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COMPARISON OF URINARY SCENTS OF TWO RELATED MOUSE  
SPECIES, *Mus spicilegus* and *Mus domesticus*

HELENA A. SOINI<sup>1</sup>, DONALD WIESLER<sup>1</sup>, SACHIKO KOYAMA<sup>1</sup>, CHRISTOPHE  
FÉRON<sup>2</sup>, CLAUDE BAUDOIN<sup>2</sup> and MILOS V. NOVOTNY<sup>1,\*</sup>

<sup>1</sup>*Indiana University, Institute for Pheromone Research, Department of Chemistry,  
Bloomington, IN 47405, USA*

<sup>2</sup>*Laboratoire d'Ethologie Expérimentale et Comparée, Université Paris 13, 99 Avenue Jean-  
Baptiste Clément, F-93430 Villetaneuse, France*

**Abstract** - Whereas the house mouse (*Mus domesticus*) has been studied extensively in terms of physiology/behavior and their pheromonal attributes, the evolutionary related mound-building mouse (*Mus spicilegus*) has received attention only recently due to its divergent behavioral traits related to olfaction. To this date, no chemical studies on urinary volatile compounds were performed on *M. spicilegus*. The rationale for our investigations has been to determine any potential differences in urinary volatiles of intact and castrated *M. spicilegus* male and explore further whether this species could utilize the same or structurally similar pheromones as the male house mouse, *Mus domesticus*. The use of capillary gas chromatography/mass spectrometry (GC-MS) together with sorptive stir bar extraction sampling enabled such quantitative comparisons between the intact and castrated *M. spicilegus* urinary profiles. Additionally, through GC-MS and atomic emission (sulfur-selective) detection, we were able to identify distinct qualitative molecular differences between intact *M. spicilegus* and *M. domesticus*. On one hand, a series of volatile and odoriferous lactones and the presence of coumarin were the unique features of *M. spicilegus*, as was the notable absence of 2-sec-butyl-4,5-dihydrothiazole (a prominent *M. domesticus* male pheromone) and other sulfur-containing compounds. Castration of *M. spicilegus* males eliminated several substances, including  $\delta$ -hexalactone and  $\gamma$ -octalactone, and decreased substantially additional compounds, suggesting their possible role in chemical communication. On the other hand, some other *M. domesticus* pheromones were also found in *M. spicilegus* urine. These comparative chemical analyses support the notion of metabolic similarities as well as the uniqueness of some volatiles for *M. spicilegus*, which may have a distinct physiological function in reproduction and behavior.

**Key Words** - *Mus spicilegus*, *Mus domesticus*, urinary volatile profile, gas chromatography/mass spectrometry, stir bar extraction, pheromones.

\*Correspondence: Milos V. Novotny, e -mail: [novotny@indiana.edu](mailto:novotny@indiana.edu)

## INTRODUCTION

There is an evolutionary connection between the common house mouse (*Mus domesticus*) and the mound-building mouse (*Mus spicilegus*) species, with two pairs of close species and subspecies in the phylogenetic tree, *M. musculus domesticus* and *M. m. musculus* in one pair and *M. spicilegus* and *M. macedonicus* in the second one (Bonhomme et al., 1984; Sage et al., 1993). The *M. domesticus* living environment is interwoven to human habitats, whereas *M. spicilegus* species is feral, and in some cases, at least during the summer period, *M. spicilegus* may live in a close contact with *M. m. musculus* (Orsini et al., 1983; Sokolov et al. 1998; Simeonovska-Nikolova 2007 ). Additionally, in their social structure and behavior, the *M. spicilegus* have developed far apart from the *M. domesticus* species. Unlike *M. domesticus*, *M. spicilegus* are monogamous (Patris and Baudoin, 1998; Dobson and Baudoin, 2002; Baudoin et al., 2005; Gouat and Féron, 2005) and build co-operatively colonial mounds for overwintering (Orsini et al., 1983; Garza et al., 1997; Poteaux et al., 2008). Moreover, *M. spicilegus* males display intense paternal care (Patris and Baudoin, 2000; Féron and Gouat, 2007).

The *M. spicilegus* species appear to rely on olfaction in their social behavior and mate selection (Patris and Baudoin, 1998; Patris et al, 2002; Heth et al., 2002, 2003). In this species, social and kin recognition through olfactory cues have been observed in several studies (Gouat et al., 1998; Dobson and Baudoin, 2002; Baudoin et al., 2005; Busquet and Baudoin, 2005; Todrank et al., 2005; Colombelli-Negrel and Gouat, 2006). The major mammalian scent sources for chemical communication are sex glands, other skin glands or urine. The salivary androgen-binding proteins (Laukaitis et al., 1997; Talley et al., 2001) and tear fluid peptides (Kimoto et al., 2005) have also been reported as sources of chemical messengers in for *M. domesticus*. In the house mouse, the urine-mediated chemical signals have been relatively well-characterized over the years (for a review, see Novotny, 2003; Hurst and

Beynon, 2004). To date, the chemical constituents of various scent sources for the *M. spicilegus* species, used in communication, are still unknown. Behavioral tests have revealed that the odor cues (expected to originate mainly from urine) are used in communication (Patris and Baudoin, 1998; Féron and Gheusi, 2003; Busquet and Baudoin, 2005; Colombelli-Negrel and Gouat, 2006). Thus far, there have been no complementary reports on the chemical nature of such olfactory cues.

The first purpose of this study has been to structurally characterize and quantify individual chemical constituents of the urinary volatile profiles for the intact male *M. spicilegus* against those of castrated animals. The second goal was to compare the findings qualitatively to the previously well characterized male *M. domesticus* urinary compounds.

A quantitative comparison of the intact and castrated *M. spicilegus* male volatile profiles was performed to explore the metabolic end-products which could be produced under the testosterone control. Some of the endocrinologically controlled urinary constituents may act as chemical communication means for various reproductive and social functions. Additionally, the urinary volatile profiles of female and male *M. spicilegus* individuals were qualitatively compared in order to explore the gender roles related to the urine-mediated chemical communication, such as those used in mate selection and individual recognition. This chemical characterization is expected to provide some clues to the observed differences in social and behavioral characteristics between the two species.

To facilitate this study, we used the stir bar aqueous extraction method (Baltussen et al., 1999, 2002), which was followed by solventless sample introduction into a gas chromatograph-mass spectrometer (GC-MS) instrument. This methodology is compatible with screening for volatile organic compounds at low concentrations in biological samples and is well suited for a compound identification and quantitative comparisons (Soini et al., 2005). In addition, a combination of gas chromatography with atomic emission detection

(GC-AED) was utilized for the highly sensitive sulfur compound profiling. In this report, we also take our previously determined characteristics of the male *M. domesticus* (ICR, C57Black/B6 and C57Black/B10) urinary volatile components and compare them qualitatively with the new *M. spicilegus* chemical information obtained in this study. Analytical approaches for the *M. domesticus* and *M. spicilegus* samples were identical. All analyses were performed in the same laboratory.

## **METHODS AND MATERIALS**

*Experimental Animals* *Mus spicilegus* mice were fifth-generation animals from a population collected in Gyöngyös, Hungary, in October 1999. From the time of their collection, the genealogy of every individual was known and all breeding pairs had been formed in a way to avoid inbreeding. They were bred at the University of Paris 13 under laboratory conditions ( $20\pm 1^\circ\text{C}$ ) with a 14 : 10 h light : dark cycle. Food (mouse pellets type M20, Special Diet Services, Witham, Essex, U.K.), water and bedding material (sawdust and cotton) were provided. Mice were weaned at 28 days of age and housed in same-sex sibling groups from 35 days of age. Males and females were 3–6 months old when they were used as urine donors.

For the male-female mouse comparisons, 6 males and 6 females were isolated in standard polycarbonate cages (26 x 16 cm and 14 cm high) 1 week before urine collection in order to eliminate the social dominance effect (Féron and Baudoin, 1993, 1998). With the male presence being required to induce sexual receptivity in *M. spicilegus* females (Féron and Gheusi, 2003), we assumed that all the isolated females were in anoestrus.

For the comparisons between intact and castrated males, isolation occurred 3 weeks prior to urine collection. Twenty males were isolated in standard polycarbonate cages (26 x 16 cm, 14 cm high). Six of them were then castrated under anesthesia induced by an

intraperitoneal injection of a mixture of ketamine (100 mg/kg per mouse) and xylazine (5 mg/kg per mouse).

Male *M. domesticus* urinary volatile profile from inbred ICR albino mice, C57BL /B6 and C57BL /B10 black mice (Jackson Laboratories, Bar Harbor, ME) were used for qualitative comparisons. The data obtained for *M. domesticus* have been previously reported by Harvey et al., 1989 and Novotny et al., 2007.

*Urine Collection* Animals were introduced individually into a clean polycarbonate cage and surveyed for urination during 5 minutes at maximum. Fresh individual urine was then quickly collected with a syringe and frozen (-20°C). A preliminary study showed that this was the best process fitted to these very sensitive mice.

*Ethical Note* The experiments complied with the current French laws (authorization 93-0033 for C. Féron; Laboratory approval was secured from the Prefecture of Seine Saint Denis (prefectorial decree 02-2651), complying with the Association for the Study of Animal Behaviour/Animal Behaviour Society Guidelines for the Use of Animals in Research.

*Reagents and Analytical Methods* All compound identifications were verified through the authentic standards whenever available. Standard compounds were either purchased from Aldrich Chemical Company (Milwaukee, WI) or synthesized in our laboratory according to the previously described synthetic methods for dehydro-*exo*-brevicomine (Wiesler et al., 1984), *sec*-butyl-4,5-dihydrothiazole (North and Pattenden, 1990) and 6-hydroxy-6-methyl-3-heptanone (Novotny et al., 1999). Twister™ stir bars (10 mm in length, 0.5 mm film thickness, 24-μL polydimethylsiloxane (PDMS) volume) were used as the sorptive extraction devices. They were purchased from Gerstel GmbH (Mülheim an der Ruhr, Germany).

Volatile and semivolatile compounds were extracted from 0.2 ml of urine in 20-ml capped glass vials for 60 min with a Twister™ stir bar. The urine samples were first diluted with 2.0 ml water (high-purity OmniSolv® water, EM Science, Gibbstown, NJ). As an internal



standard, 8 ng of 7-tridecanone (Aldrich, Milwaukee, WI) was added in 10  $\mu$ l of ethanol to each vial. Stirring speed was 800+ rpm on the Variomag Multipoint HP 15 stirplate (H+P Labortechnik, Oberschleissheim, Germany). Prior to extraction, all glassware was washed with acetone and dried at 80° C. After extraction, the stir bars were rinsed with a small amount of distilled water, dried gently on a paper tissue, and placed in the TDSA autosampler tube (a product of Gerstel GmbH, Mülheim an der Ruhr, Germany) for the gas-chromatographic (GC) or GC-MS analysis. GC equipment for the sulfur compound analysis consisted of an Agilent GC Model 6890 instrument with an Atomic Emission Detector (AED) (Model G2350A from Agilent Technologies, Wilmington, DE) and a Thermal Desorption Autosampler (TDSA, Gerstel). The separation capillary was HP-5MS (30 m x 0.25 mm, i.d., 0.25  $\mu$ m film thickness) from Agilent. Samples were thermally desorbed in a TDSA automated system, followed by injection into the column with a cooled injection assembly, CIS-4. TDSA operated in a splitless mode. Temperature program for desorption was 20° C (0.5 min), then 60° C/min to 280° C (10 min). Temperature of the transfer line was set at 280° C. CIS was cooled with liquid nitrogen to -60° C. After desorption and cryotrapping, CIS was heated at 12° C/s to 280° C with the hold time of 10 min. CIS inlet was operated in the solvent-vent mode, a vent pressure of 14 psi, a vent flow of 30 ml/min, and a purge flow of 50 ml/min. The temperature program in the GC operation was 40° C for 5 min, then increasing to 200° C at the rate of 2° C/min. The final temperature was held for 10 min. The carrier gas head pressure was 14 psi (flow rate, 1.2 ml/min). The GC unit was operated in the constant- flow mode. The emission lines for carbon (193 nm), sulfur (181 nm) and nitrogen (174 nm) were monitored during the atomic plasma emission detection.

The GC-MS instrument used for the compound identification was the Agilent 6890N gas chromatograph connected to the 5973i MSD mass spectrometer (Agilent Technologies). The GC column was a narrow-bore capillary with 180  $\mu$ m, i.d. x 20 m DB-5MS (0.18  $\mu$ m

film thickness, Agilent Technologies, Wilmington, DE). The inlet head pressure was 12.5 psi for the helium flow of 0.7 ml/min. The system operated in the constant-flow mode. The temperature program was 50 °C for 2 min, followed by a ramp of 4 °C /min to 200 °C (hold time 1 min). Positive electron ionization (EI, 70 eV) mode was used with the scanning rate of 4.51 scans/sec over the mass range of 35-350 amu. The MSD transfer line temperature was set at 280 °C. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively. The TDSA-CIS sample introduction setting was identical to that described above in connection with the GC-AED system, except that the CIS trapping temperature was set at -80 °C.

*Quantitative Evaluations and Statistical Analyses* As the basis for quantitative comparisons of urinary chromatographic profiles of the fresh urine collected on plates, the peak area integration was performed and peak areas were normalized by dividing with the peak area of internal standard (7-tridecanone) for each separated component. Either GC-MS total-ion chromatograms (TIC) or selected-ion chromatograms (SIC) obtained after the post-run modification of TICs were used for calculations. Normalized peak areas were statistically evaluated for the intact and castrated mouse groups. Student t test was employed for pairwise comparisons. The sulfur compound profiles from the GC-AED were compared in a qualitative manner between the intact and castrated males. The quantitative data obtained in this study for the male *M. spicilegus* urine samples was compared semi-quantitatively with the female *M. spicilegus* urine and qualitatively with the male *M. domesticus* urine data obtained in this laboratory previously.

## **RESULTS**

Male *M. spicilegus* urinary volatile compound profiles by GC-MS featured more than one hundred components. Approximately 60 compounds showed sufficient spectral intensity and

purity for the quantitative comparisons. Among these compounds, 46 were identified or partially identified while 10 remain unknown. The compound list is shown in Table 1.

The characteristic feature for the profiles from *M. spicilegus* was the prominent presence of  $\gamma$ - and  $\delta$ - lactones, ketones, alcohols and acids. Characteristic urinary components found in *M. domesticus* are three dihydrofuran compounds (MW 126) originating from a puberty accelerating pheromone, 6-hydroxy-6-methyl-3-heptanone (Novotny et al., 1999) and its lactol form (see Table 1 for 5,5-dimethyl-2-ethyl-4,5-dihydrofuran, Z-5,5-dimethyl-2-ethylidenetetrahydrofuran, E-5,5-dimethyl-2-ethylidenetetrahydrofuran). These three furan derivatives were present in male *M. spicilegus* at the level comparable to the *M. domesticus* data reported earlier (Harvey et al., 1989; Novotny et al., 2007). In the castrated *M. spicilegus*, the levels were significantly lower ( $P < 0.002$ ) than in intact *M. spicilegus* males. Another *M. domesticus* pheromone compound, dehydro-*exo*-brevicommin, also present in female urine in small amounts (Harvey et al., 1989; Jemiolo et al., 1991), was found at lower levels in the intact and castrated male and also in female *M. spicilegus* urine (data not shown). Castration did not change dehydro-*exo*-brevicommin levels in *M. spicilegus* significantly in contrast to the suppressed levels of dehydro-*exo*-brevicommin in the samples from castrated *M. domesticus* (Novotny et al., 1980; Harvey et al., 1989). Trace levels of the dominant male mouse pheromone for *M. domesticus*,  $\beta$ -farnesene (Harvey et al., 1989), were detected in the intact male *M. spicilegus* urine, but were not seen in female or castrated animals. N-(methylthio)methylaniline was the only identified sulfur compound in the GC-MS TIC profile shown in *M. spicilegus* profiles, being more abundant in the intact male mouse urine when compared to the mouse urine of castrates ( $P < 0.01$ ). The representative structures of the main compounds distinguishing the two species are shown in Fig.1.

Castration of *M. spicilegus* males affected the volatile profiles in three ways: certain compounds, apparently under endocrine control, disappeared, while the levels of other

compounds increased, and a set of previously undetected compounds became apparent (see Table 1). Fig. 2 illustrates the representative urinary volatile GC-MS TIC profiles for intact and castrated *M. spicilegus* males and intact male *M. domesticus*. In addition to  $\beta$ -farnesene, castration removed 2-heptanone,  $\delta$ -hexalactone, N-phenylformanilide and  $\gamma$ -octalactone from the set of urinary volatiles. Some compound levels decreased significantly after castration, including  $\gamma$ -hexalactone ( $P < 0.02$ ) and N-(methylthio)methylaniline ( $P < 0.01$ ) as shown in Table 1. In contrast, after castration, levels of 2-coumaranone, a unique compound for *M. spicilegus*, increased significantly ( $P < 0.001$ ) as demonstrated in Fig. 3. The absolute amounts of 2-coumaranone in castrated male urine were about  $300 \text{ ng/ml} \pm 200 \text{ ng/ml}$  (SD,  $n=6$ ), while in the intact mouse urine, the levels were just  $2 \text{ ng/ml} \pm 2 \text{ ng/ml}$  (SD,  $n=14$ ). In addition, other constituents, including several lactones, were found at higher levels, and new compounds such as coumarin and several late-eluting lactones appeared in the urine of castrated males (see Table 1). Fig. 4 shows a comparison of selected compound levels affected by castration. Individual variation for some of the compounds appeared relatively large ( $>70\%$ , RSD), while some compounds varied only within the range of 12-30% (RSD) among the sampled individuals. Typically, the variation due to the sampling method was only 5-10% (RSD).

Qualitative comparisons in the GC-AED sulfur-selective profiles did not reveal any clear differences between intact and castrated animals. Relatively low-level (sub-picogram), sulfur-containing compounds (data not shown) were traced, but could not be identified structurally due to their extremely small representation.

Several of the previously reported *M. domesticus* urinary compounds (Novotny et al., 1990a; 1990b, Novotny et al., 2007) were also present in *M. spicilegus* profiles including three characteristic dihydrofurans. It is notable that the male dominance signaling pheromone

compound 2-sec-butyl-4,5-dihydrothiazole and its “structural relative,” 2-isopropyl-4,5-dihydrothiazole for *M. domesticus* (Novotny et al., 1985), were not detected in the *M. spicilegus* urine. Linear sulfur compounds such as dimethyl disulfide, bis(methylthio)methane and methyl(methylthio)methyl disulfide, typical for *M. domesticus* (Novotny et al., 2007), were also absent in *M. spicilegus* GC-MS urinary volatile profiles. Furthermore, very few *M. spicilegus* urinary ketones were present compared to those identified in *M. domesticus* urine (Novotny et al., 2007).

## DISCUSSION

Since castrated male *M. spicilegus* did not show  $\beta$ -farnesene 2-heptanone,  $\delta$ -hexalactone, N-phenylformanilide and  $\gamma$ -octalactone among the urinary volatiles, it is suggested that the metabolic pathways involving these compounds may be under endocrine control. Behavioral tests using these compounds would be necessary to show what are the possible chemosignaling and physiological functions facilitated by these compounds for male and female *M. spicilegus*. Castration affected also most significantly the lactone structural types, thus demonstrating an endocrine feedback for the lactone biosynthesis. There is a strong possibility that some of the lactones may be mediators for chemical communication with the reproduction function. Furthermore, lactone levels were particularly varied among the individual intact males, so that the composition within the lactone compound profile could be related to individual recognition. In their pure form, many of the lactones exhibit fruit- or berry-like aromas (Gatfield et al., 1993), which are relatively subtle odors in human perception, as opposed to the pungent smell in the *M. domesticus* male urine caused by the sulfur-containing compounds. The same unique lactone compounds were found in the female *M. spicilegus* urine in our qualitative screening (unpublished experiments).

Lactone biosynthesis involves C-18 hydroxyl fatty acids as precursors, which undergo  $\beta$ -oxidation steps followed by lactonization (Albrecht et al., 1992). Additionally, 9,10-oleic acid has been reported as a precursor for  $\gamma$ -dodecalactones in the yeast cultures (Haffner and Tressl, 1996). This may imply that the metabolism of fatty acids leading to urinary lactone end-products could play a prominent role in the *M. spicilegus* metabolism as opposed to *M. domesticus* which show very little presence of urinary lactones. The occurrence of urinary lactones has been previously reported in female and male pine voles (*Microtus pinetorum*) (Boyer et al., 1989). In female pine voles,  $\gamma$ -octalactone exhibited the greatest urinary level changes among the volatile compounds after estrogen implantation or ovariectomy.

Other biological sources for lactones have been reported for insects and microorganisms, such as the cephalic gland  $\gamma$ -octalactone of the giant honeybee workers (*Apis laboriosa*) (Blum et al., 2000) and  $\delta$ - and  $\gamma$ -lactones emitted by marine *Alphaproteobacteria* (Dickschat et al., 2005). In these two studies, lactones were hypothesized as potential chemical signaling compounds within the giant honeybee colony and bacterial culture, respectively.

Urinary ketone displays in intact male *M. spicilegus* were interestingly sparse (Table 1). *M. domesticus* urinary ketones (e.g., 2-heptanone, 6-methyl-5-hepten-3-one, 5-hepten-2-one) have been previously found to correlate with the major histocompatibility complex (MHC) mouse haplotypes (Novotny et al., 2007).

*M. spicilegus* originate from Eastern Europe. Several diagnostic genetic loci separate *M. spicilegus* and *M. domesticus* species (Bonhomme et al., 1984; reviewed in Sokolov et al., 1998). The genes in these loci code for several proteins, which are enzymatically active. The alcohol and malate dehydrogenases, esterases, carbonic anhydrase, mannose and glucose phosphate isomerases are among those proteins. Consequently, some of the distinguishing

coded enzymes may impact the metabolic pathways leading to the excretion of urinary substances.

In addition to the lactones, urinary coumarin appears as an interesting “metabolic marker compound” for the *M. spicilegus* species. The presence of coumarin and abundance of 2-coumaranone in the urine of castrated *M. spicilegus* male also represent distinguishing metabolic pathways from *M. domesticus*. Coumarin could originate from different plants and herbs (reviewed in Bourgaud et al., 2006). In the mammalian systems, coumarin generally exhibits toxic effects and is oxidatively metabolized (detoxified) by the cytochrome P450 mono-oxygenase enzyme system in the liver microsomes (Creaven et al., 1965; Lewis and Lake, 2002). The specific enzyme for coumarin elimination through 7-hydroxylation is CYP2A5 in mouse (Miles et al., 1990). The CYP2A5 enzyme is also known to be inhibited by lactones and 2-coumaranone (Juvonen et al., 1991; 2000). In a study within the *M. domesticus* strains, P450 enzymes have been found genetically altered among these strains (Wood, 1979). Furthermore, a single autosomal gene locus Gpi-1 (glucose phosphate isomerase-1) was found responsible for the differential hydroxylase activity of P450 (Wood and Taylor, 1979). Since Gpi-1 was also found as one of the distinguishing genetic loci between *M. spicilegus* and *M. domesticus* (Bonhomme et al., 1984), the vastly different urinary volatile profiles, as seen here, could be due in part to a differential P450 oxidase activity among other metabolic routes. For example, the distinguishing Es-2 loci controlling esterases have been found especially active in the kidney (Ruddle et al., 1969), and thus likely to affect some of the urinary metabolite excretion. However, separate genetic studies and metabolic mapping are necessary to link the genetic sources for the observed metabolic profile differences between *M. spicilegus* and *M. domesticus*, as exemplified by the urinary coumarin levels observed in this study. Furthermore, it seems desirable to investigate how

could these genetically induced changes in the urinary volatile constituents facilitate chemical communication and social behavior in the “scent world” of the *M. spicilegus* species.

## CONCLUSIONS

Quantitative comparisons of the urinary volatile profiles for male *M. spicilegus* mice reveal several compounds which have previously shown biological activity as male pheromones in the *M. domesticus* species. These similarities suggest that the two mouse species carry a certain genetic linkage that may be utilized in chemosignaling. On the other hand, the total absence of the prominent *M. domesticus* male aggression pheromone, 2-*sec*-butyl-4,5-dihydrothiazole, in the *M. spicilegus* urine and the presence of unique  $\delta$ - and  $\gamma$ -lactones and coumarin seem to indicate that the species have developed distinctly separate metabolic pathways involving urinary constituents. As castration of *M. spicilegus* males removed  $\delta$ -hexalactone and  $\gamma$ -octalactone among the identified urinary constituents, their testosterone control suggests a possible involvement in chemical communication within the species. Behavioral tests are in progress to define possible roles of several urinary volatile organic compounds in *M. spicilegus* chemical communication.



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## Figure Legends

Fig.1. Distinguishing chemical structures of volatile compounds in *M. spicilegus* and *M. domesticus* male urine.

Fig 2. Representative urinary (GC-MS total ion current) profiles of A: intact and B: castrated male *M. spicilegus*. C: A comparative profile for male intact *M. domesticus*.

Fig. 3. Urinary 2-coumaranone levels before and after castration.

Fig. 4. Effect of castration: compound levels significantly reduced in urine after castration. DHF (1-3) dihydrofuran compounds, as indicated in Table 1.

TABLE 1. COMPARISON OF MALE MUS SPICILEGUS URINARY COMPOUNDS IN INTACT ( I ) AND CASTRATED ( C ) SAMPLES

Compound	Retention time (min)	Comparison of levels I vs C*	Student t-test P*
5,5-dimethyl-2-ethyl-4,5-dihydrofuran (DHF 1)	3.52	I > C	0.001
Z-5,5-dimethyl-2-ethylidenetetrahydrofuran(DHF 2)	4.97	I > C	0.001
2-heptanone	5.24	C=0	
E-5,5-dimethyl-2-ethylidenetetrahydrofuran (DHF 3)	5.72	I > C	0.002
p-cymene	9.13	I > C	0.028
3-octen-2-one	9.60		
dehydro- <i>exo</i> -brevicommin (DHB)	9.93		
$\gamma$ -hexalactone**	9.99	I > C	0.023
acetophenone	10.46	I > C	0.013
o-toluidine	10.61		
$\delta$ -hexalactone**	11.37	C=0	
undecane	11.76	I > C	0.01
nonanal	11.91	I > C	0.018
phenylacetone	12.55		
$\alpha$ lactone**	13.38	I > C	0.001
undecalactone**	13.63	I > C	0.032
4-ethylphenol**	13.95	I > C	0.003
$\alpha$ methyl toluate**	14.28		
octanoic acid	14.39		
decanal	15.42		
unidentified m/z 121 **	15.63		
N-phenylformanilide	15.65	C=0	
unidentified m/z 140**	15.83		
2-coumaranone**	16.24	C > I	0.001
$\alpha$ lactone**	16.96	I=0	
$\gamma$ -octalactone**	17.16	C=0	
$\alpha$ lactone**	17.29	C > I	0.012
nonanoic acid	17.64		
1-decanol	17.68		
$\delta$ -nonyl- $\delta$ -valerolactone**	17.82		
$\alpha$ ketone	17.9	I > C	0.036
indole	18.16		
coumarin**	18.35	I=0	
$\gamma$ -nonenolactone**	19.77	C > I	0.048
$\alpha$ lactone**	20.45	I=0	
$\alpha$ an acid**	20.90		
undecanol	21.05		
N-( methylthio)methylaniline	21.92	I > C	0.01

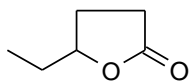


Compound	Rt (min)	Comparison of levels I vs C*	Student t-test P*
dodecanal	22.15		
geranylacetone	23.31		
$\beta$ -farnesene	23.47	C=0	
m/z 125 $\delta$ -lactone**	23.68	I > C	0.003
unknown**	23.78	I=0	
unknown**	23.97	I=0	
dodecanol	24.19	I > C	0.028
unknown**	24.99		
unknown**	25.51	I=0	
dodecanoic acid	26.87		
an acid**	27.34	I=0	
unknown**	33.66	I=0	
N,N-dimethyldodecanamide**	34.30		
unknown**	34.69	I=0	
palmitic acid	37.37	I > C	0.042
unknown**	38.30	I=0	
unknown**	38.82	I=0	

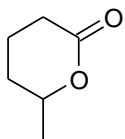
\* statistical significance for the compound level differences between intact (I) and castrated (C) male *M. spicilegus* urine (significance  $P < 0.05$  accepted)

\*\* unique urinary compounds for *M. spicilegus*, not found in *M. domesticus*

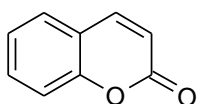
***M. spicilegus***



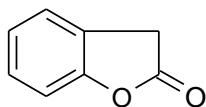
$\gamma$ -hexalactone



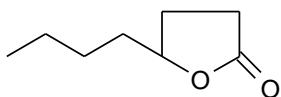
$\delta$ -hexalactone



coumarin

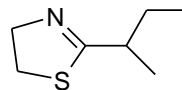


2-coumaranone

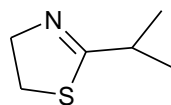


$\gamma$ -octalactone

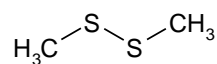
***M. domesticus***



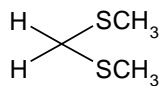
2-sec-butyl-4,5-dihydrothiazole (SBT)



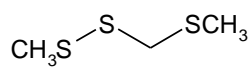
2-isopropyl-4,5-dihydrothiazole (IPT)



dimethyl disulfide



bis(methylthio)methane



methyl (methylthio)methyl disulfide

**Fig.1.**

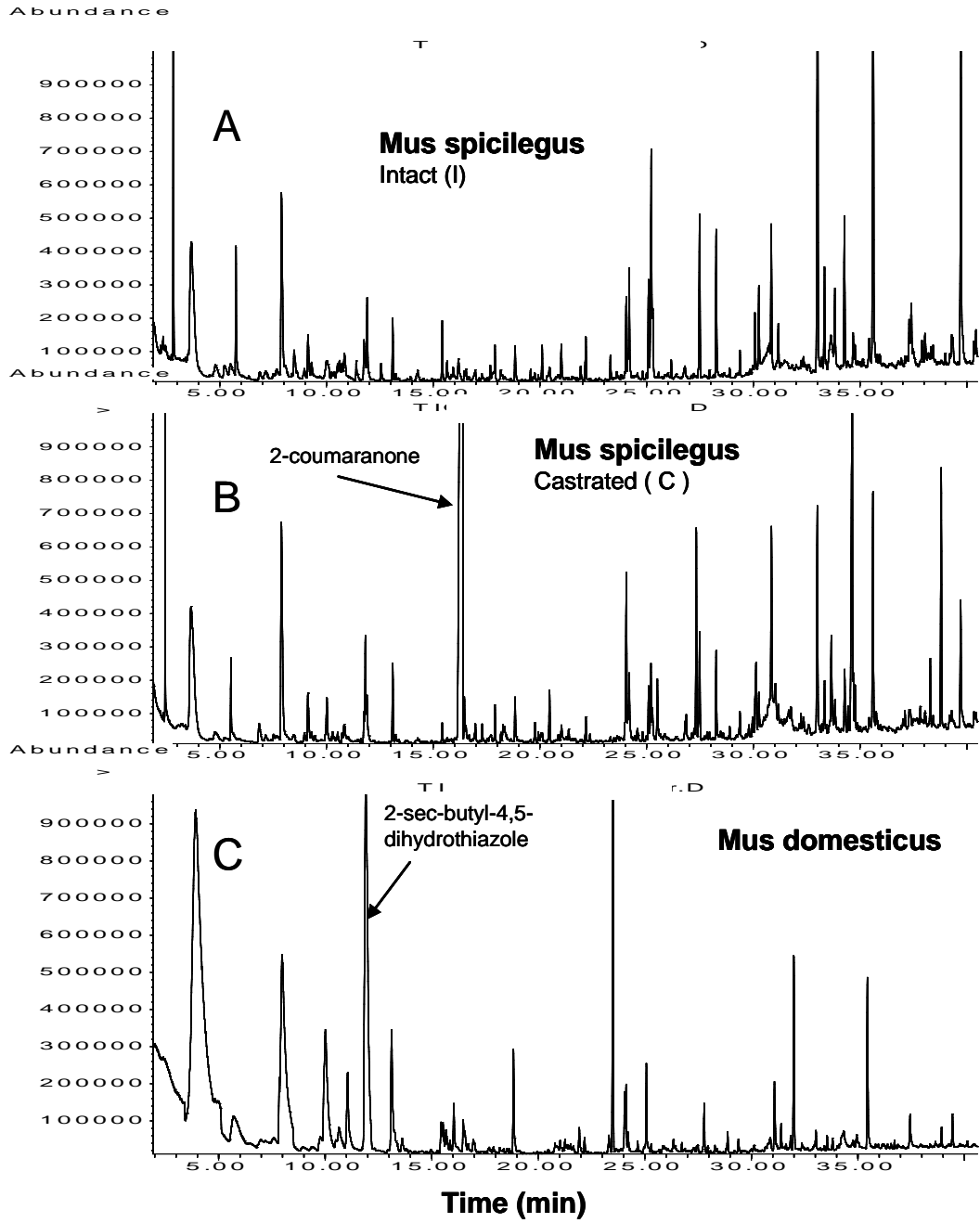
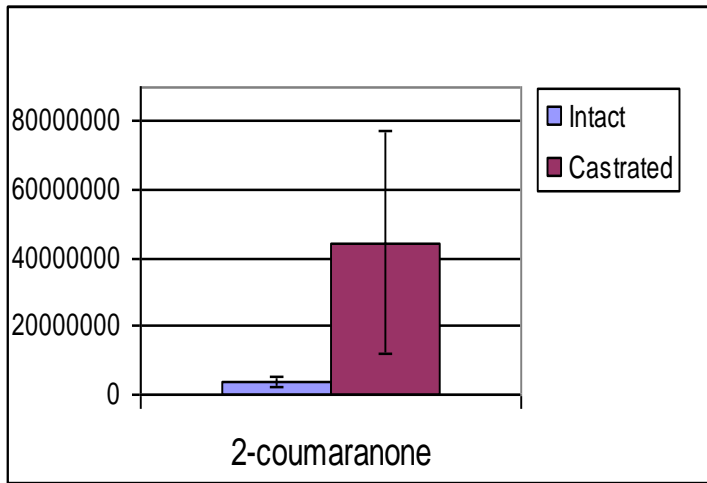
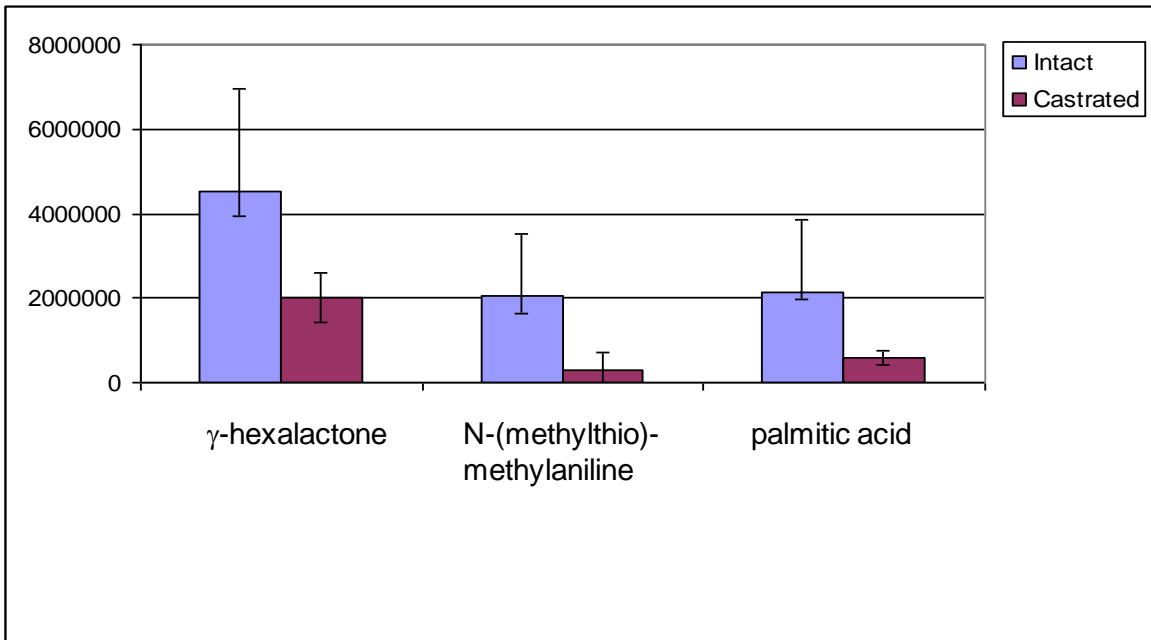
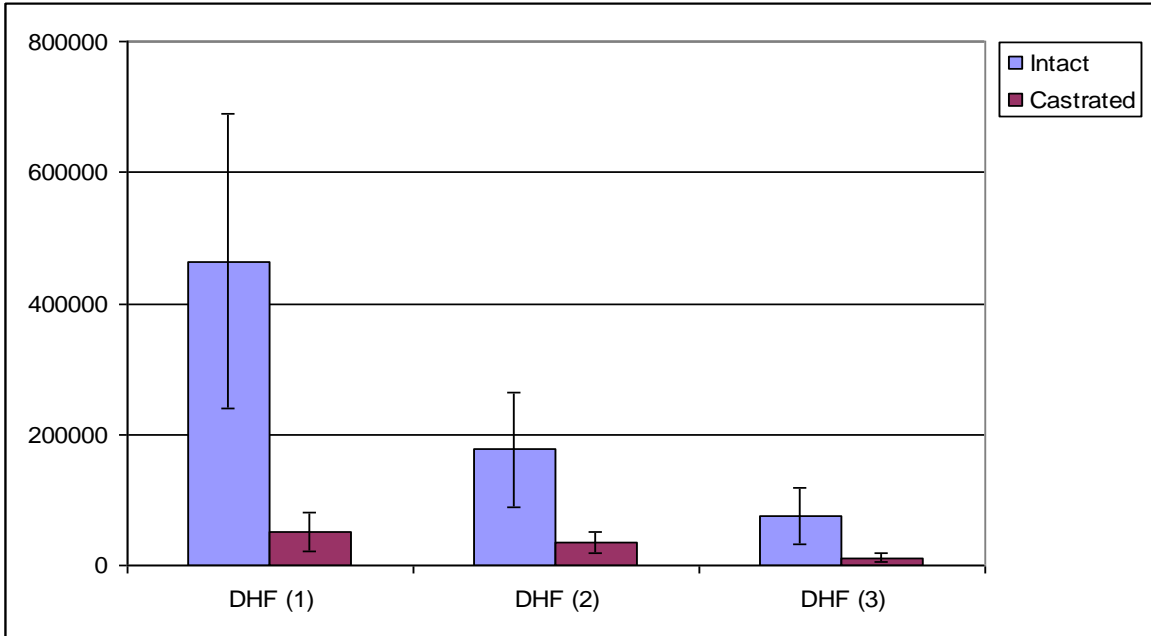


Fig. 2



**Fig. 3**



**Fig. 4**