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Rank-dependency of major urinary protein excretion in two house mouse subspecies

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Abstract. Chemical communication is important for many social mammals. Scent-related gene clusters have undergone extraordinary expansion in some species, such as the house mouse (*Mus musculus*). One such family encodes major urinary proteins (MUPs). MUPs can provide recipients with complex information about the signaller and potentially serve as honest signals of social rank. In this study, we examined the development of overall MUP production in two mouse subspecies in the context of establishing their social hierarchy during the critical period between weaning and 100 days of age. We used fraternal pairs as simple social units, where dominant/subordinate ranks were naturally established between two brothers raised together, to test the hypothesis that dominant males of both taxa excrete higher amounts of MUPs in their urine than subordinates. The results were compared to data on ontogeny of steroid hormone levels gathered in the same individuals during an earlier experiment. Higher MUP levels in dominant males were only corroborated in one subspecies (*domesticus*), whereas *musculus* males revealed similar MUP quantities irrespective of rank. These results are consistent with the notion that these closely related taxa adopted different strategies for establishing social hierarchy.

Key words: chemical communication, *Mus musculus musculus*, *Mus musculus domesticus*, social rank, testosterone, urine

Introduction

Socially living animals are involved in an ongoing competition for resources and mating opportunities, where success depends on their social status; thus, establishing and maintaining a high rank within the group is one of the salient factors determining the fitness of social animals (Dewsbury 1982, Ellis 1995, Shivani et al. 2022). The outcome of such competitions depends on many internal and external

factors (VanderWerf 2008, Chelliah & Sukumar 2013, Lardy et al. 2013), including honest signalling of competitive abilities (Zahavi 1977, Dawkins & Krebs 1978, Hurst et al. 2001a).

Communication, therefore, is an inevitable prerequisite for social life; however, social interactions also bring about demands on increasing the complexity of information transmitted. Generally, the more complex the social life, the more diverse the communication among group

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members (Rogers & Kaplan 2002, Bradbury & Vehrencamp 2011, Freeberg et al. 2012). In macrosomatic mammals, information is transmitted predominately via olfaction (Eisenberg & Kleiman 1972, Mundy 2006).

Olfactory cues can provide complex information on taxon and individual identity, sex, age, health and social status (Johnston 2003, Arakawa et al. 2008, Hurst & Beynon 2013). The scent-related gene families of some species have undergone extraordinary expansion, encoding both olfactory signals and their receptors. An example of such a mammal is the house mouse (*Mus musculus*) (Chamero et al. 2012, Karn & Laukaitis 2012, Sheehan et al. 2019). Two subspecies, the eastern house mouse (*M. m. musculus*) and western house mouse (*M. m. domesticus*), meet in Europe, forming a narrow hybrid zone from the Black Sea up to Scandinavia (Baird & Macholán 2012, Macholán et al. 2019), and thus offer a unique model for diverse evolutionary studies (Macholán et al. 2012).

There are several mediators of olfactory signals in mice, one of the most important, and certainly the most studied, being urine. Among myriads of urine components, many of which can participate in chemical communication, mouse major urinary proteins (MUPs) play a significant role (Hurst & Beynon 2004). These relatively small (18–19 kDa) lipocalin proteins, produced and excreted in considerable quantities, are important as carriers of pheromonally-active ligands (Novotny 2003, Phelan et al. 2014, Stopková et al. 2021). However, since MUPs can produce highly heterogeneous combinations of protein isoforms in the urine, it has been suggested that they modulate the behaviour and physiology of the recipients (Beynon & Hurst 2003, Chamero et al. 2007, Roberts et al. 2010, 2012, Nelson et al. 2015). Regulation of MUP expression, initially considered to be controlled entirely by testosterone, is now known to be under multihormonal control, involving both growth hormone and thyroxine (Knopf et al. 1983, Noain et al. 2013, Penn et al. 2022). However, the precise mechanism remains to be identified.

MUP quantity and profile can vary substantially during life (Thoß et al. 2015) and have been shown to be socially condition-dependent in males (Stopka et al. 2007, 2012, Thoß et al. 2015, 2019, Luzynski et al. 2021). Moreover, some MUP isoforms have been identified as indicators of dominance (Nelson et al. 2015, Lee et al. 2017; but see Hurst et al. 2001b) or promoters of aggressive behaviour and predictors of aggression-related behavioural strategies (Rusu et al. 2008). Owing to the assumed metabolic costs of MUP

production, only high-quality males are thought to produce such proteins in sufficient quantities (Gosling et al. 2000, Sharrow et al. 2002, Greve et al. 2022); consequently, MUP levels in the urine may serve as honest signals of competitive ability and dominance (Rich & Hurst 1998, Garratt et al. 2011, 2012, Nelson et al. 2015; but see Számadó 2011, Penn et al. 2022).

Despite several decades of MUP research, most studies have been limited to inbred laboratory strains or a single mouse subspecies. However, since the two European subspecies differ significantly in MUP amount and profile (Stopková et al. 2007, Hurst et al. 2017) as well as testosterone and corticosterone production dynamics (Hiadlovská et al. 2015, Daniszová et al. 2017) and social structure and hierarchy development (Hiadlovská et al. 2021, Mikula et al. 2022), simultaneous studies embracing both taxa could be highly important. The compelling findings of such comparative studies consistently suggest a more rapid establishment of social hierarchy in *M. m. domesticus* males compared to *M. m. musculus* males. Moreover, as shown by Hiadlovská et al. (2015), steroid hormone levels and body growth rates were only significantly different between dominant and subordinate males in *M. m. domesticus* and not *M. m. musculus*. If MUPs are involved in establishing mouse social hierarchy, therefore, we may expect significant differences in overall MUP levels between dominant and subordinate *domesticus* males shortly after reaching adulthood and an absence of these differences in *musculus* males.

In this study, we examine the ontogeny of overall MUP production in the two house mouse subspecies in relation to the development of their social hierarchy. We capitalise on an earlier experiment (Hiadlovská et al. 2015, 2021), where we used the same fraternal pairs as simple social units (Rusu & Krackow 2005, Krackow & König 2008, Rusu et al. 2008) to pinpoint the onset of aggression and sexual maturity during a crucial part of the male lifespan, i.e. from weaning to adulthood. In the study, hierarchical dominant/subordinate ranks were naturally established between two brothers raised together. At the same time, we also gathered data on growth and levels of steroid hormones (Hiadlovská et al. 2015).

The main goal of the present work was to test the hypothesis that dominant males of both taxa excrete higher amounts of MUP in their urine than their respective subordinates. The advantage of the experiment of Hiadlovská et al. (2015) utilised in



this study is that the design allows the investigation of several behavioural and physiological traits simultaneously, allowing us to address how MUP production reflects both important life-history milestones (sexual maturity and the onset of aggressive behaviour) and assess the possible correlation between MUP excretion and the dynamics of testosterone production during the period under study.

Material and Methods

Wild *M. m. musculus* and *M. m. domesticus* mice were sampled well outside the hybrid zone between the two subspecies, i.e. more than 30 km from the zone centre (Table S1). Males and females from the same locality were mated, and the resulting offspring weaned at 20 days. Twenty pairs of brothers, two per locality, were then established and kept under housing conditions described in detail in Hiadlovská et al. (2015; see also Supplementary material). Urine was sampled at five-day intervals from 20 days of age. After approx. 50 days of age, the intervals were extended to 5-7 days. After collection, the samples were stored at -20°C until processing.

Fur clips were taken from all males, allowing us to distinguish between the brothers. Each time a male was handled, the position of the testes (abdominal or scrotal) was assessed to determine its reproductive status. We considered the point at which we first observed testes in the scrotum as the best possible approximation of the onset of a male's sexual maturity. During each control, we checked the mice for signs of bites or body scars (Van Loo et al. 2000, 2001, 2003). If there were no signs of injuries, both males were put in a clean cage ('neutral arena' test), and their behaviour was video-recorded for 20 min, starting from the first intentional body contact. If no sign of agonistic display was observed within the 20-min period, the males were rechecked for bites or injuries the next day. When aggression was detected, they were separated with a wire mesh that allowed them to continue making visual, acoustic and olfactory contacts, thus preserving the social context. As described above, the first detection of any sign of past aggression was considered the onset of aggression (Hiadlovská et al. 2015; see also Fig. 1 in Hiadlovská et al. 2021). Maintenance of the respective social status was verified during two additional confrontations after separating the brothers at 85-89 and 95-99 days of age. Males were considered dominant if they had fewer scars or injuries (Van Loo et al. 2001, 2003) and/or consistently showed more

offensive agonistic behaviour than their brothers during all dyadic interactions (for more details, see Hiadlovská et al. 2015, 2021, and Supplementary material).

Hiadlovská et al. (2015) showed that the dominant *domesticus* males were heavier than subordinate males, whereas no difference was found in *musculus* males. When we collected body mass data only before the first dyadic test, this distinction is still apparent, with the dominant *domesticus* males being heavier than their subordinate brothers in as many as 90% of cases, while weights of the dominant *musculus* males were only higher in 50% of cases (*domesticus*: mean dominant = 18.33 g, mean subordinate = 16.72 g; t-test: $t = 1.573$, $df = 18$, $P = 0.1332$; *musculus*: mean dominant = 16.70 g, mean subordinate = 16.52 g; $t = -0.221$, $df = 18$, $P = 0.8274$). It should be pointed out here that although the results are not significant for either subspecies, this was due to the small sample size in *domesticus*, power analysis suggesting that P would be significant at $\alpha = 0.05$ if the number of fraternal pairs were increased from 10 to 17. In each case, the two subspecies were tested in the same room in the absence of other animals of either sex. Individuals whose rank could not be assigned unequivocally were excluded from the study.

Prior to MUP analysis, the urine samples were thawed, stirred, briefly centrifuged, and an appropriate amount (max. 4 μl) added to Laemmli Sample Buffer (Bio-Rad Laboratories). The resulting mixture was then heated to 95°C for 5 min. Separation was carried out using denaturing polyacrylamide gel electrophoresis (SDS-PAGE) using 4-15% Mini-PROTEAN[®] TGX[™] Precast Gels (Bio-Rad). A concentration gradient of carbonic anhydrase (29 kDa), used as a marker for SDS-PAGE (Sigma-Aldrich), was loaded onto the gels along with the samples to allow absolute quantification of the separated proteins. Electrophoresis was then run under a constant voltage of 170 V, the gels stained with Bio-Safe[™] Coomassie Stain (Bio-Rad) and their images were captured using a GS-900 Calibrated Densitometer (Bio-Rad). Protein bands of interest were then quantified using the Image Lab[™] Software (Bio-Rad), where the band volume is defined as the total intensity of pixels within the volume boundary. Protein concentrations were then calculated from a carbonic anhydrase calibration curve (Janotová & Stopka 2011). As urine dilution can vary between individuals and/or over time, we used a creatinine assay to normalise all concentrations in the samples with the LKreatinin Kit (BioVendor). Based on the creatinine concentration, the dilution coefficient was

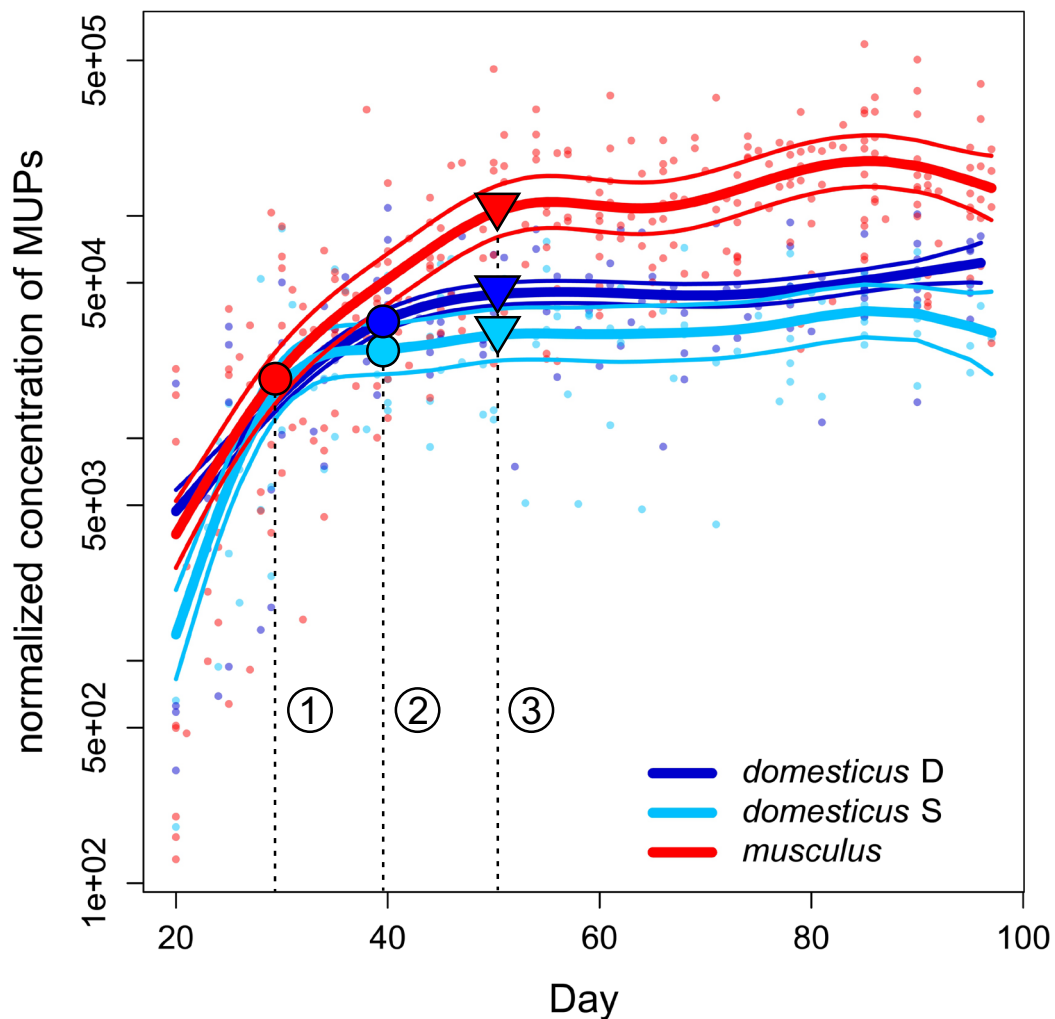


Fig. 1. Normalised MUP concentrations (ng/ml) in *musculus* and *domesticus* males measured between 20 and 100 days of age. The data were fitted using the M3d model, assuming a separate thin-plate spline curve for dominant *domesticus* males (dark blue), subordinate *domesticus* males (light blue) and *musculus* males irrespective of rank (red); thin curves correspond to \pm standard error confidence intervals; 1) sexual maturity of *musculus* males, 2) sexual maturity of *domesticus* males, 3) onset of aggressive behaviour in both subspecies (Hiadlovská et al. 2015).

calculated as described in Stopka et al. (2007; see also Supplementary material). Corrected volumes were obtained by dividing each MUP concentration by the dilution coefficient; these normalised values represent the instantaneous MUP level in a given sample.

Changes in MUP concentration were modelled with generalised additive models (Hastie & Tibshirani 1990, Wood 2006), where age (in days; as a predictor) was linked to MUP concentration using a thin-plate spline smoother. The residuals were assumed to be gamma-distributed, and a logarithmic link was used to model the expected MUP production. The error distribution was assumed to be skewed as concentrations were always positive, though they could be very close to zero. The use of gamma distribution (instead of Gaussian with the logarithmic link) was suggested

by comparison of diagnostic plots (residuals *vs.* fitted values). In addition, we considered the non-independence of MUP measurements by using two additional smoothers modelling random effects of individual (each measurement was part of an individual ontogenetic series) and pair (each male had one brother).

Several models were considered, with different combinations of subspecies and rank as predictors (Supplementary material). The simplest model (M1) assumed a single basal curve common to all males, while alternative models assumed two to four separate curves (Table 1). The models were compared using the Akaike information criterion (AIC; Akaike 1974), with the difference (Δ AIC) of six units being considered substantial evidence in favour of the better-fitting model (Burnham & Anderson 2002).



Table 1. Values for Δ AIC (difference in log-likelihood units from the best model) and Akaike weights for nine models: M1 = one curve for all males; M2 = no difference between ranks within subspecies, different curves for the subspecies; M2dd = two curves, one for dominant *domesticus* males and one for all other categories; M2ds = two curves, subordinate *domesticus* males separate; M2md = two curves, dominant *musculus* males separate; M2ms = two curves, subordinate *musculus* males separate; M3d = one joint curve for *musculus*, two rank-specific curves for *domesticus*; M3m = one joint curve for *domesticus*, two rank-specific curves for *musculus*; M4 = distinct curves both for subspecies and ranks.

Model	Δ AIC	AkaikeW
M1	48.905	0
M2	1.787	0.28
M2dd	38.541	0
M2ds	27.298	0
M2md	44.827	0
M2ms	32.553	0
M3d	0.000	0.68
M3m	8.141	0.01
M4	6.666	0.02

The relative merits of competing models were further evaluated using their Akaike weights, expressed as relative likelihoods. When normalised such that the sum was one, they could be interpreted as probabilities of being closest to the truth among the models considered (e.g. Johnson & Omland 2004).

We also tested whether there was a correlation between MUP and testosterone concentrations (see Hiadlovská et al. 2015 for a description of the method and results) by calculating the Pearson correlation coefficient in 16 bins grouping individuals of similar age, with no individual being present twice in the same bin (binning was used to obtain a sufficiently high sample size). As we expected (and tested for) a positive correlation between MUP and testosterone levels, we performed one-tailed t-tests of significance with sequential Bonferroni correction (Holm 1979) to take multiple tests into account.

Results

Two MUP level change models fitted the data considerably better than others, i.e. M3d, assuming one curve for *M. m. musculus* and two rank-specific curves for *M. m. domesticus*, and M2, based on two curves, one for each subspecies irrespective of rank (Table 1). The difference between the two models was relatively low (Δ AIC = 1.787); however, Akaike weights indicated that the more complex M3d

model was closer to the truth with a $2.4 \times$ higher probability than the M2 model (Table 1). Thus, total MUP production in urine was only associated with social status in *M. m. domesticus* males, where dominant brothers displayed slightly higher MUP concentrations than their subordinate counterparts. In contrast, *M. m. musculus* males showed no support for rank-specific differences. In all models except M1, *M. m. musculus* showed consistently higher total MUP levels than *M. m. domesticus* (Fig. 1).

Both the M3d model (Fig. 1) and M2 model (not shown) showed a steep increase in MUP production, starting from nearly undetectable levels immediately after weaning at 20 days. This increase then slowed considerably, first in subordinate *domesticus* (approx. 35 days), followed by dominant *domesticus* (about 10 days later). Conversely, *musculus* males of both ranks reached a plateau as late as 50 days of age or more (Fig. 1). Thus, the curve for *musculus* males (red) began to separate from the other two (*domesticus*, light and dark blue) around day 40 (Fig. 1)

We found no consistent association between MUP and testosterone concentrations in either taxon or rank, with Pearson correlations ranging between -0.22 and 0.55 with no apparent trend through time. Only the maximum observed correlation in *M. m. musculus* (at approx. 90 days) was significantly different from zero; however, this was inflated by the presence of a single outlier and dropped to non-significance when this value was removed.

Discussion

In this study, we addressed the hypothesis that dominant house mouse males excrete higher amounts of MUP in their urine than subordinates. This hypothesis was only corroborated in the western European subspecies *M. m. domesticus*, whereas *M. m. musculus* males revealed similar MUP quantities irrespective of rank throughout the period examined. In both subspecies, MUP excretion rose more-or-less linearly from the beginning of our experiment (20 days of age) until the curves reached a plateau. Interestingly, this plateau emerged at approx. 55 days of age in *musculus* males, i.e. the increase continued at least 15 days after attaining sexual maturity (Fig. 1; see also Hiadlovská et al. 2015). This pattern appeared similar to that observed by Thoß et al. (2015; Fig. 2A); however, after standardising the MUP concentrations with their corresponding creatinine concentrations, no trend was apparent in the data of Thoß et al. (2015; Fig. 2B). It should also



be noted that a direct comparison between Thoß et al. (2015) and our results is difficult as we standardised MUP concentrations in a slightly different manner (see Supplementary material).

Dominant *domesticus* males slowed the increase in urinary MUP excretion approx. five days after puberty. This short period is due both to later sexual maturity and a shorter rise phase of MUP production in *M. m. domesticus* compared to *M. m. musculus* (Fig. 1). In contrast, subordinate *domesticus* males reached the plateau some five days *before* sexual maturity, the turn being most abrupt relative to dominant *domesticus* and all *musculus* males (Fig. 1). Importantly, the spurt in MUP excretion ended either before (*M. m. domesticus*) or around (*M. m. musculus*) the onset of aggression at around 50 days of age in both subspecies. This finding could suggest that urinary MUPs can co-determine the process of setting up a hierarchy (Rusu et al. 2008), though most likely in a subspecies-specific manner.

Conversely, Thoß et al. (2019) noted that differences in MUP excretion between dominant and subordinate *musculus* males emerged after and not before establishing social status, indicating that social behaviour affected gene expression, and not vice versa, at least under their experimental setup. It is possible that exposing the mice to social stress triggers changes in the expression of various genes, including those encoding MUPs and steroid hormones. Indeed, Hiadlovská et al. (2015) found brief trends of increasing corticosterone production in dominant *domesticus* males, and *musculus* males of both ranks, after the onset of aggression (Hiadlovská et al. 2015: Fig. 5). However, these trends started some ten days before outset and, more importantly, corticosterone excretion decreased both before and after this period in subordinate *domesticus* males. When the experimental setup allowed subordinate males to avoid contact with dominant males, this was interpreted as an indication of an already-fixed social hierarchy in the subspecies (Hiadlovská et al. 2015). Consequently, direct comparisons of the results of Thoß et al. (2019), as well as those of Carlitz et al. (2019) and Luzynski et al. (2021), with the present study are complicated by the fact that the data were gathered under different experimental conditions, those of Thoß, Carlitz, Luzynski and their collaborators employing semi-natural enclosures. The differences between results also suggest that the complexity of the social environment plays a role in modulating both the social hierarchy and signal production.

While the rank-dependency we revealed in *M. m. domesticus* males accords with results reported by Guo et al. (2015), Nelson et al. (2015) and Lee et al. (2017), Hurst et al. (2001b) found no effect of social status on MUP excretion. Previous studies have noted that elevated MUP levels in dominant males correspond with higher daily excretion of urine (Drickamer 1995, 2001) and the number of scent marks they leave (Desjardins et al. 1973). Male scent-marking (Thonhauser et al. 2013) and total urinary protein volumes (Luzynski et al. 2021) have also been shown to be positively correlated with reproductive success. It should also be noted that subordinate males displayed higher creatinine levels than dominant *domesticus* and all *musculus* males, suggesting they reabsorb more water when producing urine (Fig. S1). This urine-concentrating tactic of subordinates is assumed to allow them to urinate as little as possible to avoid aggressive responses from dominant territory holders (Desjardins et al. 1973, Hurst 1990, Jemiolo et al. 1992).

Contrary to *M. m. domesticus*, we found no evidence of a significant influence of social status on MUP excretion in *musculus* males. This result appears to contradict Janotová & Stopka (2011), who found significantly higher MUP excretion in *musculus* males who later appeared as winners of a dyadic contest with another male. Similar outcomes were reported by Thoß et al. (2019), who revealed significant up-regulation in *musculus* dominants after they were introduced to the social environment in a semi-natural enclosure. In this context, it is advisable to explore whether and to what extent different experimental conditions influenced the results, especially contact with females in Janotová & Stopka (2011) and Thoß et al. (2019) *vs.* the absence of females in Hiadlovská et al. (2015, 2021) and the present study. This experience was either presumptive, i.e. before capture, in Janotová & Stopka (2011), or explicit, i.e. under semi-natural conditions, in Thoß et al. (2019). Furthermore, in both these studies, the males were sexually mature (over 180 days of age in the latter case), whereas our experiment covered a continuous process of developing social hierarchy over a critical life-history period.

The results of the present study also confirmed a significantly higher total MUP excretion in adult *musculus* males than in *domesticus* males, as reported previously by Stopková et al. (2007) and Hurst et al. (2017). This result may correspond to the preference of *musculus* females for con-subspecific mates, contrasting with the absence of a mate preference of either subspecies in *M. m. domesticus* (Christophe &



Baudoin 1998, Smadja & Ganem 2002, 2005, Bímová et al. 2005, Vošlajerová Bímová et al. 2011). It has been proposed that MUPs may play an important role in a subspecies recognition system (Hurst & Beynon 2004, Stopka et al. 2012, Hurst et al. 2017) and that higher MUP expression in *M. m. musculus* may reflect more robust intersubspecific discrimination in this subspecies. MUPs have also been suggested as playing a dual role, functioning simultaneously as a signal for subspecies recognition and as an individual signature (but see Thoß et al. 2016). Selection may then favour intrasubspecific homogeneity and a high level of polymorphism (Hurst et al. 2017). In such a situation, there may be conflict in decisions between a hetero-subspecific male with a higher MUP volume (indicating higher individual quality) and a con-subspecific male, thereby avoiding undesirable inter-subspecific mating. As pointed out by Janotová & Stopka (2011), it is possible that MUP concentrations also affect mate choice in *M. m. domesticus*, so this ambiguity need not be limited to one subspecies.

The absence of a difference in MUP excretion between ranks in *musculus* males could be related to prolonged male contests over social status. Thus, we compared our MUP data with testosterone levels measured in the same individuals by Hiadlovská et al. (2015). Admittedly, an association between MUP and testosterone production is still ambiguous. Early experiments concluded that testosterone directly regulates MUP expression (Rümke & Thung 1964, Hastie et al. 1979); however, in those studies, the high production of urinary proteins was induced by the application of unnaturally high levels of testosterone. In comparison, other authors (e.g. Nelson et al. 2013) have found that MUP up-regulation is independent of higher levels of circulating testosterone. In our study, the correlation between individual-level testosterone and MUP excretion proved non-significant. On the other hand, a comparison of our MUP pattern (Fig. 1) with testosterone levels across the same period (Fig. S2B) revealed a conspicuous resemblance in *musculus* males, with the MUP and testosterone curves exhibiting a similar course with an elevation between 50 and 60 days of age followed by a drop and subsequent rise. Moreover, since adult *musculus* males revealed consistently higher levels of both testosterone and MUPs than adult *domesticus* males (Stopková et al. 2007, Hiadlovská et al. 2015, this study), some relation between their production cannot be ruled out.

Recently, Carlitz et al. (2019) found a continuous increase in steroid hormones, including testosterone,

with age (and body weight) in *domesticus* mice kept in semi-natural enclosures. This pattern appears to contradict the results of Hiadlovská et al. (2015; see also Fig. S2B); however, in addition to diverse experimental designs, several other differences may explain this discrepancy. First, hormone levels in faeces (Hiadlovská et al. 2015) are likely to undergo higher fluctuations than those in hair (Carlitz et al. 2019), which more closely reflect the physiological changes of an individual. Second, the time interval considered in Hiadlovská et al. (2015, 2021) and this study is more than two times shorter than that of Carlitz et al. (2019). Finally, the method used for fitting the data in the latter study does not enable the capture of more complex hormone excretion dynamics.

There may be another, more general, mechanism underlying both of these, based on the regulatory role of pituitary growth hormone (GH; Waxman & O'Connor 2006, Noaín et al. 2013, Penn et al. 2022). While neither GH nor body mass were found to have any direct, significant effect on MUP production by Nelson et al. (2013, 2015) and Luzynski et al. (2021), Penn et al. (2022) showed that MUP expression was controlled by the pulsatile secretion of growth hormone, in contrast to a nearly continuous release of GH in females. Thus, the association between MUP production and dominance may be more complex than thought. For example, two of the MUP ligands, dehydro-exo-brevicommin and (S)-2-sec-butyl-4,5-dihydrothiazole, are involved in signalling aggressiveness and dominance (Jemiolo et al. 1985, Novotny et al. 1985, Harvey et al. 1989), and these pheromones have been found in higher concentrations in the urine of *M. m. domesticus* males than *M. m. musculus* males (Mucignat-Caretta et al. 2010). Thus, we cannot rule out the possibility that dominant and subordinate *musculus* brothers, competing for higher social status, produce similar levels of MUPs but that their signals differ in the level of individual bound volatiles.

To conclude, the hypothesis that dominant males excrete more MUPs in their urine was only confirmed in *M. m. domesticus*, with no rank-dependency confirmed in *M. m. musculus*. In both subspecies, MUP levels increased steeply until reaching a plateau; however, unlike subordinate and dominant *domesticus* males, where the rise stopped about five days before and after reaching sexual maturity, respectively, MUP levels continued to increase in *musculus* males of both ranks as long as two weeks after maturing, and close to the onset of aggression.



In addition, there was a noticeable secondary peak between 70 and 80 days of age in the *M. m. musculus* curve (Fig. S2A). Notably, this peak coincided with that for testosterone in the same individuals (Fig. S2B; see also Hiadlovská et al. 2015). Even though the correlation between MUP and testosterone levels was non-significant in this study, the striking similarity between the MUP and testosterone curves, including the secondary peak appearing at the same time during *M. m. musculus* development, suggests the existence of a general pattern associated with continuing contests over social position well beyond the onset of male-male aggression (and likely beyond 100 days of age) in this subspecies. Though the results of this study are, admittedly, based on a straightforward experimental design that differs from natural conditions, and the evidence is somewhat circumstantial, the data are fully congruent with the results of Hiadlovská et al. (2015, 2021) and Mikula et al. (2022), adding another facet to the overall picture of different population structures and dynamics of establishing social hierarchy in two European house mouse taxa.

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Author Contributions

Z. Hiadlovská and B. Vošlajerová Bímová designed the study; B. Vošlajerová Bímová and M. Macholán acquired funding; K. Daniszová, P. Hamplová, and Z. Hiadlovská performed the experiments, analysed the behavioural data and collected samples; K. Daniszová, K. Janotová and M. Kašný conducted the proteomic analyses and interpreted the data; O. Mikula and M. Macholán carried out the statistical analyses; K. Daniszová, Z. Hiadlovská, M. Macholán, O. Mikula and B. Vošlajerová Bímová wrote the paper. All authors approved the final version of the manuscript.



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Supplementary online material

Table S1. List of sampling localities of *M. m. musculus* and *M. m. domesticus* mice used as the parental generation for the experimental animals under study (see also Fig. 1). *N* = number of pairs examined.

Fig. S1. Creatinine concentrations in *musculus* and *domesticus* males measured between 20 and 100 days of age, fitted with the M2ds model, which best fit the data (one distinct curve for subordinate *domesticus* males (light blue), one curve for all other males (grey)).

Fig. S2. Normalised MUP concentrations (ng/ml), as in Fig. 1, but the y-axis is not in log-scale (A). Predicted curves for testosterone levels for the same individuals (B); light, thin curves depict 95% confidence intervals (data adapted from Hiadlovská et al. 2015).

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