneously over a 10-h period; each stimulus was deposited on a separate Benchkote-covered tiled 15 cm square placed against a different side wall of a male's enclosure, allocated by random number, as described in experiment 1.

(1) Two 50-µl streaks of fresh urinary protein (pool I) deposited in the centre of the tile plus a separate 5-µl streak of menadione solution (4 mg/ml menadione in 100% ethanol) near one edge of the tile, all deposited 30 min before testing.

(2) Two 50-µl streaks of urinary protein deposited in the centre of the tile and left for 7 days in a clean laboratory, plus a separate 5-µl streak of menadione solution freshly deposited near one edge of the tile 30 min prior to testing.

(3) Fresh urinary protein (190 µl) was mixed with 10 µl of menadione solution. After leaving the mixture for 5 min to allow ligand displacement, we deposited two 50-µl streaks and one 5-µl streak in the same pattern as on the other tiles and left them for 30 min to allow displaced ligands to disperse prior to testing.

(4) Two 50-µl streaks of fresh column buffer deposited in the centre of the tile plus a separate 5-µl streak of menadione solution near one edge of the tile, deposited 30 min before testing.

A small streak of menadione solution in ethanol was thus present on all stimulus tiles in case this stimulated any increase in scent marking and investigation. Investigatory responses were videorecorded for the first 30 min of exposure. We removed tiles temporarily after 30 min to measure the initial number of urine marks deposited, then returned them for a further 9.5 h to measure longer-term marking behaviour as in experiment 1. Again, only countermarking over the 10-h period is reported here as marks were deposited at a low level on all introduced tiles initially and countermarking became apparent only over this longer period (see above). Biochemical analyses were carried out on replicate samples of the stimulus deposits as outlined in experiment 1. At the end of the study, we kept the males in captivity for further behavioural tests.

### *Data analysis*

First we tested whether urinary protein generally stimulated more marking than the control buffer by comparing the number of marks deposited on the three protein stimuli with the buffer control using a specific Wilcoxon matched-sets test (Meddis 1984). We then compared responses between the urinary protein stimuli using nonspecific Wilcoxon signed-ranks exact tests since the hypotheses arising from the first experiment predicted different results: if countermarking is stimulated only by the proteins themselves, there should be no difference in response towards protein stimuli that differ in ligand concentration; if countermarking is stimulated by volatile ligands attached to proteins, or by their slow release from the proteins, there should be strong countermarking of the fresh intact protein–ligand fraction but much less or no countermarking when ligands have evaporated naturally or been displaced by menadione. All data are presented as $\bar{X} \pm 1$ SE ($N$=12).

## Results

The number of urine marks deposited over a 10-h period confirmed that the three urinary protein stimuli generally stimulated significantly more countermarking than the control column buffer (Fig. 5a; Wilcoxon matched-sets test: $Z = -2.32$, $P<0.025$). There was also a difference in the level of countermarking on the three protein stimuli, suggesting that qualitative differences in the marks played a role in stimulating countermarking. However, it is apparent from Fig. 5a that this was not in the direction expected. Males deposited more marks when the protein had been air dried and aged for 7 days than when it was fresh ($Z = -2.28$, $P<0.025$). After 7 days, the protein samples deposited on Benchkote substrate still retained ca. 60% of the original thiazole and 15% of the brevicomin, when compared with samples deposited initially (Fig. 4). The absence of any change in the ligand levels in deposits over the previous 5 days indicated a stable association with the substrate, and that no further volatiles were being released into the air. Mixing the protein fraction with menadione had the effect consistent with its ability to act as a ligand displacer (Robertson et al. 1998): thiazole was reduced immediately to 45% and brevicomin to 10% of the initial values. By 24 h, the residual ligand levels had stabilized at 35 and 0%, respectively (Fig. 6). Although the difference in the numbers of marks deposited when the ligands had been displaced by menadione compared with the intact protein–ligand complex did not reach statistical significance, marking again tended to be higher rather than lower when ligands had been displaced ($Z = -1.84$, $P<0.10$; Fig. 5a).

In this experiment, mice consistently visited all four tiles within the first 2–3 min of introduction, so there were no significant differences in how quickly they visited the protein compared with the control tile. Their lowest latencies tended to be towards the menadione treatment, where volatiles had been displaced from MUPs just prior to the test and were thus likely to have been at the highest concentration in the surrounding air (latency

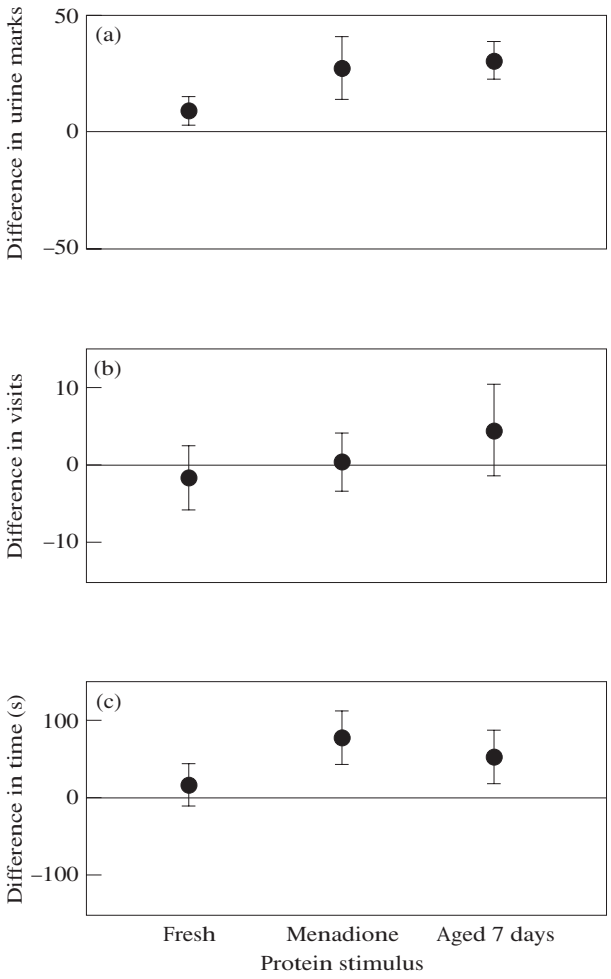


**Figure 5.** Response of male mice to urine pool I containing all urinary proteins (see Fig. 1) when presented fresh, when mixed with menadione or 7 days after deposition, relative to the response to a control tile presented simultaneously. (a) Number of urine marks; (b) number of visits; (c) duration of visits. In each case the data show the response to the protein stimulus minus the response to the control so that positive values represent a greater response to the protein stimulus than to an equivalent unmarked surface. Values are $\bar{X}$±SE ($N$=12).

to visit menadione: 62 ± 23 s; 7-day-aged protein: 104 ± 73 s; fresh protein: 124 ± 59 s; control: 135 ± 42 s). Mice were also stimulated to visit all four tiles frequently in this test, including the control tile (control tile in experiment 2: 17 ± 2.4 visits; fresh control tile in experiment 1: 9.4 ± 1.5 visits; 24-h-aged control tile in experiment 1: 9.6 ± 2.5 visits; the frequency of visits to all tiles in experiment 2 was very similar to the high number of visits to fresh or aged protein pools in experiment 1). It is thus likely that the presence of protein stimuli on three out of four introduced tiles stimulated a general rather than specific increase in investigation of the tiles (Fig. 5b). Males did, however, spend more time on the urinary protein stimuli on average than on the buffer control ($Z$= − 2.31, $P$<0.01). They also tended to spend more time investigating the 7-day-aged and the menadione-treated protein fractions than the fresh protein stimulus (Fig. 5c),

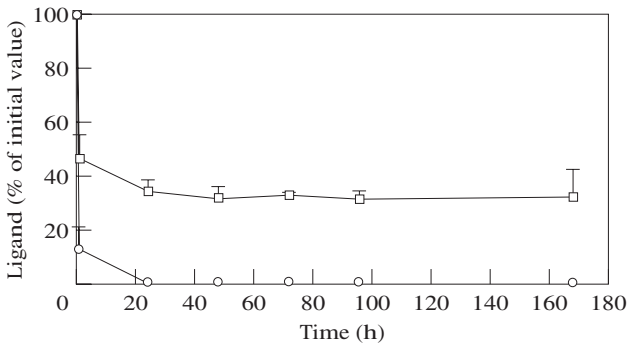


**Figure 6.** Loss of MUP-associated ligands from menadione-treated, desalted MUPs dried on to Benchkote substrate. A portion (190 µl) of pool I material (see Fig. 1) was treated with 10 µl of a solution of menadione (4 mg/ml in ethanol). An aliquot (75 µl) was subsequently applied to Benchkote (absorbent side) and allowed to dry. At different times after application of samples, the residual 2-*sec* butyl-4,5-dihydrothiazole (□) and 3,4-dehydro-*exo*-brevicomin (○) were measured by GC/MS. The residual level of these ligands is expressed relative to a sample of similarly treated pool I material that was spotted on to Benchkote and assayed immediately. Values are $\bar{X}$±SE ($N$=4).

reflecting their countermarking preference, although differences between the protein stimuli were not statistically significant.

## Discussion

Our results suggest that countermarking is not a response to volatile male signalling molecules being released from the urinary proteins. It is a response to the nonvolatile urinary proteins themselves, or to the nonvolatile protein–ligand complexes that remain in protein deposits. Indeed, volatile ligands (or their release into the air) appeared to have a slight inhibitory effect on the extent of countermarking when fresh protein was presented alongside other protein deposits that had much lower levels of male ligands, although all of the protein deposits were countermarked more than the control novel substrate. Volatile ligands thus did not inhibit countermarking per se and, in our first experiment, males countermarked very strongly a fresh protein deposit that had high ligand levels when this was the only protein stimulus introduced into their territory. In our second experiment, by contrast, the countermarking response was split between a choice of three different protein stimuli.

This apparent response to the proteins (or to nonvolatile protein–ligand complexes) rather than to the volatile male signalling molecules is rather surprising in view of the nonvolatility of protein as an odour stimulus, and suggests that the characteristic approach and very close investigation of scent deposits that mice always show (unless prevented from contacting the scent source) may be important for detecting the stimulus, either through nasal contact with the scent deposit or by allowing proteins to be drawn up into the nasal cavity in an aerosol or in particulate form. Krieger et al. (1999) have recently shown that receptors in the vomeronasal organ

respond specifically to major urinary proteins in rats, even when no volatile signalling molecules are bound to these MUPs. Removal of the vomeronasal organ considerably reduces the normal marking behaviour of male mice (Maruniak et al. 1986), suggesting that competitive scent mark advertisement may operate through the vomeronasal system.

The strength of male countermarking appeared to correspond to the amount of time mice spent investigating the protein deposit. The apparent preference for countermarking deposits that contained comparatively few volatile ligands may thus have arisen incidentally because information was harder to gather from these deposits, thus males spent longer investigating them. Alternatively, males may have preferred to countermark proteins that had comparatively few ligands because the main MUP ligands are positively associated with the aggressive status of a competitor (Apps et al. 1988; Harvey et al. 1989; Novotny et al. 1990; Jemiolo et al. 1992). The low level of these ligands in the aged and menadione-treated protein deposits might thus suggest that the signal came from a more subordinate or less aggressive male and Hurst (1990) showed that dominant males tend to mark more strongly in response to urine from unfamiliar subordinates than in response to urine from another dominant male.

## GENERAL DISCUSSION

At first sight, we might expect males to respond more strongly to scent marks that indicate a challenge from a highly aggressive intruder likely to threaten the resident male's own dominance. However, there are greater risks associated with challenging a strong competitor and, not surprisingly, animals will usually tackle lower quality subordinate intruders much more readily (e.g. Rowe & Redfern 1969; Poole & Morgan 1975; Hurst 1990). Introduction of urine from a subordinate male into a dominant male's territory stimulates a rapid increase in the territory owner's aggression and heavy countermarking (Hurst 1990) while unfamiliar dominant male urine stimulates a more cautious investigatory response and less countermarking (Hurst 1990, 1993). Indeed, Hurst et al. (1998) showed that volatile male ligands emanating from the proteins in fresh scent marks deposited by an intruder stimulate an initially cautious approach towards the unfamiliar scent marks by the territory owner. Importantly, however, territory owners do not avoid a challenger's marks, which are always investigated closely (see also Hurst 1990, 1993). Such caution in approach was not apparent in this study. Indeed, the latency to approach urinary protein was less than towards the clean control tile in experiment 1, suggesting that males were attracted to investigate by volatile ligands released from MUPs. This is likely to be due to an important difference in the way that intruder scents were presented in the two studies. Hurst et al. (1998) deliberately placed the intruder's scent in a site that the resident would normally visit (its nest site) and measured hesitancy as the resident approached. In our present experiment, stimuli were placed around the edges of a male's enclosure, sites that the resident would not normally visit very quickly. The emission of airborne volatiles that attracted the resident to investigate would thus have resulted in tiles being visited earlier than those that did not emit odours of interest, even if the male's approach to the tile was initially cautious. Mucignat-Caretta & Caretta (1999) have also shown that the volatiles emitted from proteins in male mouse urine will induce animals to overcome their natural reluctance to enter a brightly lit area to investigate, although only after a first investigation of the scent-marked area.

The emission of airborne volatiles from MUPs in the scent marks thus draws attention to the marks, stimulating closer investigation. Since the thiazole, brevicomin and farnesenes are produced only by adult males, these volatiles will also provide information on the nature of the scent depositor. In experiment 2, mice showed a short latency to visit all tiles. This might have been due to the sudden release of a large quantity of ligands into the air from the menadione-treated scent mark when we introduced the tiles, making it difficult for the mice to detect a clear gradient towards a site gradually emitting these odours. They thus may have visited all tiles quickly and frequently, including the control tile. Note that the generally shorter latencies in the second experiment were not due simply to prior experience by those males ($N$=7) that were used in both experiments 1 and 2. All subjects had previously encountered scent-marked tiles during pre-experiment pilot tests to check their response to whole urine (see Methods).

In addition to conveying information concerning the sex and competitive status of the depositor, competitive scent signals need to communicate the advertiser's identity. This would allow females and competitors to recognize the male advertising his competitive dominance, but would also allow males to distinguish between a competitor's scent marks and their own so that they can recognize and countermark any challenges. More than 99% of the protein in mouse urine consists of MUPs. These small urinary proteins are species specific (Sampsell & Held 1985), show a very high degree of polymorphism between individuals in wild mouse populations (Robertson et al. 1996; Pes et al. 1999) and are produced at a much higher concentration by males than by females (Finlayson et al. 1963). They thus have the potential to convey information on individual identity, either directly via appropriate receptors (Krieger et al. 1999) or through differential affinities for ligands (Beynon et al. 1999). Using inbred mice, Robertson et al. (1993) showed that different MUP allelomorphs bind different ratios of brevicomin and thiazole. In addition, the ligands attached to urinary proteins may also vary between individuals. The major histocompatibility complex (MHC) genes, which code for cell surface proteins involved in recognition of an individual's own tissue in the immune response, have been strongly implicated as a major source of individual identity odours in rodent urine (reviewed by Yamazaki et al. 1992; Brown 1995), although other genes also contribute to an individual's unique odour (e.g. Yamazaki et al. 1986; Schellinck et al. 1993; Eggert et al. 1996). MHC-determined odours are carried exclusively in the protein

fraction of mouse urine (Singer et al. 1993) and consist of a complex mixture of volatiles bound to the urinary proteins (MUPs and possibly fractionated MHC membrane proteins; Singer et al. 1993, 1997). Such protein-volatile complexes in urine may thus be important in allowing mice to discriminate an intruder's scent marks from their own, stimulating them to countermark. However, although mice can discriminate between individuals using airborne volatiles gradually released from urinary proteins (Singer et al. 1993), we found that protein deposits were still highly effective in eliciting countermarking 7 days after deposition, when volatiles no longer appeared to be released. This suggests that mice were not responding to airborne volatiles released from the proteins but detected and responded to either the proteins themselves, or the nonvolatile protein–ligand complexes that remained in the deposits.

When animals encounter airborne volatiles from individuals of different MHC (or other genetic) types, they usually approach the odour source to investigate more closely (see for example Brown et al. 1987; Ninomiya & Brown 1995). However, volatile 'individual identity' cues are highly susceptible to disruption by environmental influences. Animals can apparently change identity by eating a different food type (Schellinck et al. 1997), for example, or if a change occurs in their bacterial gut flora (reviewed by Brown 1995), and conspecifics are then unable to recognize the volatiles emanating from an otherwise familiar individual. Under natural conditions, where environments are considerably more complex and variable than carefully regulated laboratory conditions, such a mechanism for signalling individual identity would seem to be highly unreliable. The expression of proteins, on the other hand, is largely under genetic determination and is unlikely to be strongly influenced by such environmental effects. We suggest, therefore, that volatiles emanating from the urinary proteins may act to alert animals to the presence of interesting odours in their local environment, stimulating close investigation of the scent source where nonvolatile proteins or protein–ligand complexes may provide more reliable information on the species and individual identity of the depositor.

It seems surprising that resident males responded so strongly to aged scent deposits, which would have presented no current competitive challenge to their dominance. Female mice are able to discriminate between fresh and 24-h-aged scents when discriminating between the scent marks and countermarks deposited by two competing males (Rich & Hurst 1999), and the ability to be able to detect whether scent marks are fresh or aged would seem essential if animals are to use such signals to assess the current presence and dominance status of individuals. Proteins (with or without ligands retained over prolonged periods) are nonvolatile and relatively stable (unpublished data), and will thus remain in the environment for prolonged periods. This would make them unreliable signals of an individual's current presence and status (a factor that can change within minutes in the event of a successful challenge from a competitor) in the absence of more volatile components that are affected by ageing. It is notable, however, that in this study we examined responses to the protein fraction in isolation from other more volatile urinary constituents which might provide important additional information concerning the age of the deposit.

In conclusion, the protein fraction of male mouse urine contains the chemical signals that stimulate competitive countermarking. This fraction consists predominantly of the major urinary proteins and their ligands, including most of the male volatile signalling molecules. The emission of airborne signalling molecules from these proteins attracts mice to investigate an intruder's scent marks, providing information that the scent mark is from another competitive male mouse. Close investigation of the nonvolatile proteins or protein–ligand complexes stimulates the countermarking response, perhaps because this provides further information that the scent mark is not one of the resident male's own but is within his territory. The protein fraction does not appear to contain information on the age of the deposit, however, since mice responded just as strongly to aged as to fresh protein deposits.

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