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The Expansion of House Mouse Major Urinary Protein Genes Probably Did Not Facilitate Commensalism with Humans

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Abstract: Mouse wild-derived strains (WDS) combine the advantages of classical laboratory stocks and wild animals, and thus appear as promising tools for diverse biomedical and evolutionary studies. We employed 18 WDS representing three non-synanthropic species (*Mus spretus*, *M. spicilegus*, and *M. macedonicus*) and three house mouse subspecies (*M. musculus musculus*, *M. m. domesticus*, *M. m. castaneus*) which are all important human commensals to explore whether the number of major urinary protein (MUP) genes and their final protein levels in the urine are correlated with the level of commensalism. Contrary to expectations, MUP copy number (CN) and protein excretion in the strains derived from *M. m. castaneus*, which is supposed to be the strongest commensal, were not significantly different from the non-commensal species. Regardless of an overall tendency for higher MUP amounts in taxa with higher CN, there is no significant correlation at the strain level. Our study thus suggests that the expansion of the *Mup* cluster, which appeared before the house mouse diversification, is unlikely to facilitate commensalism with humans in the three house mouse subspecies. Finally, we found considerable variation among con(sub)specific WDS, warning against generalisations of results based on a few strains.

Keywords: copy number variation; ddPCR; MUP excretion; *Mus musculus*; proteomics; synanthropy

1. Introduction

Progress in life sciences is inevitably contingent on biological models. Besides well-known and extensively employed invertebrates such as the nematode *Caenorhabditis elegans* or *Drosophila* fruit flies, the house mouse (*Mus musculus*) is undoubtedly one of the most widespread model organisms. This synanthropic mammal species consists of three widespread subspecies: *musculus*, *domesticus*, and *castaneus*, which diverged approximately 350–500 thousand years ago [1–5]. House mice are significant human commensals, while other species involved in the same clade ('*M. musculus* group': *M. spretus*, *M. macedonicus*, *M. spicilegus*, and *M. cypriacus*), live for the most part outside the synanthropic niche [6,7].

Like most other models, mice have several advantages, such as ease of breeding and manipulation, a relatively short generation time, and the ability to reproduce all year round. The whole-genome sequence was published only a year after that of humans [8], and large databases of well-annotated sequences and diverse molecular markers with known positions in the genome are now available. Since house mice are highly tolerant of inbreeding, numerous inbred laboratory stocks have been established during the last century. However, these 'classical laboratory strains' (CLS) have several substantial limitations.

First, these mice represent an artificial taxon. In the 1980s, it appeared that most CLS, until then considered domesticated *M. m. domesticus*, carry *M. m. musculus* Y chromosomes [9–11]. Later, it was shown that CLS's genome is a mixture of all three subspecies, though the contribution of *domesticus* prevails [12–14]. Second, the genetic and phenotypic diversity of CLS is severely restricted. For example, despite a wide array of existing stocks, their ancestry basically traces back to a single female

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[12,15–17]. Therefore, mouse inbred strains cannot encompass the whole variation in the wild and do not represent natural conditions.

Consequently, scientists have started to pay attention to wild mice and more natural settings in addressing genetic, physiological, behavioural or ecological questions, e.g. using seminatural enclosures [18–23] or barns [24–26]. However, the scale of natural variation covered by the mice seeding those experiments has necessarily been limited. Moreover, high variability contradicts the requirements for experimental reproducibility (see [13] for a review of the strengths and limitations of CLS and wild mice).

A promising compromise between the availability of sufficient variation and reproducibility is inbred stocks directly derived from mice captured in natural populations [17,27–29]. Contrary to CLS, the geographic origin and pedigree of these wild-derived strains (WDS) are precisely known. Although they cannot equal wild mice in the level of variation they harbour, this deficiency can be alleviated by increasing the number of strains employed. A current study of 101 WDS representing five species (including the three *M. musculus* subspecies) and eight natural Y chromosome consomic strains has revealed substantially higher variation than CLS: for example, WDS displayed as many as 2483% single-nucleotide polymorphisms (SNP) compared to CLS [17]. Therefore, WDS appear to be excellent tools for diverse genetic, behavioural, and ecological studies, allowing miscellaneous hypothesis testing.

Among especially opportune candidates for such testing are genes involved in olfaction. The house mouse is extraordinary because it possesses ~1200 olfactory receptors and ~250 pheromone receptors in its genome [30–32]. The high numbers of receptors mirror a complex mix of chemical signals involved in mouse communication. This mix includes products of multigene families such as secretoglobins (also known as androgen binding proteins, ABP), major histocompatibility complex (MHC), exocrine gland-secreted proteins (ESP), odorant-binding proteins (OBP), and major urinary proteins (MUP) (see [33–35] for review), detected either by the main olfactory epithelium or vomeronasal organ.

This study is focused on major urinary proteins, which play a prominent role in mouse chemical communication [36]. These relatively small (18–20 kDa) barrel-shaped lipocalin proteins bind low-molecular-mass ligands, including volatile pheromones, which are released to the external environment through urine, saliva, tears, and other secretions. Because of this bond, the release is slow, and the effect of the ligands is thus protracted [37,38]. However, at least some of them can, on their own, also modulate the recipients' behaviour and physiology [39–44]. The most important are urinary MUPs (uMUPs) which are expressed in the liver and secreted through the kidneys into the urine [45–47]. The uMUPs production is sexually dimorphic: males excrete 3–10 times more uMUPs than females [43,48,49].

In house mice, the *Mup* cluster consists of at least 21 genes and 20 pseudogenes, which are tandemly arrayed across an ~2 Mb long stretch of chromosome 4. There are two classes of *Mup* genes that differ by sequence similarity and expression profiles. Evolutionarily older is the peripheral class, characterised by <82% mature protein sequence identity. The central cluster is younger and highly homogeneous (>97% mature protein sequence identity), suggesting a very recent series of duplications or gene conversions [50–52]. According to Sheehan et al. (2019), the central *Mup* cluster has undergone two phases of expansion: a minor one in the common ancestor of *M. musculus*, *M. spretus*, *M. macedonicus*, and *M. spicilegus* and a second, larger expansion predating the diversification of the three *M. musculus* subspecies.

The timing of the second central *Mup* expansion indicates that this event could have facilitated the evolution of human commensalism in house mice about 10,000 years ago [52] since the ability to advertise more complex information in urinary scent marks can be beneficial in dense populations observed in synanthropic circumstances [53]. High population densities associated with the synanthropic niche should increase interaction rates among mice, making information-rich urinary marks highly important. Therefore, although the expansion of the central *Mup* gene cluster predated human commensalism, its further diversification could have been positively selected by the synanthropic bond [52]. On the other hand, we may assume that the *Mup* genes, like other large gene

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families, are prone to high within-species copy number variation (CNV) [54,55]. Unfortunately, a large part of this variation remains undetected despite years of high-throughput sequencing. One of the reasons for this gap is difficulties with correctly assembling large repeated regions [56,57], whereas other approaches like qPCR may lack adequate accuracy [58]. Moreover, most previous studies have been limited to a couple of CLS, such as the reference genome mouse C57BL/6, while information on the number and arrangement of *Mup* paralogs in wild populations and/or other mouse species is rather scarce. This gene cluster may thus harbour an important portion of undetected variation [50,51].

As mentioned above, the number of *Mup* genes positively correlates with the level of synanthropy in the *M. musculus* group, with higher CN in the commensal house mouse than in other, non-commensal species [52]. Can this association be extended to different house mouse subspecies or populations? For example, according to Payne et al. (2001) [59] and Beynon et al. (2002) [60], a free-living ('feral') island *M. m. domesticus* population revealed higher MUP profile similarity and lower diversity than the mainland, farm-living populations. Similarly, Cheetham et al. (2009) [49] reported reduced MUP profile complexity and diversity in lab strains compared to wild mice. Although the former case is likely to be in large part related to population size differences, and all three studies were focused on MUP electrophoretic band profiles rather than the number of *Mup* genes, these data indicate we may expect differences in total CN and expression not only between synanthropic and non-synanthropic mouse species but also between subspecies and populations of *M. musculus* itself. Despite the fact that house mice are ecologically very flexible [61,62], *M. m. domesticus* is generally believed to be more tightly associated with humans than *M. m. musculus* [61,63–65]. Populations of the former subspecies were also shown to be more strongly structured into local breeding units or demes [66]. Much less is known about the Asian subspecies *M. m. castaneus*, but it is also known to be highly commensal [61,67–69] or even more commensal than *M. m. domesticus* [61,63,65], being „man's closest indoor associate among undomesticated mammals" [70] (p. 20).

In this study, we test for a potential association between the level of synanthropy and the number of *Mup* gene copies within the *Mus musculus* species group using the droplet digital PCR method. These data are complemented with measurements of MUP levels in the urine. Wild individuals are not suitable for this purpose because each mouse is genetically distinct (and, for some part, heterozygous), which hampers generalisations. Therefore, here we employ 18 WDS representing three house mouse subspecies and three other species of *Mus*. We show that notwithstanding a general trend for higher CN in *M. musculus* than in its non-commensal relatives, CNV does not fully reflect the strength of synanthropy. Moreover, though the total numbers of the urinary *Mup* gene copies were found to be correlated with their overall uMUP levels in the urinary proteome, CN is a poor predictor of the final level of uMUP excretion in individual strains. Finally, we show that variation among strains derived from different subspecies does not exceed variation within the subspecies. We thus confirm the high genetic variability harboured by WDS and hence their great value for many fields of life sciences.

2. Materials and Methods

2.1. Mice

Genomic DNA was extracted from the liver as described in Supplementary Material. In total, we analysed 52 individuals of 18 WDS, representing *M. m. musculus* (5 WDS), *M. m. domesticus* (5 WDS), *M. m. castaneus*, *M. spretus*, *M. macedonicus*, and *M. spicilegus* (2 WDS each) (Table 1, Figure 1).

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Figure 1. Map of Europe with locations of the founding populations of the WDS employed in this study: brown circles: *Mus spretus*, light green circles: *M. spicilegus*, dark green: *M. macedonicus*, red circles: *M. musculus musculus*, blue circles: *M. m. domesticus*; the violet line schematically depicts the hybrid zone between *M. m. musculus* and *M. m. domesticus*. Missing are two *M. m. castaneus* stocks, CIM from Masinagudi, India, and CKN from Nairobi, Kenya.

Table 1. List of wild-derived strains used in this study. More details on the WDS can be found at https://housemice.cz/en. *N* = number of individuals per strain (CNV/expression); note that CNV was estimated regardless of sex, whereas an equal number of males and females per strain was used for expression measurements.

Mus musculus								
musculus			*domesticus*			*castaneus*		
Strain	Country	*N*	Strain	Country	*N*	Strain	Country	*N*
BUSNA	Czechia	3/6	DDO	Denmark	3/6	CIM	India	3/6
MBK	Bulgaria	3/6	DROS	Bulgaria	3/6	CKN	Kenya	3/6
MDH	Denmark	3/6	SCHUNT	Germany	3/6			
MPB	Poland	3/6	STRA	Germany	3/6			
STUF	Czechia	3/6	WLA	France	3/6			
M. spretus			*M. spicilegus*			*M. macedonicus*		
Strain	Country	*N*	Strain	Country	*N*	Strain	Country	*N*
SEB	Spain	3/6	ZRU	Ukraine	3/6	XBS	Bulgaria	3/6
SMON	France	3/6	ZPB	Bulgaria	2/2	MACSO	Bulgaria	2/2

2.2. CNV

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Copy numbers were scored using the QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA). We designed an MUP-specific assay consisting of two primers and a fluorescent probe (Supplemental Figure S1, Supplementary Material) using C57BL/6J sequence and primer design tools (Geneious Prime 9.1.5, Biomatters). The validity of the assay was checked first with NCBI Primer-BLAST (Supplemental Table S1) and then using C57BL/6J genomic DNA as a template. All samples were run in triplicates (technical replicates) and processed in the Quantasoft environment provided with the QX200 ddPCR System, and the resulting values were rounded. The resulting numbers were halved to get haploid CN estimates. Values for the biological replicates were then averaged.

2.3. Proteomic analysis

We have followed a protocol described in [71]. In short, all urine samples were precipitated with ice-cold acetone and centrifuged at 14000 rpm for 10 minutes at 0°C. The protein concentration of each lysate was determined using the BCA assay kit (Fisher Scientific). Peptides cleaved with trypsin were desalted on a Michrom C18 column. Nano Reversed phase columns were used (EASY-Spray column, 50 cm x 75 μm ID, PepMap C18, 2 μm particles, 100 Å pore size). Eluting peptide cations were converted to gas-phase ions by electrospray ionisation and analysed on a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo) with the same parameters as described in [71–73]. LC-MS data were pre-processed with MaxQuant software (version 1.6.34) [74]. The false discovery rate (FDR) was set to 1% for both proteins and peptides, and we specified a minimum peptide length of seven amino acids. The Andromeda search engine was used for the MS/MS spectra mapping against our modified Uniprot *Mus musculus* database, containing 44,900 entries. We modified our databases such that all MUP sequences were removed, and instead of them, we added a complete list of MUPs from the Ensembl database [75]. Quantifications were performed using label-free algorithms [74] with a combination of unique and razor peptides.

2.4. Statistics

All data sets were tested for normal distribution. When no significant deviation from normality was proved, parametric tests (analysis of variance, Tukey HSD, Student's *t*-test, Pearson's correlation) were used; otherwise, non-parametric tests (Kruskal-Wallis, Mann-Whitney, median test) were applied. Statistica v. 14 [76] was employed for all the statistical analyses.

3. Results

3.1. CNV

Variation within the technical (within-individual) replicates, expressed as Poisson errors provided by the QX200 software, was very low (Supplemental Table S2) regardless of the number of copies (r =- 0.102; p = 0.4916). Nevertheless, the variance between individuals within WDS was significantly increasing with CN (Figure 2), i.e. the higher CN, the greater the difference between individuals of the same strain (r = 0.541; p = 0.0204).

ANOVA revealed highly significant CN variation among the taxa (F = 44.649, d.f. = 5, p = 0.0000). As expected, non-commensal WDS had the lowest CN captured by our assay, with average values ranging from 4.40 (MACSO) to 9.65 (ZPB) but mostly between 5 and 6 (Supplemental Table S2). In contrast, higher CN values were found in the commensal subspecies: the ranges of *Mup* copies were 22–35 in *M. m. musculus* (mean = 25.94, SE = 1.354), 17–40 in *M. m. domesticus* (mean = 26.11; SE = 2.093), and 7–14 in *M. m. castaneus* (mean = 10.62, SE = 1.486). According to the Tukey HSD post-hoc test, the difference between *musculus* and *domesticus* was not significant (p = 1.0000), while both the subspecies had significantly higher CN than all other WDS, including *castaneus* (p = 0.0001). Although *castaneus* also had generally higher CN than the non-commensal species, these differences were not significant (p = 0.2960–0.8542 depending on comparison). Finally, differences between the non-commensal species were not significant (p = 0.9535–1.0000 depending on comparison) (Table S4, Supplementary Material).

As shown in Figure 2, CN values varied widely among the strains (F = 194.660, d.f. = 17, p = 0.0000), although the variation could only be tested in two commensal *M. musculus* subspecies,

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musculus and *domesticus*, for which a sufficient number of WDS was measured. We gauged it as differences in average CN between WDS within subspecies vs. differences between WDS of different subspecies. Student's *t*-tests showed no significant differences both in *musculus* and *domesticus* (p = 0.5012 and 0.1403, respectively), and the two subspecies did not significantly differ from each other in this respect (p = 0.0919). In summary, intrasubspecific variation appeared similar in the two subspecies, and it was not different from intersubspecific variation in both of them.

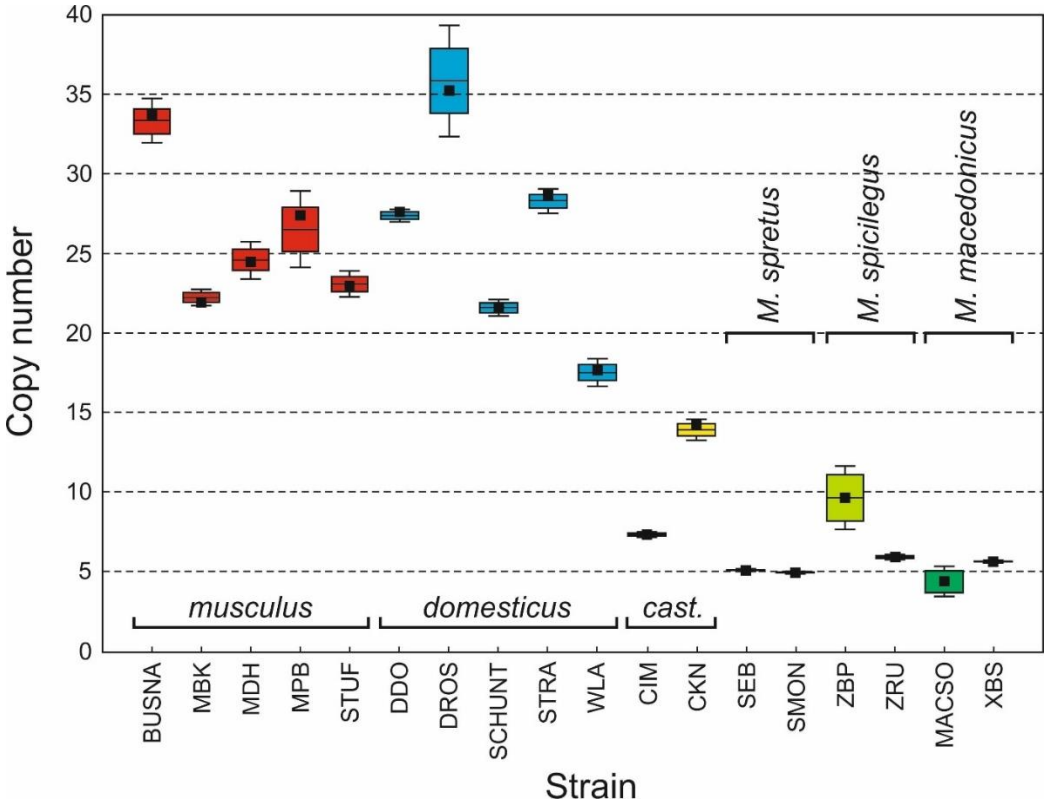

Figure 2. Box plot of copy numbers estimated for each WDS; horizontal line: mean, box: standard error, whiskers: standard deviation, black squares: median; *cast*.: *M. m. castaneus*.

3.2. Total urinary MUP levels

Like in CNV, the total uMUP levels varied considerably among WDS but, contrary to CNV, also within the strains, especially in *M. m. musculus* and *M. m. domesticus*, in both sexes (Figures 3 and 4; Supplemental Table S6). Kruskal-Wallis and median tests of variance among strains yielded slightly different results, yet all were close to the significance limits (males: Kruskal-Wallis H(17,50) = 26.973, p = 0.0585; median test Chi-square = 31.333, d.f. = 17, p = 0.0182; females: Kruskal-Wallis H(17,50) = 32.548, p = 0.0129; median test Chi-square = 26.000, d.f. = 17, p = 0.0745). When taxa, instead of WDS, were compared, all results were significant (males: Kruskal-Wallis H(5,50) = 21.135, p = 0.0008; median test Chi-square = 20.667, d.f. = 5, p = 0.0009; females: Kruskal-Wallis H(5,50) = 25.0177, p = 0.0001; median test Chi-square = 14,333, d.f. = 5, p = 0.0136). Kruskal-Wallis pairwise comparisons only showed significant differences between *musculus* and *M. spicilegus* in males (p = 0.0458) and between *domesticus* and *M. spretus* and *M. spicilegus* in both sexes (males: p = 0.0213 and 0.0039, respectively; females: 0.0045 and 0.0008, respectively; Table S5, Supplementary Material). The same results were revealed using pairwise Mann-Whitney tests after Bonferroni adjustment of the alpha value.

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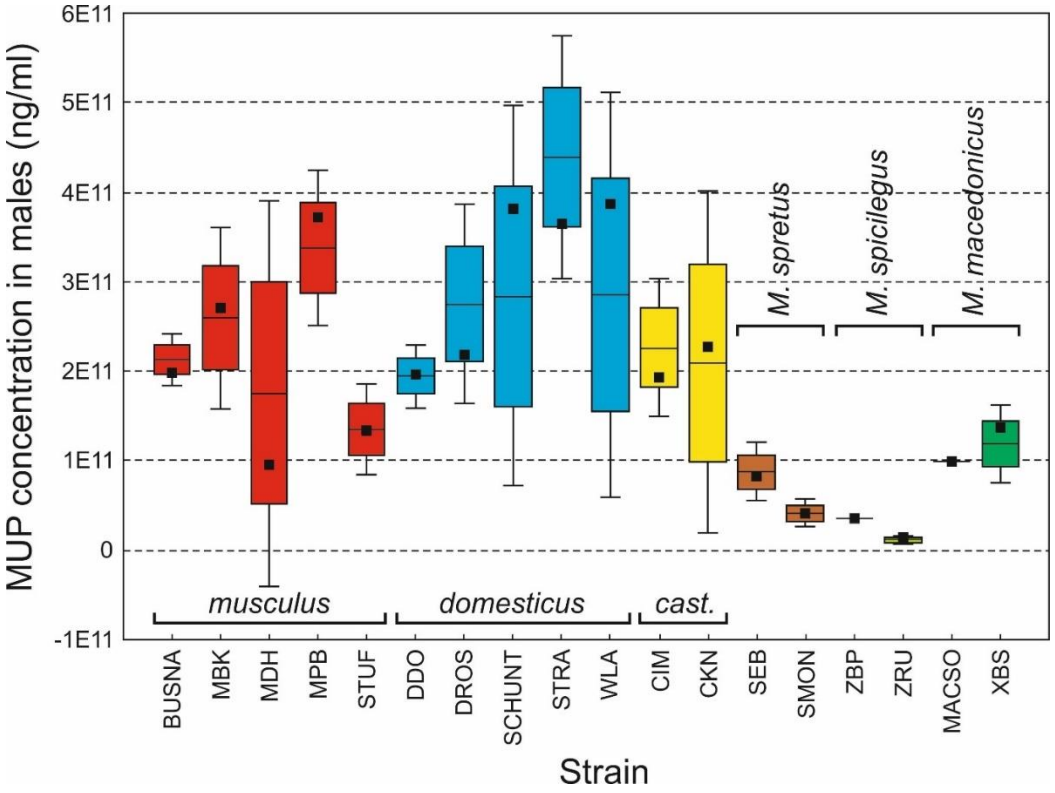

Figure 3. Box plot of total urinary MUP levels estimated for males of each WDS; horizontal line: mean, box: standard error, whiskers: standard deviation, black squares: median; *cast*.: *M. m. castaneus*.

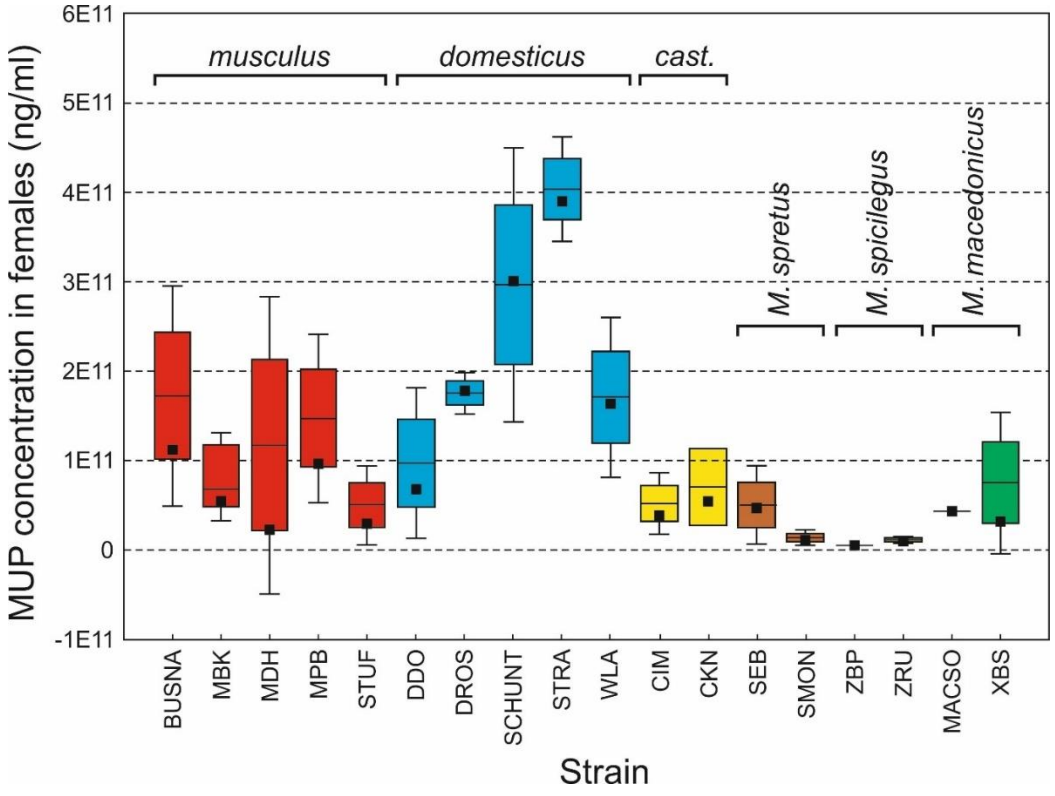

Figure 4. Box plot of total urinary MUP levels estimated for females of each WDS; horizontal line: mean, box: standard error, whiskers: standard deviation, black squares: median; cast.: M. m. castaneus.

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Variation was again assessed only in *M. m. musculus* and *M. m. domesticus* as differences between WDS within and between the subspecies. Significant differences between intrasubspecific and intersubspecific variation were found in neither subspecies nor sex ($p > 0.05$ in all cases). This means that mean differences between the consubspecific strains within *musculus* and *domesticus* are comparable to those between heterosubspecific strains. When we compared intrasubspecific variation between the subspecies, we got a non-significant result for males (*t*-test: $p = 0.9556$) but significant for females, with higher variation in *domesticus* than *musculus* ($p = 0.0154$).

To what extent does variation in the total uMUP amount reflect variation in CN? When all strains are pooled, the protein levels appear significantly correlated with CN (males: $r = 0.699$, $p = 0.0015$; females: $r = 0.625$, $p = 0.0056$). However, these results can be false positives caused by underlying general differences between the taxa, as shown in a hypothetical example in Supplemental Figure S2. Indeed, when the two taxa with sufficiently high N were tested separately, all results were insignificant (*musculus*, males: $r = 0.118$, $p = 0.8505$; females: $r = 0.859$, $p = 0.0621$; *domesticus*, males: $r = 0.037$, $p = 0.9523$; females: $r = 0.046$, $p = 0.9420$). In summary, uMUP CN is a poor predictor of the final protein excretion at the level of WDS within the two subspecies.

4. Discussion

In this study, we showed that notwithstanding a general trend for higher CN in *M. musculus* than in its non-commensal relatives, CNV does not fully reflect the strength of synanthropy since CN in *M. m. castaneus* is not, on average, significantly different from non-commensal species *M. spretus*, *M. macedonicus*, and *M. spicilegus*. Moreover, CN was a poor predictor of the total uMUP excretion at the level of individual WDS. Finally, we showed that variation among strains derived from different subspecies does not exceed variation within the subspecies.

Low CN in *M. macedonicus*, *M. spicilegus*, and *M. spretus* is consistent with the previously published results [52], suggesting that these non-commensal members of the *M. musculus* species group have undergone only one round of expansion of the central *Mup* gene cluster, while an additional expansion occurred in the three commensal house mouse subspecies prior to their diversification [52]. Higher CN can potentially render a wider room for finer diversification of chemical signals [77]. As pointed out by Hurst (1987) [53], Pocock et al. (2004) [78], and others, an ability to render detailed individuality information in the urine or other excreted fluids should be beneficial for enhanced interactions among animals in dense house mouse populations. Higher information complexity may thus have facilitated the evolution of human commensalism [52]. A similar association between social structure and the level of individuality was described in marmots [79]. It has also been shown that people from small hometowns (i.e. from lower-density populations) have a poorer face-learning ability than individuals from large hometowns (i.e. from higher-density populations) [80]. Moreover, a large part of this variation is genetically determined [81,82].

In this respect, the strikingly low copy number of *Mup* genes in the southeastern Asian house mouse subspecies *M. m. castaneus* is surprising since this taxon is commonly considered to be strongly synanthropic [61,65,67–70], with feral populations extremely rare (e.g. in Micronesia [63]). It should also be noted that all the *M. musculus*-derived strains used in this study, including the two *castaneus* WDS (CIM, CKN), were established from commensal populations living in similar synanthropic habitats. Our study thus shows that *Mup* CN is not correlated with the level of commensalism within the house mouse. This conclusion is further corroborated by the extensive CN variance within each of the subspecies, indicating relaxed or weak selective pressure for high CN.

The discrepancy between CN and final protein excretion is not surprising for a couple of reasons. First, in addition to functional *Mup* paralogs, CN captured through ddPCR is likely to also involve several pseudogenes, despite our effort to amplify functional genes only. Second, the expression of individual MUPs is under complex endocrine control involving testosterone, thyroxine, and growth hormone [83,84]. Therefore, it can be down-regulated or up-regulated depending on health, food supply, age, and other conditions [85,86] or as a consequence of social interactions [87], though the latter potential influence may have been prevented or at least reduced by individual housing of the mice under study. We should also keep in mind that we analysed raw data without creatinine

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correction [86,88], and differential water intake before urine collection thus may have introduced another source of 'noise'. It has been suggested that uMUPs are able to provide individual identity information in urine markings (e.g. [36,50,53,89–92]). This notion has recently been challenged by Thoß et al. [85,93], who argue that individual MUP excretion profiles are dynamic rather than stable over time as required for the 'barcode hypothesis', implying a further cause of MUP excretion variability. In any case, regardless of which of the two contradictory hypotheses is correct, we should expect higher variation within WDS in the total MUP levels than in the total *Mup* CN, in agreement with the results of this study.

At the level of individual taxa, the overall uMUP excretion corresponds to CN. In the three non-commensal species, the data are consistent with those published earlier [52,84], not only by the apparently, though not always significantly, lower uMUP levels relative to *M. m. musculus* and *M. m. domesticus* but also by differences within the non-commensal group, with uMUP excretion being highest in *M. macedonicus* and lowest in *M. spicilegus* in both sexes (Figures 3 and 4). By contrast, while previous studies [48,52,94] reported higher MUP levels in *M. m. musculus* males than in *M. m. domesticus* males, we found no significant differences between them. The cause of this discrepancy is unclear; however, it would be interesting to explore whether and how different mouse subspecies respond to varying social contexts, which was, in this case, complete social isolation. On the other hand, our data are consistent with the lack of difference between these subspecies in females reported by Stopková et al. (2007) [48], Hurst et al. (2017) [94], and Sheehan et al. (2019) [52]. In fact, despite high variance among the consubspecific strains, we revealed slightly higher uMUP excretion in *domesticus* females than in *musculus* females, in agreement with Stopková et al. (2007) [48] and Sheehan et al. (2019) [52].

5. Conclusions

We confirmed substantial genetic variability harboured in WDS and hence their great value for many fields of life sciences, including copy number variation and its medicinal and evolutionary consequences. Chemical communication is highly important for many mammals [95,96]. Therefore, some mammal species, such as the house mouse, have undergone extraordinary expansion of genes encoding olfactory signals and their receptors [30–32]. Mouse olfactory signals involve products of various multigene families [33–35,97], which are excreted into several body fluids, mainly into the urine. As a crucial part of urine scent marks [36,98], major urinary proteins have long been focused on by a vast array of studies (e.g. [36,52,84,90]). However, many of them employed highly inbred laboratory strains which had been created by artificial selection and thus substantially differ from wild mice in numerous traits [84]. Other surveys have been limited to a single subspecies. This all makes generalisations of such studies difficult.

Wild-derived strains combine the advantages of CLS (reproducibility due to inbredisation) and wild animals (natural variability). Here, we showed tremendous diversity in MUP gene copy number and total protein levels in the urine captured in mouse WDS, confirming the great value of these stocks for genetic, biomedical, and evolutionary studies [17]. However, we should keep in mind that 'riding two horses at once', i.e. combining reproducibility with diversity, also imposes higher demands on the number and proper choice of employed WDS. Namely, they should both compensate for the haphazardness of fixation of individual traits by the inbredisation process *and* cover as much of the natural variation as possible. The first demand can be met by choosing more than one WDS from the same local area, whereas the second calls for using stocks derived from populations scattered across a great deal of a (sub)species range.

To conclude, our analysis of the number of *Mup* genes and levels of their final protein product in the urine, using 18 stocks derived from commensal and non-commensal taxa of the *Mus musculus* species group, suggests that the second expansion of the central *Mup* cluster is unlikely to facilitate commensalism with humans in the three house mouse subspecies.

Supplementary Materials: The following supporting information can be downloaded at:.

Author Contributions: Conceptualization, M.M.; formal analysis, M.M.; investigation, K.D. and Z.H.; writing—original draft preparation, M.M.; writing—review and editing, K.D. and Z.H.; project administration, M.M.; funding acquisition, M.M. All authors have read and agreed to the published version of the manuscript.

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