Chapter 3
Urinary Lipocalins in Rodenta: is there a Generic Model?

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Abstract It is increasingly clear that mediation of chemical signals is not the exclusive domain of low molecular volatile or water soluble metabolites. Pheromone binding proteins play an important role in mediating the activity of low molecular weight compounds, while proteins and peptides can also act as information molecules in their own right. Understanding of the role played by proteins in scents has been derived largely from the study of Major Urinary Proteins (MUPs) in the mouse (*Mus musculus domesticus*) and the rat (*Rattus norvegicus*). As part of an ongoing programme to explore the diversity and complexity of urinary proteins in rodents, we have applied a proteomics-based approach to the analysis of urinary proteins from a wider range of rodents. These data suggest that many species express proteins in their urine that are structurally similar to the MUPs, although there is considerable diversity in concentration, in sexual dimorphism and in polymorphic complexity. This is likely to reflect a high degree of species-specificity in communication and the information that these proteins provide in scent signals.

3.1 Introduction

Early views of pheromone chemistry were shaped in part by precedents derived from the insect world. Thus, semiochemicals were considered to be low molecular weight, volatile molecules that were transmitted through the atmosphere from sender to receiver. The (somewhat alliterative) "simple, single signal" model has served well, but in higher animals it is necessary to invoke additional complexity. First, it becomes more critical that the receiver of the signal is able to identify the individual that transmitted the signal together with its status. The ability to recognise individual conspecifics and/or kin and associate this with information about that individual's status and behaviour is likely to be critical to most social interactions

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within vertebrate species, including competitor assessment, mate assessment, and the development of relationships within social groups. Scents thus need to provide a range of information that is clearly discriminable, and require different qualities to transmit variable information about an animal's current status (e.g. social status, reproductive status, health status) and invariant information about the animal's identity (species, sex, relatedness, individual). Second, as scents are often deposited in the environment in the form of scent marks or odour plumes to provide information over a period of time, some information in scent needs to be sustained, whilst other components will reflect temporal changes as the scent ages.

It might be expected, *a priori*, that information about the variable qualities of an animal is encoded via metabolites that provide for plasticity of expression, while invariant information about identity is more directly encoded in the genome. The most obvious candidates for directly encoded components that signal owner identity are proteins derived directly from the scent owner's genes, or peptides generated indirectly by proteolytic degradation of proteins encoded in the genome (note that any such peptides would need to be distinguishable from degradation products derived from food sources or from infectious agents that would not provide invariant identity information). Indeed, the emergent literature is providing increasing evidence for the presence of proteins in scent marks, and the ability of the vomeronasal system to respond to proteins or short peptides.

The family of proteins most commonly associated with the processes of chemical communication are the lipocalins, a large and diverse family of small extracellular β-barrel proteins with a hydrophobic calyx suitable for the transportation of small hydrophobic molecules (Akerstrom, Flower and Salier 2000). Although there is a low pairwise conservation of the specific amino acid sequence of lipocalins (often $< 20\%$), the structure of these proteins is a highly conserved eight-stranded antiparallel β-barrel with an internal hydrophobic calyx. The structure of most lipocalins is stabilised by a disulphide bond linking the main β-barrel to the carboxyl terminus of the protein. Lipocalins exhibit a wide specificity of natural ligand binding as the dimensions of the hydrophobic calyx are highly variable and the parts of the protein sequence responsible for ligand binding can tolerate a wide variety of amino acid side chains (Skerra 2000). Individual lipocalins are classified according to a number of highly conserved short sequences or typical structurally conserved regions (SCRs; Flower 1996).

3.2 A protein-based experimental approach

The lipocalin family is characterised by a high rate of evolution and substantial sequence divergence. As such, a genome based approach to lipocalin identification in other species is less satisfactory as sequences derived from genomic data from the rat or the mouse are unlikely to generate useful probes, for example, for PCR amplification of genomic or cDNA. Moreover, the known genomes are populated with many lipocalin genes, not all of which are involved in chemical communication, or are expressed in scent secretions. As such, our approach has

been protein based, targeting the emerging methodologies of proteomics to the proteinaceous components of scent marks. The advantages of such an approach are that the proteins are observed directly in the scent secretion, it is possible to quantify and examine the complexity of the scent proteins and, by mass spectrometry, to assess the heterogeneity, sequence conservation and primary sequence data for each protein. Once primary sequence data are obtained, even for short runs of amino acids, the sequences can be used to search databases using alignment tools such as BLAST or to specify the sequence of PCR primers. This approach has been exemplified by our work on urinary lipocalins from the house mouse (Darwish Marie, Veggerby, Robertson, Gaskell, Hubbard, Martinsen, Hurst and Beynon 2001; Beynon, Veggerby, Payne, Robertson, Gaskell, Humphries and Hurst 2002; Beynon and Hurst 2004; Armstrong, Robertson, Cheetham, Hurst and Beynon 2005; Robertson, Hurst, Searle, Gunduz and Beynon 2007), which has provided a paradigm for the analysis of similar proteins from other species.

3.3 Urinary lipocalins in *Mus musculus domesticus*

House mice (*Mus musculus domesticus*), thought to have become commensal some 10,000 years ago, live in territorial social groups in which the ranges of many individuals overlap (Hurst 1987a; Barnard, Hurst and Aldhous 1991). In this species the urinary protein concentration can reach ~30 mg/ml in males, and over 99% of the protein content is attributable to members of the lipocalin family: the major urinary proteins or MUPs (Beynon and Hurst 2003; Beynon and Hurst 2004; Hurst and Beynon 2004). MUPs are the product of a multigene family of approximately 30 genes and pseudogenes located on chromosome 4 (Bennett, Lalley, Barth and Hastie 1982; Bishop, Clark, Clissold, Hainey and Francke 1982). Urinary MUPs are synthesised in the liver and secreted into serum where they are rapidly excreted by the kidneys. The synthesis of MUPs in the liver is sex-dependent, resulting in a urinary protein concentration three to four times higher in post-pubescent male mice than female mice (Beynon & Hurst 2004), and an even more pronounced sexual dimorphism in expression in the closely related sub-species *Mus musculus musculus* (Stopkova, Stopka, Janotova and Jedelsky 2007). The sexual dimorphism extends beyond the total amount of protein—there are several proteins that are expressed in a male-specific pattern (Armstrong et al. 2005). The expression of MUP mRNA has also been detected in a number of secretory tissues including the submaxillary, lachrymal, mammary, parotid, sublingual and nasal glands (Shahan, Denaro, Gilmartin, Shi and Derman 1987; Utsumi, Ohno, Kawasaki, Tamura, Kubo and Tohyama 1999). In urine, multiple MUPs are expressed simultaneously, leading to complex protein profiles. These profiles are highly polymorphic in wild-caught mice, such that the overall MUP pattern expressed by each unrelated individual is unique although the polymorphism is not evident in inbred strains that are geneticially homogenous (Robertson, Cox, Gaskell, Evershed and Beynon 1996; Robertson, Hurst, Bolgar, Gaskell and Beynon 1997; Beynon et al. 2002).

MUPs bind pheromones within the hydrophobic calyx of their structure (Bocskei, Groom, Flower, Wright, Phillips, Cavaggioni, Findlay and North 1992; Zidek, Stone, Lato, Pagel, Miao, Ellington and Novotny 1999; Timm, Baker, Mueller, Zidek and Novotny 2001), and delay their release from scents into the air (Robertson, Beynon and Evershed 1993). A number of pheromones in mouse urine show sex or status-specific expression. These have a number of reproductive priming and behavioural effects including acceleration of female puberty onset (Novotny, Jemiolo, Wiesler, Ma, Harvey, Xu, Xie and Carmack 1999) or puberty delay (Novotny, Jemiolo, Harvey and et 1986), extension of oestrus (Jemiolo, Harvey and Novotny 1986), inter-male aggression and male-female attraction (Jemiolo, Alberts, Sochinski-Wiggins, Harvey and Novotny 1985; Novotny, Harvey, Jemiolo and Alberts 1985).

Two pheromonally active ligands in mouse urine, 2,3-dehydro-exo-brevicomin (brevicomin) and 2-sec-butyl-4,5-dihydrothiazole (thiazole) (Bacchini, Gaetani and Cavaggioni 1992; Novotny, Ma, Wiesler and Zidek 1999) are associated with urinary MUPs following purification. In addition to the role of binding the pheromonally active ligands *in vivo*, which may be important for transporting pheromones to receptors in the vomeronasal organ, MUPs extend the duration of scent signals by delaying the release of thiazole and brevicomin from urine marks after deposition (Robertson et al. 1993; Hurst, Robertson, Tolladay and Beynon 1998).

Individual mice express a combinatorial pattern of MUPs (typically at least 7–12 isoforms) reflecting multiple allelic variants and multiple expressed loci (Robertson et al. 1997). Among wild mice, individuals each express a different pattern even when captured from the same population (Payne, Malone, Humphries, Bradbrook, Veggerby, Beynon and Hurst 2001; Beynon et al. 2002), with the exception of very closely related animals that have inherited the same haplotypes from their parents (a 25% chance among outbred sibs, similar to MHC type sharing). The extreme heterogeneity in the sequence of MUPs is mostly confined to strands B, C and D and the intervening turns of the ß-barrel structure (Beynon et al. 2002).

Recent work has indicated a number of potential chemical communication roles for MUPs, as opposed to their ligands, in deposited urine (Beynon and Hurst 2004). There is persuasive evidence that the MUP themselves are a source of olfactory signals; stimulating increased competitive scent marking (Humphries, Robertson, Beynon and Hurst 1999), puberty acceleration (Mucignat Caretta, Caretta and Cavaggioni 1995) and pregnancy block (Peele, Salazar, Mimmack, Keverne and Brennan 2003). More critically, it is clear that the pattern of MUPs expressed in the urine encodes an individual ownership signal that allows individuals to distinguish their own scent marks from those of other males (Hurst, Payne, Nevison, Marie, Humphries, Robertson, Cavaggioni and Beynon 2001), and allows females to recognise individual males (Cheetham, Thom, Jury, Ollier, Beynon and Hurst 2007). Although airborne volatiles emanating from scent marks induce mice to investigate the scent more closely, they only countermark when they can contact the scent (Nevison, Armstrong, Beynon, Humphries and Hurst 2003) and then only when the scent contains MUPs that are different from their own (Hurst, Beynon, Humphries, Malone, Nevison, Payne, Robertson and Veggerby 2001). This suggests that

owner recognition involves detection of involatile MUPs through the vomeronasal system.

Evolution of a MUP expression profile as a signal of individuality and kinship is appealing, given the high sequence heterogeneity, stable expression patterns and non-volatile nature of proteins. The high concentration of MUPs in urine and the resistance of the β-barrel structure to denaturation or degradation are consistent with a dual role of delivery and slow release of volatile signals, and stable encoding of identity of the owner. It is increasingly important to explore the nature, complexity and use of urinary lipocalins in other species, to assess the extent to which the subtleties of the process in the house mouse may be generalised. In the remainder of this chapter, we report an overview of our recent work on other rodent species

3.4 Urinary lipocalins in *Mus macedonicus*

Three other *Mus* species (*M. macedonicus, M. spretus and M. spicilegus*) are closely related to and occur sympatrically with *M. m. domesticus* in Europe and the Middle East. These species live independently of humans, utilizing more scattered food resources and thus live at much lower densities. We therefore sought to characterise MUPs from *M. macedonicus* for comparison with the well characterised MUPs from *M. m. domesticus*. Urine from *M. macedonics* individuals demonstrated a MUP-sized band on gel electrophoresis. However, when the samples were analysed by mass spectrometry, the urine from each male *M. macedonicus* contained a single major protein species of mass 18742Da and all individuals were the same, in marked contrast to *M. m. domesticus*. A combination of peptide mass fingerprinting and tandem mass spectrometry/*de novo* sequencing revealed that this protein was a kernel lipocalin, containing all three SCRs (Flower 1996), and differed by only seven amino acid changes to the most similar protein that has been characterized from *M. m. domesticus*. All of the amino acid changes were located at the surface of the molecule and molecular modeling of the predicted protein of the *M. macedonicus* sequence demonstrated that the amino acid substitutions had little effect on the tertiary structure—this protein was indubitably a MUP (Robertson et al. 2007). At present, we lack data on *M. macedonicus* females.

3.5 Urinary lipocalins in *Mus spretus*

In common with *M. macedonicus*, urine from male *M. spretus* also demonstrated a MUP-sized band following gel electrophoresis. The proteins within this band were analysed by high resolution anion exchange chromatography and electrospray ionisation mass spectrometry (ESI-MS). The former technique produced an elution profile consisting of just three peaks, in contrast to both the more complex patterns observed previously from *M. m. domesticus* and the single major peak in *M. macedonicus*. Furthermore, similar analyses from five individual males resulted in near

identical profiles, in terms of relative peak area and chromatographic retention time. The molecular mass of the proteins within the anion exchange peaks was subsequently determined by ESI-MS. Each peak was found to contain a single protein, the masses of which were 18666Da, 18687Da and 18758Da, with the 18758Da protein being the most abundant. A peptide mass fingerprinting experiment performed on the 18758Da protein confirmed that it shared considerable sequence identity to MUPs from *M. m. domesticus* but also contained some differences in the amino acid chain. *De-novo* sequencing of two Lys-C peptides from the 18758Da *M. spretus* MUP characterised two such changes: these were $A_{103}T$ and $E_{49}D$ (*M. m. domesticus/M.spretus*). Both substitutions involve amino acid residues on the surface of the molecule and in the light of the *M. macedonicus* investigation, are thought to have little effect on the structure. The MUP status in *M. spretus* females is not yet known but under investigation.

3.6 Urinary lipocalins in the Norway rat, *Rattus norvegicus*

Rattus norvegicus developed within the rodent family Muridae about 5–6 million years ago (Verneau, Catzeflis and Furano 1998) and now is a commensal presence virtually worldwide. As in the *Mus* species, *Rattus norvegicus* excrete a great deal of protein in their urine (20 mg/day for mature males), most of which is lipocalin formerly known as α_{2U} globulin (Chatterjee, Hopkins, Dutchak and Roy 1979) but which is now more properly referred to as rat major urinary protein (rat MUP). Rat MUPs are tissue and sex specific proteins under complex multihormonal and developmental control (Kulkarni, Gubits and Feigelson 1985). They migrate to a similar position as mouse MUPs on SDS PAGE gels and are structurally very similar (Bocskei et al. 1992). Rat MUPs bind small hydrophobic ligands (Lehman-McKeeman, Caudill, Rodriguez and Eddy 1998), although no endogenous ligand has been identified as yet, and male urine has been implicated in puberty acceleration in female rats (Vandenbergh 1976) and the timing of lactational estrous in dams (Schank and McClintock 1997). The rat MUPs belong to a multigene family with more than 20 closely related isoforms (McFadyen, Addison and Locke 1999; McFadyen and Locke 2000). As with the house mouse, rat MUPs are expressed in salivary, lachrymal and mammary glands, but the highest concentration and complexity is found in preputial glands which do not secrete MUPs in mice. Further, only male rats express MUPs in liver, corresponding to the male-specific expression of urinary MUPs in this species (MacInnes, Nozik and Kurtz 1986).

Most previous work on rat MUPs has been conducted with inbred or relatively inbred laboratory rat strains that are likely to exhibit considerably reduced phenotypic variation relative to the wild population, as we see in mice. As an initial exploration of MUP expression, we analysed urine from nine wild-caught male rats captured from several different populations in northern UK by isoelectric focusing electrophoresis (IEF). The protein banding pattern was very similar between individuals, consisting of two major and several minor bands. Peptide mass

fingerprinting (PMF) of the two main bands revealed them to be strong matches to rat MUPs. Electrospray ionisation mass spectrometry (ESI-MS) demonstrated that the urine of each individual contained two principal proteins of 18714Da and 18730Da. The ESI-MS and PMF data allowed unambiguous identification of the two main proteins as the rat MUPs AAA40642 (18714Da) and P02761 (18730Da), both synthesised in the liver. One of the minor bands was identified as the rat MUP Q63213 (18340 Da) which is also expressed in preputial and salivary glands (Bayard, Holmquist and Vesterberg 1996; Saito, Nishikawa, Imagawa, Nishihara and Matsuo 2000). The other minor bands are novel, previously unknown rat MUPs and are currently being characterised. The overall pattern of rat urinary MUPs by IEF and ESI-MS is remarkably consistent between individuals, contrasting that of the wild caught *M. m. domesticus* urinary MUP profiles. Additional wild individuals are being investigated to see if the rat urinary MUP pattern remains invariant.

3.7 Urinary lipocalins in *Phodopus roborovskii*

The Roborovski hamster is closely related to the other dwarf hamster species-the dwarf winter white hamster (*Phodopus sungorus*) and the Djungarian hamster (*Phodopus campbelli*). All three dwarf hamster species live in extreme environments: *P. roborovskii* inhabits desert and semi-desert regions with little vegetation in Russia, China, Manchuria and Mongolia, whereas both *P. sungorus* and *P. campbelli* are native to the forest-steppe zone of central Asia. Dwarf hamsters are nocturnal and live in a system of subterranean tunnels and nests formed by burrowing. The extreme physical conditions in their natural habitats has caused dwarf hamsters to adapt physiologically to conserve heat and water, while the harsh conditions also limit the opportunities for breeding, resulting in a highly compressed reproductive cycle that enables rapid maturation of their offspring. Dwarf hamsters have adapted to the limited water availability in their natural habitat by developing a highly effective renal mechanism to concentrate urine and limit the volume of water lost. The desert environment of *P. roborovskii* is the most extreme habitat of the dwarf hamsters, consequently they are able to highly concentrate their urine to a volume significantly less than that of *P. sungorus* and *P. campbelli* (Natochin Iu, Meshcherskii, Goncharevskaia, Makarenko, Shakhmatova, Ugriumov, Feoktistova and Alonso 1994). Male dwarf hamsters respond to urine and other scents emitted by females during different reproductive states, suggesting a combined set of odours could provide precise information about female reproductive state (Lai and Johnston 1994). Males can discriminate between male and female odour, and investigate scent marks from males and females in a sex dependent manner (Reasner and Johnston 1987). The frequency of urine marking is greater in males, particularly when within a female's home area, while females mark at a constant rate irrespective of location in the habitat.

Urinary protein output was assessed by measuring total protein and creatinine concentration for six male and six female captive-bred *P. roborovskii* hamsters.

The protein:creatinine ratio was very similar for males (12.0 ± 0.8) and females (13.5 ± 0.8). The similarity between the sexes was maintained when urinary proteins were resolved by 1D SDS PAGE. For all individuals, two low molecular weight proteins were apparent, one migrating at approximately 21 kDa and a second, smaller protein migrating at approximately 6 kDa. The intensity and the relative abundance of the 21 kDa and 6kDa bands were remarkably consistent across individuals. Proteins were subjected to in-gel digestion with trypsin, followed by MALDI-ToF mass spectrometry of the resultant peptides. The mass spectrum of the tryptic peptides from the male and female 21 kDa protein were virtually identical, demonstrating that the 21 kDa protein in male and female urine is likely to consist of the same protein(s). Similarly, the 6kDa protein yields the same mass spectrum in both sexes. However, the lack of similar peptides derived from the 6 kDa protein and the 21 kDa protein mean that the smaller protein is not a degradation product of the larger. Peptide mass fingerprint analysis of the 21 kDa protein did not identify any statistically significant matching protein sequences from non-redundant protein sequence databases. However, comprehensive mass spectrometric analysis and *de novo* peptide sequencing have allowed us to define virtually all of the protein sequence of the 21 kDa protein. From this, it is clear that the protein is a lipocalin (of similar length, possessing all of the SCRs), and that is has sequence and structural features that mean that it is most similar to the vaginal protein aphrodisin from the Syrian or Golden hamster *Mesocricetus auratus*, a degree of sequence similarity that permits the construction of a molecular model using aphrodisin as a template (M. J. Turton, J. L. Hurst and R. J. Beynon *unpublished data*). The 21 kDa protein was present in cage washes, in urine samples obtained by bladder massage and by direct recovery from bladder urine—it is most unlikely that this is due to vaginal fluid contamination, especially since the same protein is present in equal amounts in males!

3.8 Urinary lipocalins in the bank vole, *Clethrionomys glareolus*

The bank vole, *Clethrionomys glareolus* is the smallest of the vole species in Britain. The habitat of *C. glareolus* is woodland and thick undergrowth, where they travel along a system of worn routes either forced through the undergrowth or in shallow tunnels to avoid attack from predators (e.g. owls, stoats and weasels). *C. glareolus* is a polygamous rodent species, the mating season is early spring—summer and over winter they form a mixed sex group of 2–4 females with some of the last litter young and 1–2 males. During the mating season, this group breaks up and mature females inhabit non-overlapping solitary home ranges close to the over wintering site while males form hierarchical groups with larger home ranges that overlap (Bujalska 1973). The size of female home ranges is determined by their litter size and availability of food (Koskela, Mappes and Ylonen 1997; Kapusta and Marchlewska-Koj 1998). The increased aggression and territoriality of mature females during pregnancy and lactation decreases the size of home ranges and increases the distance between neighbouring females, preventing home range

boundaries overlapping. Male *C. glareolus* form stable dominance hierarchies in the mating season through brief fighting episodes and each inhabits a separate burrow. Some subordinates relocate to vacant areas and immature males live on the breeding territories of females. Bank voles are nocturnal animals using scent from urine, faeces and several skin glands for intraspecific communication.

Wild male *C. glareolus* scent mark their territories by depositing small urine droplets or fine traces using the long brushlike hairs on the prepuce (Johnson 1975). These scent marks appear similar to those of house mice, and indicate a specific and controlled function for marking with urine. Paired male bank voles repeatedly urine mark and over-mark in a new environment and, after the establishment of a hierarchical order, urine marking by the submissive vole is diminished while the dominant vole urine marks the subordinate's burrow and nest area, consistent with a role for the urine marks as territorial markers within a stable hierarchy (Rozenfeld 1987). Females show heightened activity and interest in marking urine and preputial secretions from dominant males. Protein in bank vole urine was identified at 13–14 kDa in sexually mature males. The expression of this protein is thought to be androgen-dependent as the protein was absent or weakly expressed in urine from females, immature males and castrated males (Kruczek and Marchlewska-Koj 1985).

We characterized the urinary protein of *C. glareolus*. There was clear evidence for a strong sexual dimorphism in adults (protein:creatinine ratio in males: 45.4 ± 3.2; females: 3.7 ± 0.9) such that males secreted approximately 10 times as much urinary protein as females. The majority of the protein in male-derived samples migrated at approximately 16 kDa, but in females a similarly sized protein was apparent when samples were concentrated. However, the peptide mass fingerprint for the two sexes yielded unique sets of peptides with very little overlap, from which we can infer that the urinary proteins of either sex are different gene products. Thus far, we have characterized the male 16 kDa protein. On intact mass analysis, two proteins, of average mass 16930Da and 16625Da were present in urine from both laboratory bred and wild caught *C. glareolus*. A detailed *de novo* sequence analysis of overlapping peptides obtained by digestion of the predominant 16930Da protein with different endopeptidases allowed assembly of over 95% of the protein sequence, and clear identification of this protein as a kernel lipocalin, in which all three SCRs were present. The primary sequence showed greatest similarity to aphrodisin, rather than MUP type sequences, and a model could be readily built using the three dimensional structure of aphrodisin as a template (M. J. Turton, J. L. Hurst and R. J. Beynon *unpublished data*).

3.9 Conclusions

Within the limitations of sample size and extent of characterization, several general statements can be made in relation to the species described here, all of which express substantial concentrations of urinary lipocalins. First, these urinary proteins appear to be widespread across species that are not very closely related and occupy

different niches but use urine for scent communication. The urinary lipocalins seem to exhibit less plasticity of sequence and structure than the broader lipocalin family, which implies that they fulfil a specific role in chemical communication. Considering those proteins that have been characterized in detail (from *Mus* species, brown rat, bank vole, Roborovski hamster), the emergent picture is of a protein between 150 and 170 amino acids that can readily be modelled onto the structures of either mouse MUP or aphrodisin. This does not of course guarantee that the proteins fold in a typical lipocalin beta barrel, but the modelling data are of sufficient quality to suggest that this is a valid presumption. Second, sexual dimorphism in expression of urinary lipocalins varies considerably between species, from a lack of any observed dimorphism in *P. roborovskii,* through greater investment in MUPs among male house mice with only some MUP isoforms being male-specific, to entirely male-specific expression of urinary MUPs in brown rats. This suggests that the role of urinary lipocalins in sexual communication is strongly species specific and MUP genes may be subject to strong sexual selection and rapid evolution.

A third compelling feature to emerge from these studies is the surprising lack of polymorphic heterogeneity in species other than *M. m. domesticus*. The pattern more commonly seen is of the expression of one or a few lipocalin variants with a similar pattern of expression between individuals of the same sex. Even though relatively small numbers of individuals have been examined, a comparable set of samples obtained from *M. m. domesticus* reveals multiple urinary MUPs expressed per individual with substantial inter-individual variation in the MUP profile. The simpler lipocalin pattern in other species examined so far means that there is inadequate polymorphism in these proteins to provide an individual ownership signal in urine. This may reflect differences in the population ecology of aboriginal species such as *M. macedonicus* where individual recognition may be less important than in commensal house mice, and might imply rapid expansion of the genome and of the role of MUPs in commensal house mice to meet a species-specific requirement for individual and kin recognition. In these mice in particular, multiple males and females live within close territorial social groups with extensive spatial overlap between neighbours such that borders need to be vigorously defended (Hurst 1987b; Barnard et al. 1991). The need to maintain territorial dominance scent marks and advertise a stable signal of ownership may then be driven by such high density populations and have led to selection for polymorphic MUP expression. However, it is also clear that MUPs and MUP-like proteins are expressed in other glands involved in scent communication, with similarities and differences between species. As yet, there has been little exploration of individual heterogeneity in these proteins and their functions in sent communication. Further exploration of urinary lipocalins will do much to expand our understanding of the role of these proteins in behavioural ecology. However, that exploration should focus as much on the proteins themselves as their putative ligands.

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