SNIFFING OUT FRIENDS AND FOES: HOW OLFACTORY SIGNALS INFLUENCE THE SOCIAL ENVIRONMENT OF MALE LABORATORY MICE

by

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Dedicated to the millions of laboratory mice and
the patients who hope to benefit from mouse models

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ABSTRACT

Home cage aggression in male laboratory mice continues to challenge preclinical researchers. It reduces animal welfare and can alter research parameters, potentially reducing the validity and reliability of study data. While simply reducing aggression would be beneficial, promoting socio-positive, affiliative behaviors would greatly improve mouse welfare as mice are a social species. Mice also use olfaction to communicate, so this sensory modality could be used as a tool to improve social interactions in the home cage. A scoping review of the literature on how mammalian odor signals impact same sex social behavior found that studies are dominated by rodent subjects, treatments from urine, and aggression measures (Chapter 1). As a whole, urine treatments had a variable effect on aggression. This review highlights that treatments from non-urinary sources are not often tested, and affiliative behavior is rarely measured.

One murine odor source worth exploring is found in used nesting material. Mice build complex nests for insulation, and it has been speculated that the nest holds odor signals that appease home cage aggression, particularly aggression triggered by cage cleaning. It has been suggested that the nest contains secretions from plantar sweat glands, but the chemical content of neither nesting material nor plantar sweat have been examined. The main goals of this dissertation are to identify the odors stored in used nesting material, determine the sources of those odors, and test them for a behavioral role.

Samples of used nesting material were collected from cages of group housed male mice. Further, plantar sweat, saliva, and urine were collected from the dominant and subordinate mouse in each cage as plausible odor sources. All samples were analyzed for protein and volatile organic compound content. Home cage aggression and affiliative behavior were also recorded to compare to odor profiles. Protein profiles showed that used nesting material contains a variety of proteins that primarily originate from plantar sweat, saliva, and urine sources (Chapter 2). A large proportion of these proteins contain messages about individual identity and bind volatile compounds that further contribute to identity cues. This suggests that the nest aids in maintaining a familiar odor environment. Analysis of volatile content showed that small compounds in the nest are also traced back to plantar sweat, saliva, and urine sources (Chapter 3). Few of the compounds have a known behavior role. However, one compound detected in nest, sweat, and saliva samples

had a negative correlation with home cage aggression and three compounds (two from sweat and one from urine) had a positive correlation with affiliative behaviors, making them potential candidates for controlled studies on social behavior.

Before testing the four candidate compounds, a challenge from the correlation study needed to be addressed. Body fluid samples were collected from individual mice based on social status, as this factor impacts production of known murine pheromones. Further, aggression is typically directed from a dominant to a subordinate mouse for territorial reasons. An aggression appeasement signal is likely to be produced by a subordinate to mitigate the dominant mouse's perceived threat. Data from the correlation study showed no odor profile differences based on social status, and the pheromones that are known to vary with social status did not differ between dominant and subordinate mice. Therefore, Chapter 4 assesses the convergent validity of several dominance measures. Over one week, home cage interactions were observed in group housed male mice. For every aggression occurrence, the aggressor and target mouse was recorded to calculate individual dominance rankings in each cage. Then, individual mice were evaluated for the following measures known to correlate with dominance: levels of urinary darcin (a murine pheromone); scores from three rounds of the tube test; and ratio of preputial gland weight to body length. Postmortem wounding was also compared. Results showed that urinary darcin and preputial gland ratio have strong convergent validity with dominance ranking based on home cage aggression.

Finally, the four candidate compounds (identified in Chapter 3) were developed into treatment solutions to assess their effect on home cage social behavior (Chapter 5). Cages of group housed male mice were randomly assigned one of five treatments (four compounds + control) and home cage aggression and affiliative behavior were recorded for one week. Postmortem wounding was recorded as a secondary aggression measure and social stress was measured through fecal corticosterone metabolites from each cage's dominant and subordinate mouse (rank based on preputial gland ratio). Treatment did not predict changes in most measures. This may be due to limitations in application or from the original correlation study, which are further discussed.

Although the final study showed null results, future research is still warranted to fine tune application methods and gain a better understanding of how odor signals impact interactions other than aggression. The relationship between olfaction and affiliative behaviors is largely unexamined and this dissertation is a first step in filling that gap.

CHAPTER 1. MODULATING CAPTIVE MAMMALIAN SOCIAL BEHAVIOR: A SCOPING REVIEW ON OLFACTORY TREATMENTS

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1.1 Abstract

Many species use olfaction as a primary form of communication. Because of this, odor signals could be a useful tool to improve captive animal welfare by reducing aggression and promoting socio-positive behavior. However, to fully gauge the potential benefits of odor manipulations, the quality of existing literature must first be evaluated. Therefore, a systematic search and scoping review was conducted to summarize prevalent methods, treatment outcomes, and modulating factors in existing literature on the effect of mammalian, intraspecies odors on non-reproductive social behavior. Results from a systematic search of three databases were included if they were published in a peer reviewed journal, used a terrestrial mammalian species, and contained original data evaluating how an odor signal from the subject species directly affected non-reproductive social behavior. All articles were screened by two researchers, data were extracted by one, and reporting quality was assessed by both using the SYRCLE risk of bias tool. Sixty-three articles were included based on this criteria. Most subjects were sexually mature, male rodents. The most common odor treatment originated from urine and aggressive behavior was measured most often. Overall, urine and saliva treatments had a variable effect on aggression, while urine most often increased scent marking and social investigation behavior. Concerningly, most articles showed unclear or high risk of bias. Data from this review highlights a need for additional research on how odor signals from sources other than urine affect behavior and how socio-positive behaviors are affected in general. Further, it emphasizes the need for more transparent reporting as the current body of literature makes it difficult to determine each experiment's quality and how much weight it should be given when interpreting outcomes pertaining to our overall understanding of olfactory communication.

1.2 Introduction

Social stress is often a cause of poor welfare among captive mammals (Broom and Johnson, 2019). For example, aggression and subsequent wounding can affect hormones and cause extra strain on an animal's immune system (Barnard et al., 1996). Consequently, social stress can lead to negative consequences in both research and production (Ferdowsian and Beck, 2011). Because stress can impact both hormonal and behavioral measures, laboratory results can be altered (Bailey, 2018; Balcombe et al., 2004). Any unexplained data variation or unexpected physiological effects can impact the validity of experiments. Further, aggression in male laboratory mice is a prevalent concern among researchers, as it also leads to injury, death, and early euthanasia (Weber et al., 2017). Ultimately, more animals are required to replace those that are lost and which is in conflict with the principles of the 3Rs (Russell and Burch, 1959). From a production perspective, aggression and social stress can present an economic burden due to increased costs and reduced product quality. This can be common among commercially housed pigs. In these systems, pigs are often regrouped based on body weight and sex, which can be an animal welfare concern as it often leads to aggressive interactions (Arey and Edwards, 1998; Rhim et al., 2015). In turn, this can cause injury, stress, and, occasionally, death from severe wounding (Camerlink et al., 2014). Aggression also decreases weight gain and the efficiency of food conversion, further reducing carcass quality (Colson et al., 2006).

One way to improve animal interactions is to utilize one of their innate communication mechanisms, such as the olfactory system (Bossert and Wilson, 1963; Eisenberg and Kleiman, 1972; Hurst, 1989). Odor signals have been shown to improve welfare and reduce stress in a variety of housing environments (Matsukawa et al., 2016; Nielsen, 2017; Takahashi et al., 2013). However, in terms of social interactions, informal searches show that most work focuses on how specific odors can promote aggression (Chamero et al., 2007; Kaur et al., 2014; Mugford and Nowell, 1971; Novotny et al., 1985). For instance, the most well-known mammalian pheromones are produced by rodents and are urine borne signals that either promote aggression or territorial scent marking (Apps et al., 2015; Arakawa et al., 2008).

In contrast, based on informal literature searches, work on odor signals that affect affiliative behavior or reduce aggression is lacking. Perhaps, this is due to terminology. Most odor treatments used to reduce stress in captivity are considered pheromones (Nielsen, 2017). However, in order for an odor to be considered a true pheromone, it must meet five criteria. These criteria

require that 1) the synthesized odor triggers the same behavioral response as the natural stimulus; 2) it is effective at a similar concentration as the natural stimulus; 3) all compounds in a combination are proven necessary for the behavior response; 4) only the proposed combination produces the desired effect; and 5) an evolved pathway for the pheromone signal is demonstrated (Wyatt, 2009). Although pheromones can be effectively used to improve animal welfare, (e.g., the use of Feliway to reduce feline stress in veterinary settings (Griffin and Levy, 2010; Pereira et al., 2016) and Dog Appeasing Pheromone to reduce stress in shelter dogs (Tod et al., 2005)) more complex odor profiles may also be beneficial, but are classified in a different manner. For instance, individual signature mixtures are crucial for social recognition (Wyatt, 2010). Specifically, preserving odors from the nests of laboratory mice has been shown to reduce aggression at cage cleaning (Van Loo et al., 2000). These occupied nests contain a variety of major urinary proteins which can be found in unique ratios for social recognition (Barabas et al., 2019; Hurst et al., 2001).

Despite the number of studies on how intraspecific odors can affect social behaviors and general animal welfare, there has not been a comprehensive overview on the current state of this field. Anecdotally, research on how odors can promote positive interactions is limited. Because of this, there is a need for a quantitative assessment on how odor signals could be utilized to reduce social stress.

A systematic search and scoping review was conducted to provide a quantitative overview of odors that may influence non-reproductive social behaviors across terrestrial mammalian species. This review has four primary objectives. First, it summarizes what species are prevalent in existing olfaction literature. Second, it documents which glandular odor sources are most often tested. Third, it documents which social behaviors are most commonly measured and finally, the review summarizes how these behaviors are influenced by odor treatments. Modulating factors are also recorded for each article to document how prevalent certain environmental and study conditions are in the existing literature. Finally, reporting quality is further evaluated using the Systematic Review Center for Laboratory animal Experimentation (SYRCLE) risk of bias tool to provide a descriptive summary of olfaction research quality.

1.3 Methods

1.3.1 Protocol

Before collecting articles, a protocol was developed and published with the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE). This protocol is publicly available (https://www.radboudumc.nl/getmedia/8c56a760-b46b-471d-a3e7-2fc408360591/OdorSystematicReviewProtocol20200917.aspx) and defines this study's *a priori* criteria for article inclusion/exclusion and article components for data extraction. Ultimately, this study had the following deviations from the published protocol: 1) articles were not restricted to those using captive animals, as studies on wild populations still provide insight on how odor signals can influence behavior; 2) the ROBINS-I criteria for non-randomized studies was not used. Instead, a modified SYRCLE risk of bias tool was used and is described below (see Risk of bias assessment). The protocol's title also differs from this manuscript since this study's objectives better fit the definition of a scoping review than a systematic review (Grant and Booth, 2009).

1.3.2 Article acquisition

Articles were obtained through electronic database searches from the first index date through August 25, 2020 and by scanning reference lists of relevant text books and a peer reviewed literature review on mammalian pheromones (Liberles, 2014; Nielsen, 2017; Wyatt, 2014a). Information specialists (DW and JY) created the search strategy and performed searches in three electronic databases (PubMed, CAB Abstracts (Web of Science platform), and Agricola (Ovid platform)) on August 25, 2020. These databases were considered sufficient for this review because PubMed provides broad coverage of the biomedical literature, including laboratory animal research, while CAB Abstracts and Agricola provide focus on veterinary medicine and animal health and welfare. The total number of results was 4143 and after removing duplicates in Endnote citation management software on August 26, 2020, the final number was 3609. The information specialists then uploaded the results into a Rayyan project (Ouzzani et al., 2016) for screening and selection by the team. See Appendix A for the full search strategy. Between the textbook and literature review sources, 2455 additional references were obtained.

1.3.3 Article screening

First, all references were screened for inclusion based on their titles and abstracts. If the title and abstract did not provide enough information to decide, the full text was retained for review. Relevant articles were selected using the following criteria:

(1) subjects were a terrestrial, mammalian species;

(2) odor treatments originating from healthy individuals of the subject species or synthetic equivalents were used;

(3) non-reproductive social behavior was measured;

(4) the direct effect of the odor on behavior was examined;

(5) the study contained a control group;

(6) behavior data were analyzed with a statistical model;

(7) the article was a published, peer reviewed study collecting original data (no review articles, book chapters, or conference proceedings);

(8) full text was written in English.

Data were excluded if the subjects underwent any type of surgical procedure or were given additional treatments; only control or baseline measurements were included.

For this review, non-reproductive social behavior was defined as aggression, affiliation, or social investigation. Additionally, studies measuring scent marking were included since this behavior is meant to mark territory and deter intruders. However, scent marking is also used for mate attraction, so only studies that measure intra-sex scent marking were included; i.e., the effects of female odors on male scent marking and vice versa were not included. Two reviewers independently screened each article (AJB and SRD). Any disagreements were settled by discussion or input from a third investigator (BNG).

1.3.4 Data extraction

One investigator (AJB) collected data from each article to meet this review's objectives. If multiple experiments were published in a single article, data from each relevant experiment were

collected separately. To address the first three objectives, the species, gland/fluid source of the odor treatment, and behavior measured in each experiment were recorded, respectively. For the fourth objective, the reported change in behavior was recorded for each experiment (increase, decrease, or no effect) based on the reported statistics in the experiment ($P<0.05$ for significance). If multiple measures of the same behavior were reported (e.g., frequency and latency), an increase or decrease was recorded if an effect was detected in at least one measure. Hormonal measures of stress and the odor treatment's impact were also documented when reported. However, most studies did not report stress measures, so these data were not analyzed. Finally, study and environmental variables were recorded for each experiment. This included subject and donor age, sex, and housing conditions; donor familiarity to the subject; reported temperature and humidity; treatment form (e.g., liquid versus diffusion treatment); the type of control used; where the treatment was administered; behavior sampling method; reported observer reliability; and type of analysis used. Descriptive statistics were calculated for all recorded measures.

1.3.5 Risk of bias assessment

Risk of bias was assessed for each article using the SYRCLE risk of bias tool (Hooijmans et al., 2014). Two investigators (AJB and SRD) reviewed each article independently and any disagreements were resolved through discussion. The SYRCLE risk of bias tool consists of ten questions to identify five sources of bias in animal research: selection, performance, detection, attrition, and reporting bias. For articles that used a traditional control vs treatment design, the SYRCLE tool was used without modification. However, if the article used a repeated measures design or a combination of designs, the SYRCLE tool was modified. Questions 1-3 were modified to ask if selection bias was minimized at each study time point and question 5 was modified to ask if researchers and care staff were blinded to treatment order. Details for each question's criteria are listed in Appendix A, Table A.1.

1.4 Results

1.4.1 Article acquisition and screening

3609 articles were acquired from the electronic database searches and 2455 additional titles were gathered from textbook and literature review references lists. However, any relevant titles from the textbooks and literature review were duplicates with titles from the electronic searches. Ultimately, 63 articles met all inclusion criteria and were assessed for qualitative description (listed in Appendix A Table A.2, publication years are visualized in Appendix A Figure A.1). Figure 1.1 outlines the article inclusion steps.

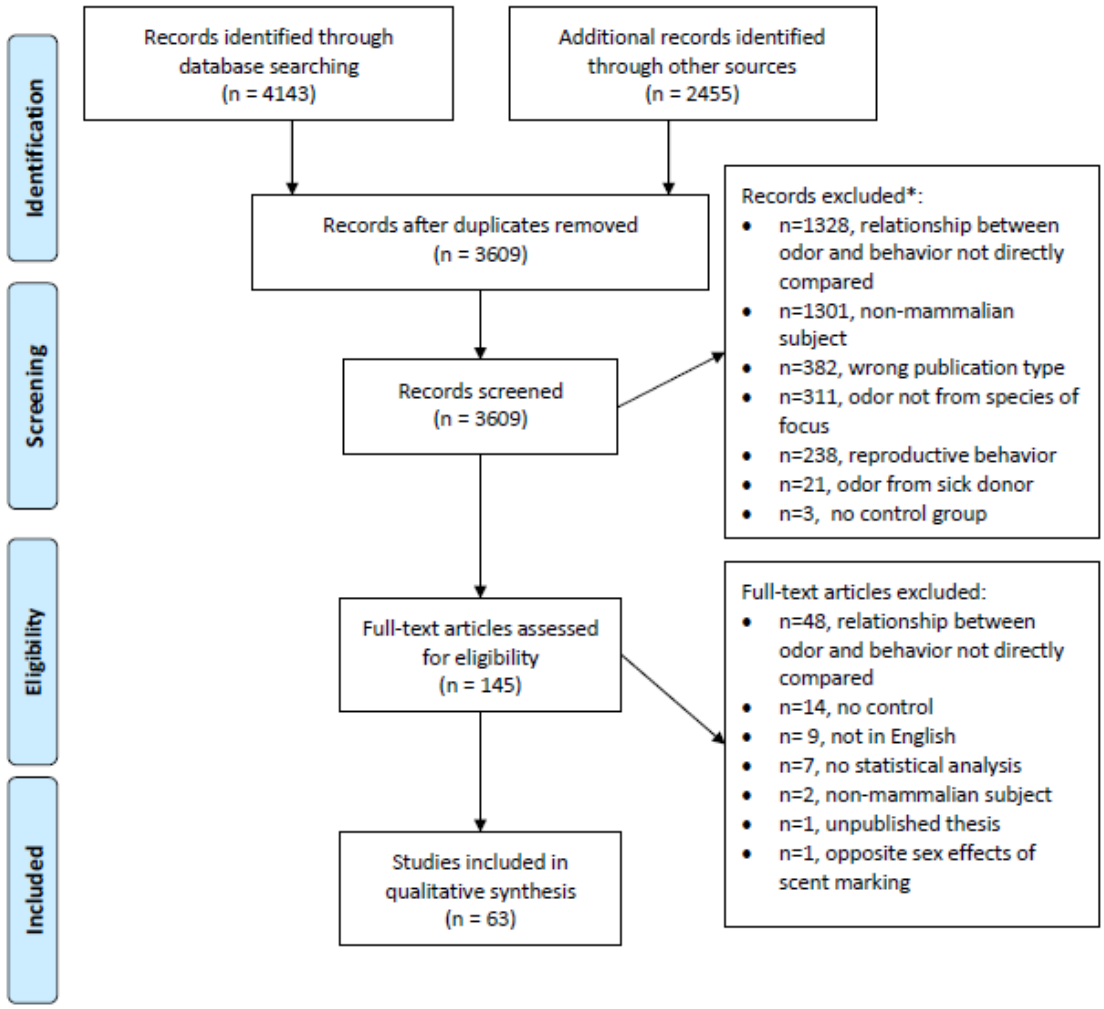

Figure 1.1. Steps for article inclusion from a systematic search of peer reviewed research on how odors impact terrestrial mammalian social behavior. *most records had multiple exclusion labels, the box total does not represent the difference between records screened and those assessed for full text eligibility.

1.4.2 Characteristics of odor literature

The 63 articles contained 96 experiments that were screened for study characteristics. Rodents were predominantly used in these experiments (80.21%), followed by porcine (9.38%) and canine (4.17%) subjects (Figure 1.2A). Most odors were tested on and collected from sexually mature, male subjects (Appendix A Table A.3;A.4). Study subjects and odor donors were relatively equally divided between solitary and social housing when reported and applicable (Appendix A Table A.3;A.4). However, whether fighting was observed in socially housed donors was not typically reported (Appendix A Table A.4). When reported, most odor donors were unfamiliar to the subject (Appendix A Table A.4). Most experiments did not report if subjects received any enrichment (Appendix A Table A.3). Since most work used rodent subjects, housing parameters specifically for rodents were also assessed. Most of the included rodent studies used static housing, but did not report bedding type (Table 1.1). When reported, most studies used wood based bedding (Table 1.1). Note: since the first publication on individually ventilated systems was released in 1994, any experiments published before that year were assumed to use static cages (Choi et al., 1994).

Table 1.1. Housing parameters for rodent subjects in odor literature

Category	Percent
Cage Style*	
complex natural housing	1.30
individually ventilated	1.30
not clearly reported	29.87
static	67.53
Bedding type	
soil + natural vegetation	1.30
straw	1.30
corn cob	3.90
wood based	38.96
not clearly reported	54.55

*if not explicitly stated, any experiments conducted before 1994 were assumed to use static cages.

Across the 96 experiments, 220 odor treatments were tested. Most treatments were whole urine or a specific urinary component (59.10%; Figure 1.2B). Gland/fluid sources that were tested

in less than ten total treatments were grouped as an “other” category and treatments that combined secretions from two or more sources were grouped as “multiple”. Each accounted for 19.10% and 16.81% of treatments, respectively. The other primary fluid source for odor treatments was saliva, making up 5% of included treatments (Figure 1.2B). Most experiments did not report temperature or humidity conditions (Table 1.2). Odor treatments were most often presented as a liquid and tested against water or controls with no treatment (Table 1.2). Most treatments were administered in an unfamiliar treatment arena or a clean home cage devoid of familiar odors (Table 1.2).

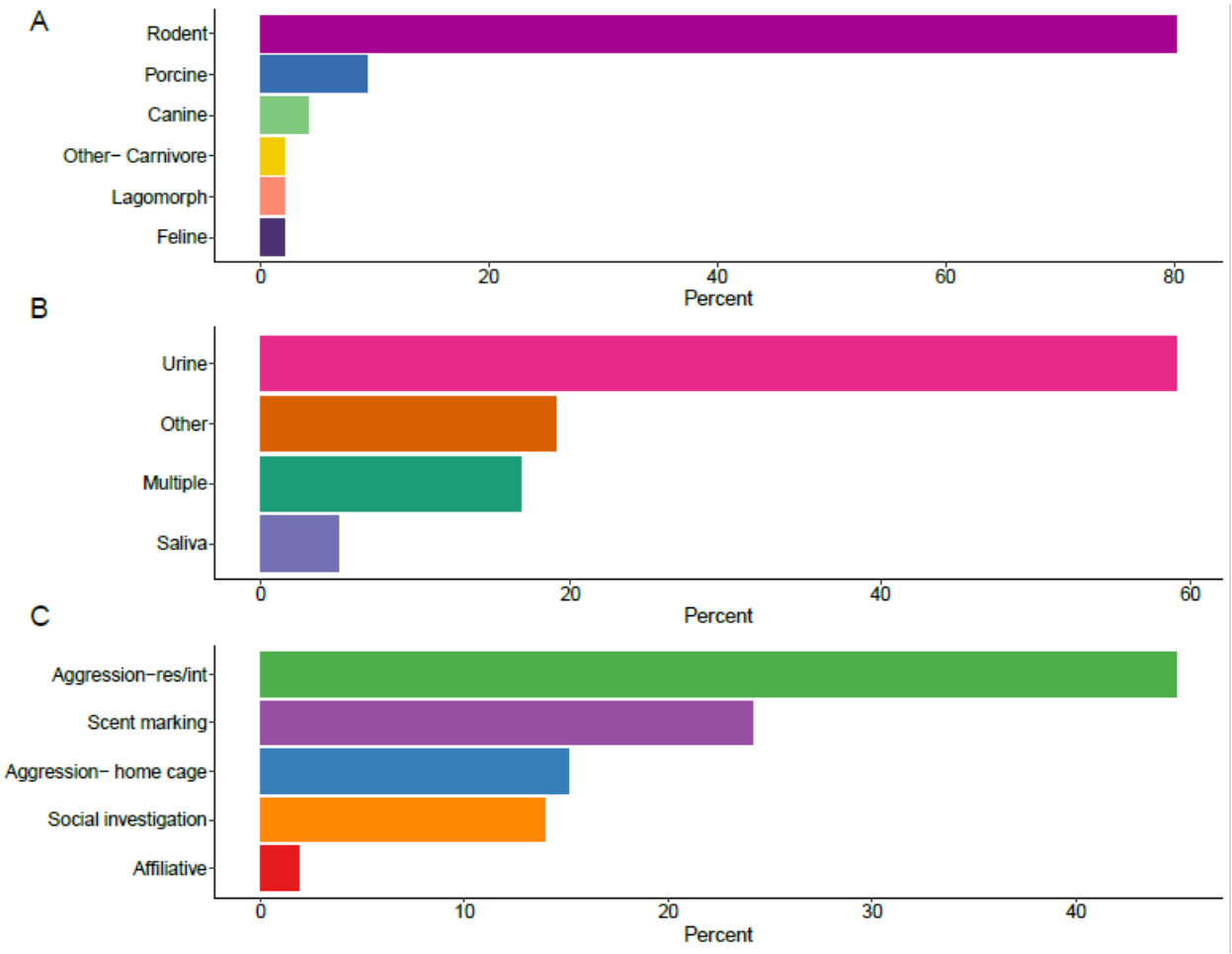

Figure 1.2. (A) Prevalent species classifications used across 96 experiments of olfactory literature. (B) Fluid sources from 220 odor treatments used in the 96 experiments. Odor sources used in less than ten reported treatments were grouped as an "other" category. "Multiple" represents treatments that were a combination of at least two different gland secretions or fluids. (C) Social behavior measured in response to the 220 odor treatments. 265 behaviors were reported since it was possible for multiple behaviors to be measured in response to a single treatment.

Table 1.2. Study and environmental variables reported in odor literature

Variable	Percent
Temperature + Humidity	
reported	36.84
not reported	63.16
Treatment Form	
odorless substrate	0.46
gel	0.46
donor rubbed on target	0.91
soiled substrate + liquid	0.91
solid	0.91
unclear	1.83
diffusion	2.28
soiled substrate	21.00
liquid	71.69
Control	
not clearly reported	0.45
alcohol	0.45
ESP1	0.91
saline	0.91
subject odor	2.73
Tris-HCl	3.18
stranger odor	4.09
PBS	5.00
vehicle[#]	9.09
water	34.55
no odor	38.64
Treatment Location	
not clearly reported	9.09
clean home enclosure	14.55
testing arena	34.55
home enclosure	41.82
Sampling Method	
hourly rate	0.76
scan sample	0.76
all occurrence	89.02
proportion of tiles marked*	0.38
number of marks*	9.09
Model Type	
parametric and nonparametric	1.89
not clearly reported	6.44
nonparametric	36.74
parametric	54.92

[#] control was the solvent used to dissolve or dilute a treatment; specific solvents varied
*recorded outcome of a behavior (e.g., area marked from scent marking, not behavior itself)

In response to the 220 odor treatments, 264 social behaviors were measured. The highest percent of behavior responses was aggression in a resident intruder paradigm (44.91%), followed by scent marking (24.15%), aggression in a home cage (15.10%), social investigation (13.96%), and affiliative behavior (1.88%; Figure 1.2C). The affiliative behaviors primarily included allo-grooming, play behavior, and resting while in contact with a conspecific. However, additional behaviors specific to African wild dogs, such as vocalizations and facial expressions, were also included (Van den Berghe et al., 2019). Most behavior was recorded using all occurrence sampling, but roughly 10% of measures were the result of a behavior, not the behavior itself (Table 1.2). For example, to quantify scent marking behavior, researchers recorded the number of tiles marked or the area of a piece of paper with marks on it. In these studies, the result of scent marking was measured, not the behavior directly. None of the included studies reported intra- or inter-observer reliability. Over half of the behavior measures were analyzed using parametric statistics (Table 1.2).

1.4.3 Odor impacts on social behavior

When assessing the direct impact of odor treatments on behavior, a gland/fluid source was only analyzed if a behavior was measured in response to at least five treatments from that source. Whole urine or urinary component treatments were most prevalent. The data are simplified into those that used a urine based odor due to the large diversity of urinary components that have been tested. Of all the aggression measured in response to urine, 42% of treatments increased it, 21% decreased it, and 37% had no effect (Figure 1.3). Of all the scent marking measured in response to urine, 62% of treatments increased it, 6% decreased it, and 32% had no effect (Figure 1.3). Of all the social investigation measured in response to urine, 74% of treatments increased it and 10% had no effect, while 16% of responses to urine weren't reported (Figure 1.3). This unreported social investigation data were included in the review, as they were one of multiple behaviors measured in a given experiment. Several experiments recorded both aggression and social investigation in response to a urine treatment, but did not report results for the latter behavior.

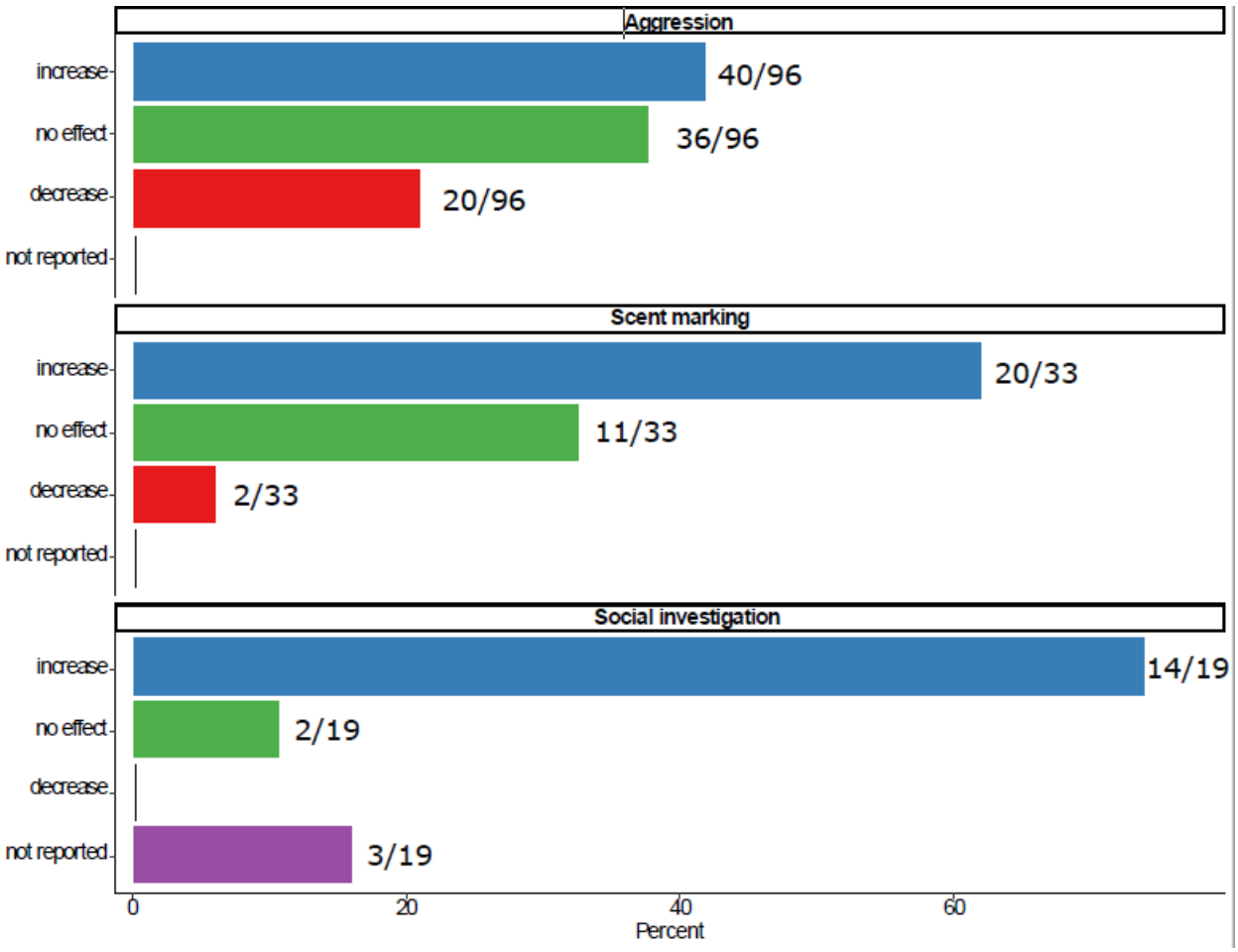

Figure 1.3. Reported effects of urine treatments on aggression, scent marking, and social investigation behavior. Data are plotted as the percent of each behavior's urine treatments demonstrating each effect, with the proportion of urine treatments listed at the end of each bar.

The other primary odor source was saliva, and there were only enough treatments to examine its effect on aggression. Of all the aggression measured in response to saliva, 45% of treatments had no effect, 36% increased it, and 18% decreased it (Figure 1.4A). Lastly, there were only enough treatments using anal gland secretions to examine its effect on scent marking. Of the scent marking measured in response to anal gland odors, 100% of treatments increased it (Figure 1.4B).

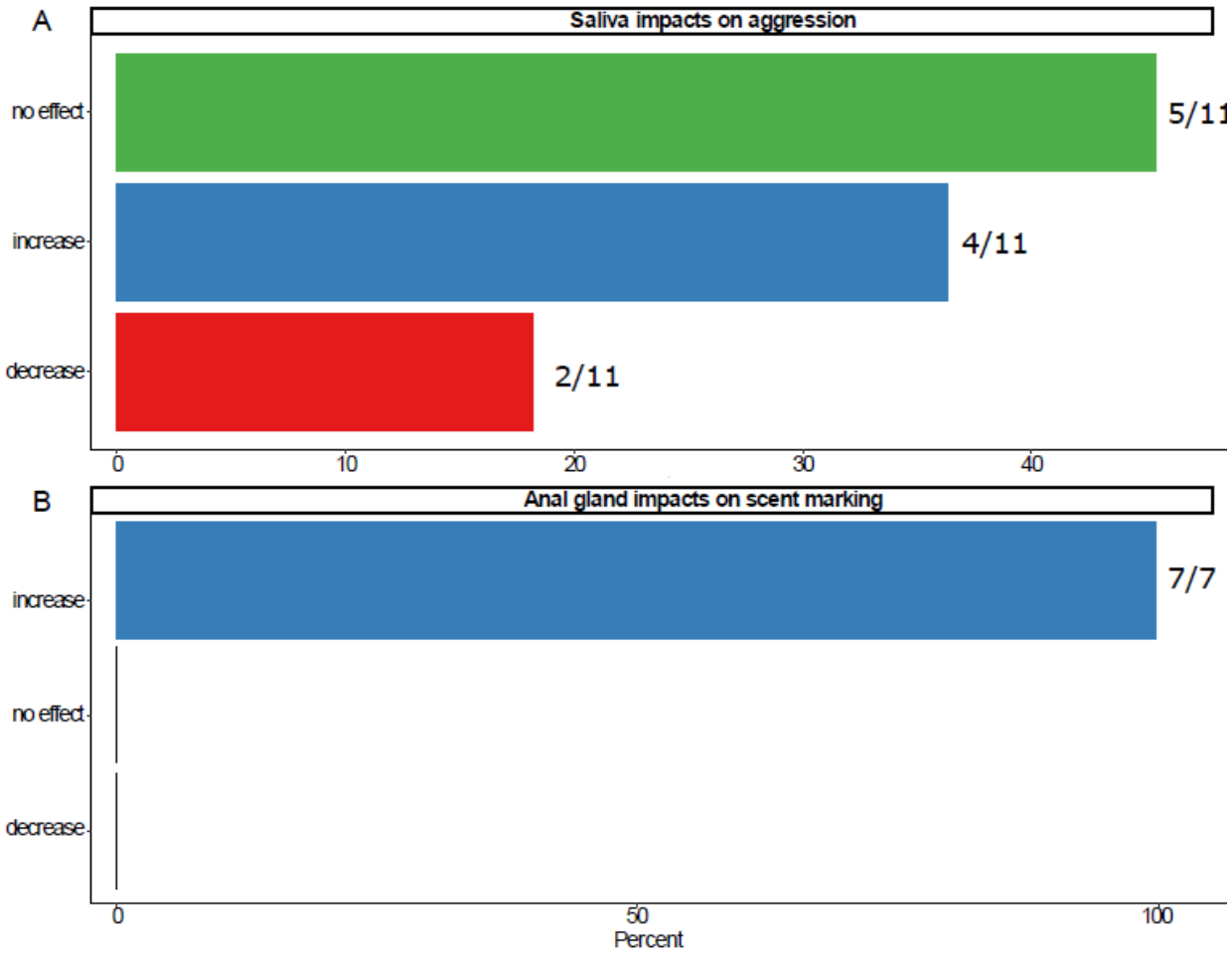

Figure 1.4. Reported effects of (A) saliva on aggression and (B) anal gland secretions on scent marking. Data are plotted as the percent of the respective odor treatments demonstrating each effect per behavior, with the proportion of odor treatments listed at the end each bar.

1.4.4 Risk of bias in odor literature

Most articles in this review had poor reporting and subsequently either high or unclear risk of bias (Table 1.3). Most articles did not report a randomization method for assigning animals to a treatment group or order (62%). In most articles, it was unclear if the researchers assessed baseline traits, concealed the treatment/treatment order, housed the animals randomly, blinded researchers, or randomly chose animals for outcome assessment. In only one third of the articles, it was clear that all experimental units were analyzed. In roughly one third of the articles, it was unclear if animal exclusion was related to the true outcome, was balanced across treatments, or if excluded values were replaced/predicted. Most articles reported results for all listed outcomes (94%). In just over half of the articles, it was clear that treatments were free of contamination and

the study was not influenced by the funders. However, in most articles it was unclear if the analyses were free of errors (88%).

Table 1.3. Reporting quality. Percent of included articles displaying low, high, and unclear risk of bias. NA denotes that a specific question did not apply.

SYRCLE question	Low	High	Unclear	NA
1. Randomized treatments/order	37.88	62.12	0	0
2. Similar baseline measures	4.55	12.12	83.33	0
2a. If no, adjustments were made	6.06	0	89.39	4.55
3. Concealed treatment/order allocations	39.39	6.06	54.55	0
4. Random housing*	1.47	0	89.71	8.82
4a. Unlikely that data were influenced By non-random housing	0	89.39	0	10.61
5. Blinded researchers	9.09	4.55	86.36	0
6. Random outcome assessment order[#]	3.03	7.58	78.79	10.61
7. Blinded outcome assessment	13.64	1.52	84.85	0
7a. Unlikely that data were influenced By lack of blinding	9.09	77.27	0	13.64
8. All samples were analyzed	33.33	18.18	48.48	0
8a. Exclusion was unlikely to be related to the true outcome	10.61	4.55	55.52	33.33
8b. Excluded data were balanced across groups	9.09	9.09	48.48	33.33
8c. Missing data were predicted appropriately	0	10.60	56.56	33.33
9. Protocol was available	0	100	0	0
9a. If protocol was not available, all outcomes were reported	93.94	4.55	1.52	0
10. Other sources of bias				
10a. Treatments were free of contamination	56.06	15.15	28.79	0
10b. Study was free of funder influence	57.58	0	42.42	0
10c. Study was free of analysis errors	7.58	4.55	87.88	0
10d. Excluded animals were replaced	0	18.18	48.48	33.33

*articles that used wild populations or privately owned subjects were marked as "NA"; [#] articles where the subject was given free choice between two or more treatments were marked as "NA".

1.5 Discussion

This review aimed to provide a descriptive, quantitative summary of how intra-species odor signals can influence non-reproductive social behavior and highlight prevalent modulating factors across experiments. A vast majority of this literature has been conducted on rodent species which likely reflects the general prevalence of mice and rats in biomedical research (Commission,

2012). Less than 10% of experiments used porcine subjects. This low percent was surprising since aggression is a top welfare concern for production pigs (Arey and Edwards, 1998). This demonstrates a need for additional studies on porcine odor signals: pigs have a strong olfactory system and social preference in piglets can be influenced by the olfactory environment (Kristensen et al., 2001). There is also need for olfaction research in companion animals since work using canine and feline subjects combined made up less than 5% of the experiments. Aggression is also a top ranking behavioral issue for dog and cat owners (Fatjó et al., 2006). Since both species use olfaction as a primary communication mechanism (Gadbois and Reeve, 2014; Vitale Shreve and Udell, 2017), it is surprising that so little literature exists on the signals that influence social behavior in these domesticated species. This is especially so considering that there is some evidence that existing pheromone products, Feliway and Dog Appeasing Pheromone, can reduce stress in veterinary and shelter environments (Pereira et al., 2016; Tod et al., 2005). However, of the included articles in this study, Feliway was only tested twice (once in companion cats (Ogata and Takeuchi, 2001) and once in lions (Martínez-Macipe et al., 2015)) and Dog Appeasing Pheromone was only tested once in African wild dogs (Van den Berghe et al., 2019). Further, the reported subjects and odor donors were primarily adults and male, leaving a knowledge gap on how scents from females and juveniles may impact social behavior. This is of specific importance since juvenile female mouse urine and maternal pig skin secretions have been shown to have an aggression reducing quality (Dixon and Mackintosh, 1976; Guy et al., 2009). Further work on signals produced by females in general could be beneficial for reducing social stress.

One hundred and thirty odor treatments reported here originated from urine, which is likely because it is the primary excretion route for signals produced as metabolic byproducts. While these byproducts themselves likely do not have a communication role, it is suggested that they are precursor compounds for pheromones (Stökl and Steiger, 2017). It is also a common transmission media and released during scent marking behavior of many species (Allen et al., 1999; Arakawa et al., 2008; Feldman, 1994). Unsurprisingly, it contains a variety of known odor signals. In mice, the primary proteins excreted in urine belong to the major urinary protein (MUP) family (Barabas et al., 2019), which are used for social recognition (Hurst et al., 2001). It also contains several known aggression promoting pheromones (Chamero et al., 2007; Kaur et al., 2014; Mugford and Nowell, 1971; Novotny et al., 1984). The diversity of tested urinary components likely explains why the summary of treatment effects on behavior was so variable. Unfortunately, there were not

enough experiments performed on a single urinary component to warrant an official meta-analysis. Perhaps this will be possible in the future with more literature on specific signals.

The summary of 11 saliva treatments also showed a variable impact on aggression. Like urine, saliva contains proteins from several families used for identification purposes in mice such as MUPs, odorant binding proteins (OBP), and androgen binding proteins (Barabas et al., 2019; Karn and Laukaitis, 2015, 2011). Further, volatile compounds are often transported in OBPs to increase the potential signal diversity found in saliva (Stopková et al., 2016). Since many of these molecules are used for recognition, it is possible that salivary signals have a context dependent effect on the receiver. Many of the saliva donors in these articles were unfamiliar to the subjects, which could provoke aggression in response to unknown odor signatures. In fact, the only experiments to report a decrease in aggression used the synthetic version of androstenone, thus removing any associated individual cues (McGlone and Morrow, 1988). Three of the five "no effect" reports on aggression also came from the same set of experiments (McGlone and Morrow, 1988). This suggests that any signals that may promote socio-positive behavior could be masked by individual signatures and warrants additional research on how salivary signals can modulate social interactions.

To further reduce the study diversity in this data set, the most reported behavior was aggression from the resident intruder paradigm. While the resident intruder test is a common assessment of aggression, it induces territoriality between unfamiliar male mice and may not be relevant for solving home cage behavior concerns (Weber et al., 2017). Home cage aggression, which directly reflects problematic behavior, was recorded in only 15% of studies. While efforts to reduce home cage aggression in mice are needed, they address the worst interactions that could be performed in captivity. Good welfare is not indicated simply by a lack of negative interactions, but the presence of socio-positive behavior. One central pillar of animal welfare focuses on promoting positive affect and pleasurable emotional states in animals (Fraser et al., 1997). In social species, this would be reflected by affiliative interactions, which accounted for less than 2% of measured behavior. Behaviors such as allo-grooming and group sleep are often performed to strengthen social bonds in rodents and primates (Brown, 1985; Di Bitetti, 1997; Fedurek and Dunbar, 2009). Play behavior is often performed socially, is done when animals are in a relaxed state, and can spread good welfare throughout a group (Held and Špinka, 2011). Based on the data

acquired here, there is a fundamental lack of knowledge on how odor signals may affect welfare through rates of affiliative behavior.

In terms of reporting quality, most articles showed either high or unclear risk of bias using the SYRCLE criteria. This makes it difficult to accurately assess study quality, and determine how much weight should be given to each when interpreting outcomes pertaining to our overall understanding of olfactory communication. This demonstrates a need for more transparent reporting and ties into several of the measures here on study modulating factors. First, temperature and humidity levels were not reported in over 60% of articles. For odor treatments, this is of concern since individual molecular effects on behavior can be impacted by temperature and humidity (Collins, 1981). Second, most studies recorded behavior using all-occurrence sampling, but none reported a metric for inter- or intra-observer reliability. It is unknown how reliable these behavior records are, making it difficult to trust the resulting data. However, this is not surprising as a previous survey of articles in a prominent animal behavior journal found that 96% of publications did not mention observer reliability (Kaufman and Rosenthal, 2009). Third, most experiments reported using parametric or nonparametric analyses. However, it was mostly unclear if there were any analysis errors: mainly, a lack of confirmation that the data met relevant model assumptions. Violating model assumptions decreases the model's accuracy and, worst case scenario, could yield misleading results (Doncaster and Davey, 2007). While most articles here were published before 2010, these data indicate a critical need for more prevalent use of the ARRIVE guidelines (du Sert et al., 2020; Kilkenny et al., 2010) even though specific factors of interest here are not explicitly listed (observer reliability or temperature and humidity).

Since most of the articles used rodent subjects, the reported bedding type was of interest. Estrogen disrupting compounds are present in corncob bedding and male *Peromyscus* mice are more aggressive when housed on it compared to cardboard bedding (Markaverich et al., 2002; Villalon Landeros et al., 2012). While corncob is influential on Peromyscus, no empirical studies exists to test if similar effects occur in *Mus musculus*. However, an epidemiological assessment of the factors driving aggression in laboratory mice found a higher fighting risk in mice housed in IVC cages with corncob than those housed in static cages on wood bedding (Theil et al., 2020). It is possible that corn cob bedding could influence baseline levels of behavior and skew any treatment effects. Aggression could be inflated to either mask any appeasement qualities, or

overemphasize them. Over half the articles in this review did not clearly report the type of bedding used, which further reduces the trust of existing rodent olfaction literature.

1.5.1 Conclusion

In summary, existing intraspecies olfaction literature is dominated by sexually mature, male rodent subjects, treatments originating from urine, and aggression focused outcomes. While urine treatments had a variable impact on behavior, the diverse amount of urinary components that were tested makes it difficult to draw strong conclusions. Finally, most articles demonstrated a high risk of reporting bias. These data demonstrates the need for more inclusive research on how odor signals from other body fluids may influence animal interactions, how socio-positive behavior is modulated by odor signals, and more transparent reporting in the field.

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CHAPTER 2. PROTEOME CHARACTERIZATION OF USED NESTING MATERIAL AND POTENITAL PROTEIN SOURCES FROM GROUP HOUSED MALE MICE, *MUS MUSCULUS*

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2.1 Abstract

Laboratory mice (*Mus musculus)* communicate a variety of social messages through olfactory cues, and it is often speculated that these cues are preserved in nesting material. Based on these speculations, a growing number of husbandry recommendations support preserving used nests at cage cleaning to maintain familiar odors in the new cage. However, the content of used nesting material has never been chemically analyzed. Here we present the first comprehensive proteome profile of used nesting material. Nests from cages of group housed male mice contain a variety of proteins that primarily originate from saliva, plantar sweat, and urine sources. Most notably, a large proportion of proteins found in used nesting material belong to major urinary protein ("MUP") and odorant binding protein ("OBP") families. Both protein families send messages about individual identity and bind volatile compounds that further contribute to identity cues. Overall, this data supports current recommendations to preserve used nesting material at cage cleaning to maintain odor familiarity.

2.2 Introduction

Mice (*Mus musculus*) are the most common species used in research and rely heavily on olfactory signals for communication (Bronson, 1971). Pheromones, a well-known type of olfactory signal, are defined by their ability to reliably trigger specific behavioral and/or physiological responses in their recipients (Wyatt, 2010). In fact, most of our current knowledge of pheromone signals is biased toward rodent species: 35 of the 62 known mammalian pheromones originate in rats or mice (Apps et al., 2015). Mice can release a variety of compounds in response to various stimuli or social situations which ultimately trigger physical or behavioral responses in their

cagemates (Brennan, 2010; Hurst, 2009; Latham and Mason, 2004; Wyatt, 2010). Most odor signals are classified as volatile organic compounds (VOCs) (Apps et al., 2015), but protein/peptide signals also play an important role in chemical communication. Several exocrine gland secreting peptides (ESP) from the lacrimal gland influence sexual behavior by triggering lordosis in females and deter unwanted advances towards juvenile males (Apps et al., 2015); major histocompatibility complex peptides are crucial for conspecific recognition and mate selection; and several members of the major urinary protein (MUP) family contribute to individual recognition and male dominance status (Wyatt, 2014b, 2010). Specifically, MUP20 (also known as "darcin") not only binds known VOC pheromones, but plays a crucial role itself in learning an individual's unique VOC profile for mating or general recognition (Kwak et al., 2012; Roberts et al., 2012). MUP20 has also been shown to promote aggression and indicate social dominance in wild derived and outbred male mice (Lee et al., 2017; Nelson et al., 2015). It has been argued that genetic homogeneity may reduce MUP20's impact on inbred strains, but results similar to wild mice have been reported in C57BL/6 males (Guo et al., 2015; Kaur et al., 2014). These effects support the argument that the behavior of any mouse strain can be influenced by odors within a single cage.

Natural mouse behavior drives them to build nests even in the laboratory setting (Latham and Mason, 2004) and it has been suggested that the nest acts as a depository for cage level olfactory signals (Van Loo et al., 2000). In fact, it is commonly suggested for vivarium technicians to preserve the old nest site during cage cleaning in order to maintain existing odor cues in the new cage (Weber et al., 2017). However, to date, no one has examined the chemical profile of the nest to confirm if odorants are deposited there and how they may affect research parameters.

Typical nesting behaviors involve manipulating the material using the mouth or paws ("Mouse Ethogram," n.d.), so it is expected that the material could hold contents from salivary and plantar glands. Saliva is a known source of several androgen and odorant binding proteins used for individual recognition (Karn and Laukaitis, 2015, 2011; Stopka et al., 2016) while the plantar glands produce an oily, sweat-like, substance that has been attributed to a variety of signaling roles such as stranger/conspecific recognition, and route tracing in new territories (Brown, 1985; Ropartz, 1977; Van Loo et al., 2000). These messages do not change over time and have a lower metabolic cost to the sender if they are long lasting. Therefore, the contents are likely nonvolatile in order to remain stable in the environment (Brennan, 2010). Like nesting material, the contents

of plantar sweat have not been analyzed. Additionally, urinary proteins may also be present in the nesting material. It has been reported that mice prefer to keep their nests free of urine and feces (Blom et al., 1993; Makowska et al., 2019), but it is possible for them to track urinary compounds onto the material due to limited cage space. The above fluids are all plausible sources of nest chemosignals either from direct material manipulation or random tracks. However, to best understand the messages that may be relayed by these signals, we need to know where they originate and how they are deposited.

A group of 5 mice, a typical laboratory housing density, in a standard sized shoebox cage has the potential to create a complex odor environment that may influence physiological and behavioral measures. However, two odor sources, nesting material and sweat, have not been the subject of chemical profiling. Therefore, the objective of this study was to characterize the protein profile of used mouse nesting material. We then compared the nest's proteome to that of plantar sweat, saliva, and urine for a more comprehensive overview of its content's plausible origins.

2.3 Methods

2.3.1 Ethics statement

Ethics statement: All methods involving animals were approved by the Purdue University Institutional Animal Care and Use Committee (protocol #1707001598) and follow federal animal guidelines.

2.3.2 Animals

This study used two cages of five male C57BL/6NCrl mice acquired from Charles River Laboratories (Wilmington, MA). All mice were specific pathogen free. Mice were approximately 8 weeks of age upon arrival and housed in open top, 11.5" x 7.25" x 4.25" mirco-isolator cages (Ancare, Bellmore, NY) with aspen wood chip bedding (NEPCO, Warrensburg, NY), 8.5g virgin kraft crinkle paper nesting material (Enviro-dri, Cleveland, Ohio), and *ad libitum* food (Envigo, Teklad 2018, Indianapolis, IN) and water treated by reverse osmosis. The mice were housed for one week under a 12:12 light: dark cycle between 20.6-22.2℃ with 28-50% relative humidity. These mice were part of a larger, behavioral study.

2.3.3 Sample collection and protein extraction

Unless otherwise noted, all samples were collected at the end of the weeklong study when mice were approximately 9 weeks of age. All fluid samples were collected from two mice per cage. Those mice were chosen based on their social ranking as determined by the tube test (Lindzey et al., 1961). Briefly, a one inch diameter tube was secured between two plexiglass arenas. After each individual mouse was acclimated to the arena, pairwise trials were conducted between cage mates in which one mouse was placed at each end of the tube and simultaneously released. After meeting in the middle, the first mouse to back out of the tube was declared the trial loser. All trials were replicated four times and the arena was cleaned with ethanol and air dried between trials. Each mouse's win percentage was determined from the number of trials he won divided by the number in which he competed. The mouse with the highest win percentage in each cage was considered the dominant, while the mouse with the lowest was the subordinate.

2.3.3.1 Nest

One sample of crinkle paper, containing 25 individual strips, was taken from each cage (N=2), since groups of mice sleep together in a communal nest. The center and periphery of the nest were sampled using metal forceps that had been previously cleaned with acetone and allowed to air dry. Since mice restructure their nests daily (Jirkof et al., 2013), it is unknown whether secretions would be equally distributed throughout the nest.

2.3.3.2 Sweat

Mice were anesthetized with compressed isoflurane throughout the procedure. Sweat samples were collected from two mice per cage (N=4) by injecting 50µL of a 1mg/mL pilocarpine hydrochloride solution (Sigma-Aldrich, St. Louis, MO) subcutaneously to one hindfoot. After losing consciousness, their feet were cleaned with ethanol, allowed to dry, and injected with the pilocarpine solution. The highest volume of sweat is produced approximately 10-20 minutes post injection(Vilches et al., 2002), so strips of 100% cotton filter paper (Ahlstrom, Helsinki, Finland) were secured to the foot for 20 minutes after injection using plastic hair clips (Conair, East Windsor, New Jersey). The clips held the filter paper in place without compromising blood flow to the foot.

After 20 minutes, individual filter paper strips were stored in 1.5mL centrifuge tubes in a -80℃ freezer until processing. Mice were euthanized following the collection of all the samples.

2.3.3.3 Saliva

The pilocarpine solution used for sweat collection also stimulates saliva production, so the acrylic anesthesia chamber floor was wiped with ethanol after the mice lost consciousness in preparation for saliva collection. Saliva was collected from the same mice used for sweat sampling (N=4) via pipette and stored in a -80℃ freezer until processing.

2.3.3.4 Urine

On day 5 of the study, urine was collected by scruffing each mouse over a clean bowl lined with aluminum foil. Gentle abdominal massage was applied when needed. Urine was collected from all mice, but only analyzed from mice sampled for sweat and saliva (N=4). Samples were stored in a -80℃ freezer until processing.

2.3.4 Sample preparation

Protein samples were prepared for analysis as reported previously (Aryal et al., 2018). Proteins were extracted from the nesting material and sweat filter paper using a 20mM TRIS-HCl, pH 7.5 extraction buffer and precipitated with 5x the sample volume of acetone. Proteins in all samples were denatured using 40µL of 8M urea and total quantities were determined using a bicinchoninic acid assay. The samples were reduced with 10mM dithiothreitol, alkylated with 20mM iodoacetamide, and digested at 37°C for 5 hours with a mass-spec grade trypsin and Lys-C mix (Promega, Madison, WI) at a minimum 1:25 enzyme to substrate ratio. Peptides were desalted using Pierce C18 spin columns (Pierce Biotechnology, Rockford, IL), eluted with 80% acetonitrile (ACN) and 0.1% formic acid (FA), and dried at room temperature in a vacuum concentrator for 1 hour. Clean, dry peptides were resuspended in 97% purified water, 3% ACN, and 0.1% FA at a final concentration of 0.2 µg/µL.

2.3.5 Liquid chromatography/ tandem mass spectroscopy (LC-MS/MS)

Samples were analyzed by reverse-phase LC-ESI-MS/MS system using the Dionex UltiMate 3000 RSLC nano System coupled to the Orbitrap Fusion Lumos Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides were loaded onto a trap column (300 μm ID × 5 mm) packed with 5 μm 100 Å PepMap C18 medium, and then separated on a reverse phase column (50-cm long × 75 μm ID) packed with 2 μm 100 Å PepMap C18 silica (Thermo Fisher Scientific, Waltham, MA). The column temperature was maintained at 50°C. All the MS measurements were performed in the positive ion mode, and using 120 min LC gradient and standard data-dependent mode(Aryal et al., 2018). MS data were acquired with a Top20 data-dependent MS/MS scan method. Instrument was calibrated at the start of each batch run and then in every 72 hours using calibration mix solution (Thermo Fisher Scientific, Waltham, MA). The performance of the instrument was also evaluated routinely using *E. coli* digest.

2.3.6 LC-MS/MS data analysis

LC-MS/MS data were analyzed using MaxQuant software (version 1.6.3.3) against the UniProtKB *Mus musculus* genome (85,159 sequences as of Feb. 2019, www.unitprot.org). Unless stated otherwise, default settings were used. We edited the following parameters for our search: 10 ppm precursor mass tolerance; trypsin/Lys-C enzyme specificity; variable modification was oxidation of methionine (M); fixed modification was carbamidomethylation of cysteine (C); false discovery rate (FDR) of 0.02; peptide spectral match (PSM) and protein identification was set to 0.01. Label free quantitation (LFQ) was selected. All quantifications were calculated by MaxQuant. After the search, peptides with MS/MS counts under 2 were removed from the dataset. Log2 LFQ values were used for analyses in R Studio (version 3.4.3) with the following packages: *tidyverse, VennDiagram, pheatmap, RColorBrewer, magrittr, corrplot, FactoMineR, factoextra,* and *cowplot*.

2.3.7 Bioinformatics analysis

All majority protein IDs were searched in the PANTHER gene database (www.pantherdb.org) and compared to the entire verified *Mus musculus* proteome (Swiss-prot, 22,262 proteins, version 14.0 April 2018). In cases where a protein had multiple IDs, only the first

two were used in the search. Classification is based on Gene Ontology (GO) for the molecular function category.

2.3.8 Data availability

All raw LC-MS/MS data are available on the Mass Spectrometry Interactive Virtual Environment (MassIVE) data repository at ftp://massive.ucsd.edu/MSV000084022.

2.4 Results

To assess the proteome content of nesting material, we housed 8 week old male C57BL/6NCrl mice in groups of five with 8.5g of crinkle paper nesting material. This form of material allows the mice to build more complex, naturalistic nests (Hess et al., 2008). We chose to collect nest samples after one week because that is a common length in between cage cleaning for static housing across animal facilities. Commonly, facilities completely replace the nest with new material at cage cleaning, so our samples represent a maximum amount of protein content to which the mice would be exposed. To trace the nest profiles to tentative protein sources, we collected sweat and saliva samples the same day as nest collection while urine samples were collected two days prior. Proteins were extracted from all four sample types, underwent tryptic digestion, and were analyzed using liquid chromatography- tandem mass spectroscopy (LC- MS/MS).

2.4.1 Global quantitation

We detected 432 proteins/protein groups across all sample types and filtered that list to the 304 proteins with at least two MS/MS peptide counts per protein in at least 2 replicates of a single sample type. Of that list, 46% (140) were common to at least 2 sample types; 10% were unique to the nest samples; 21% were unique to sweat samples; 15% were unique to saliva samples; and 8% were unique to urine samples (Figure 2.1A). Comprehensive peptide and protein lists and quantifications can be found in the Supplementary Information (Appendix B, Table B.1). Pearson correlation coefficients of $\log_2$ label free quantitation (LFQ) were highest within sample type (Figure 2.1B). Nest sample replicates had a correlation coefficient of 0.85. Average coefficients between sweat, saliva, and urine replicates were 0.69, 0.80, and 0.76 respectively. Nest samples also showed coefficients of at least 0.2 with saliva and urine samples, but had minimal correlation

with sweat samples. There was also a slight negative correlation between sweat and saliva samples, with coefficients less than -0.2 between most replicates. A principal component analysis (PCA) was used on $\log_2$ LFQ intensities for the 140 common proteins present in at least two sample types. It showed that replicates for each sample type cluster together on PC1 and PC2 (Figure 2.1C). Variation on PC1 separated all sample types while variation on PC2 separated urine and nest samples from saliva and sweat. Individual protein loading values for PC1 and PC2 are listed in Appendix B, Table B.2.

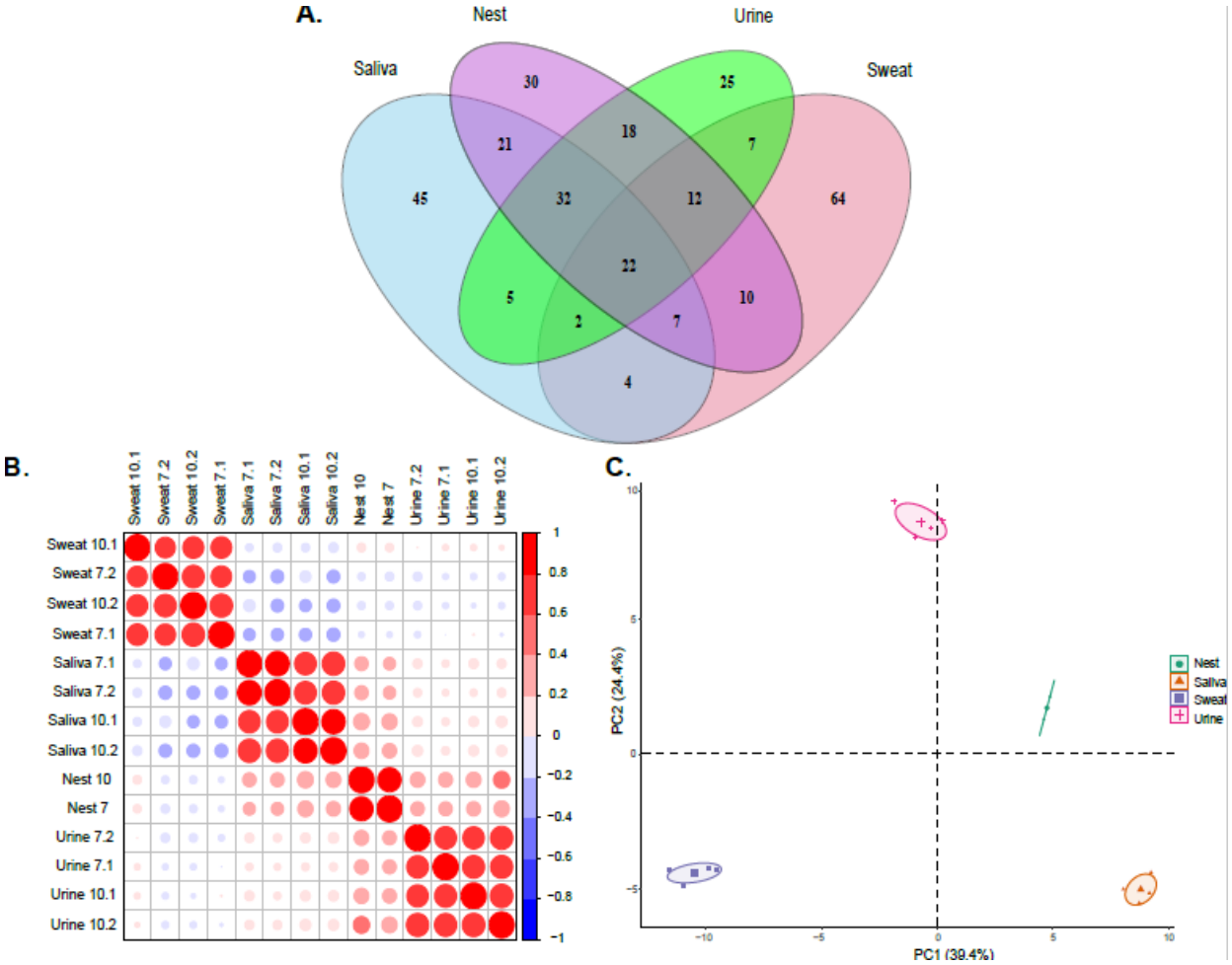

Figure 2.1. Profile analysis of nesting material, sweat, saliva, and urine proteomes. (A) Venn diagram of proteins quantified with at least 2 peptide counts in 2 replicates of a single sample type. (B) Pearson Correlation plot between replicates based on hierarchical clustering of log2 label free quantitation (LFQ) intensities. (C) Principal component analysis sample plot based on log2 LFQ intensities of 140 common proteins detected in at least two sample types; percentages in parentheses represent the explained variance for the first and second Principal Component (PC). See Appendix B Table B.2 for complete list of protein loadings.

2.4.2 Chemosensory related expression patterns

The 140 common proteins were grouped into six clusters using hierarchical clustering based on $\log_2$ LFQ z-scores (Figure 2.2). Twenty seven of these common proteins were matched to known genes with chemosignal or odorant binding function (Table 2.1) and were primarily found in three of the six protein clusters (Figure 2.2). Six proteins matched to members of secretoglobin (Scgb) family and were primarily androgen-binding protein (ABP) subunits. They showed high abundance in saliva and nest samples and overall, had low abundance in sweat samples with the following exceptions: Scgb1b27 had high abundance in two sweat replicates while Scgb2b2 had high abundance in one sweat replicate. Scgb proteins also had low abundance in urine samples with the exception of Scgb2b27 which had high abundance in two replicates and Scgb2b7 which had high abundance in all urine replicates (Figure 2.2 inset).

Peptides from several lipocalins were also detected across sample types and may function as pheromone transporters. Three odorant binding protein (OBP) had high abundance levels in saliva and nest samples and variable sweat and urine presence. Obp2a and Obp2b peptides had low abundance levels in sweat and urine samples while peptides from Obp1a had high abundance in sweat and variable abundance in urine samples (Figure 2.2 inset). Additionally, vomeromodulin and lipocalin11 had high abundance in nest and saliva samples and low abundance in sweat and urine samples. However, lipocalin11 had high abundance in one urine replicate (Figure 2.2 inset).

Nine MUP proteins, including MUP20, were also detected across all sample types. These peptides had high abundance in nest and urine samples and low abundance in saliva except for MUP5 which had high abundance in saliva samples. Overall, MUP expression in sweat samples was low with the following exceptions: peptides for MUP1; MUP7 had high abundance in sweat; peptides for MUP12 and MUP2 had high abundance in one sweat replicate respectively (Figure 2.2 inset).

Peptides for MUP4 and Scgb2b24 were also detected in nest samples and had low abundance in sweat samples. Both had variable abundance in saliva. In urine, MUP4 had variable expression while Scgb2b24 was low (Appendix B, Figure B.1). ESP15 peptides had high abundance in nest samples, but only had high abundance in one saliva replicate (Appendix B, Figure B.1). MUP10;MUP1 peptides had high abundance in all samples, but had low abundance in one saliva replicate (Appendix B, Figure B.1).

Figure 2.2. Protein abundance varies across sample types. The heatmap depicts the change in intensity for 140 proteins found in at least two different sample types. Hierarchical clustering was used to classify the proteins into six clusters. The color scale depicts log2(LFQ intensity) z-scores. Expression patterns for 21 peptides with known odor functions are emphasized in the line graphs

Figure 2.2 continued

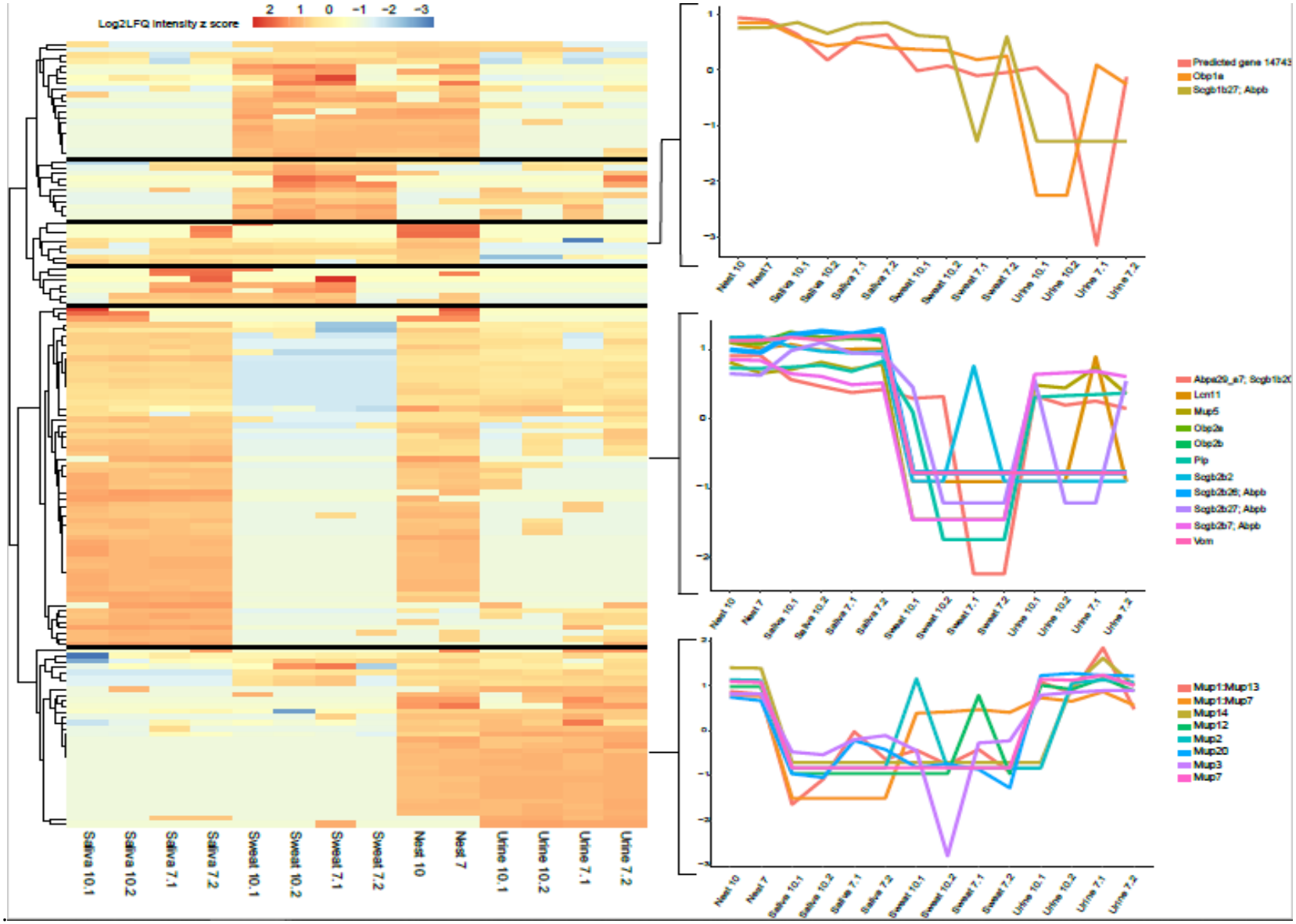

Table 2.1. Proteins with odorant related functions based on Gene Ontology (GO) searches. Detected proteins had at least 2 MS/MS counts in two replicates of a single sample type. List is limited to the first two protein IDs where applicable and organized by proteins common to at least two sample types and those unique to each sample type.

Common Proteins		
Protein ID	Protein name	Gene name
Q8R1E9;Q7TNY5	ABPBG27; Salivary androgen-binding protein beta subunit	Scgb2b27
Q3UU48;P02816	Prolactin-inducible protein homolog	Pip
A2ANT5;P11590	Major urinary protein 4	Mup4
Q9D3H2	Odorant-binding protein 1a	Obp1a
A2BHD2	Predicted gene 14743	Gm14743
O35176	Androgen binding protein A2	Scgb1b2
Q58ES8;A2CEL1	Major urinary protein 1	Mup1;Mup13
D2XZ31;E9PWZ2	Androgen binding protein A7; A20	Abpa29_a7;Scgb1b20
A2BIN1;Q4FZE8	Major urinary protein 10; Major urinary protein 1	Mup10;Mup1
Q5FW60	Major urinary protein 20	Mup20
Q3KQQ2;P04939	Major urinary protein 3	Mup3
Q91WB5;G3UXN8	Androgen binding protein A27	Scgb1b27
D2XZ37;G5E8B4	Secretoglobin family 2B member 2	Scgb2b2
P11591	Major urinary protein 5	Mup5
D3YYY1	Androgen binding protein BG7	Scgb2b7
A2BHR2	Lipocalin 11	Lcn11
P11589	Major urinary protein 2	Mup2
Q58EV3;E9QA79	Major urinary protein 1; Major urinary protein 7	Mup1;Mup7
A2CEK7	Major urinary protein 12	Mup14
Q8JZX1;Q7M745	Androgen binding protein BG26	Scgb2b26
Q8K1H9	Odorant-binding protein 2a	Obp2a
A2BHR0	Odorant-binding protein 2b	Obp2b
Q80XI7	Vomeromodulin	Vom
D2XZ39;Q7M747	Secretoglobin family 2B member 24	Scgb2b24
A8R0U8;A8R0U7	Exocrine gland secreted peptide 15	Esp15
L7MUC7	Major urinary protein 7 (Fragment)	Mup7
B8JI96	Major urinary protein 14 (Fragment)	Mup14
Unique Proteins		
Protein ID	Protein name	Gene name
Saliva		
Q24JQ8;Q62472	Vomeronasal secretory protein 2	Lcn4
Q14AJ3;Q62471	Vomeronasal secretory protein 1	Lcn3
G5E8B5;Q7M742	Secretoglobin family 1C member 1	Scgb1c1

Table 2.1 continued

Nest		
J3QK77;Q9JI02	Secretoglobin family 2B member 20	Scgb2b20
A8R0U0	Exocrine gland secreted peptide 6	Esp6
J3QJY4	Androgen binding protein A3	Scgb1b3
S4R2L0;J3QM75	Androgen binding protein BG12; Androgen binding protein BG19	Scgb2b12;Scgb2b19
Q9D3N5	RIKEN cDNA 5430402E10 gene	5430402E10Rik
S4R1X8;S4R2V3	Secretoglobin, family 2B, member 17; member 15	Scgb2b17;Scgb2b15
A0A089N3F1;D2XZ38	Androgen binding protein BG3	Abpbg3;Scgb2b3
Urine		
A9R9V7	Major Urinary Protein 21	Mup21
A2CEK6;L7N222	Major urinary protein 11; Major urinary protein 13	Mup13

Chemosignal peptides unique to each sample type are also listed in Table 2.1. In summary, submaxillary gland protein 3A and vomeronasal protein 2 were detected in all saliva replicates while vomeronasal protein 1 and Scgb1c1 were detected in two saliva replicates. MUP21 was present in all urine replicates while MUP11 was present in one urine sample. Sweat samples did not contain any unique known odor related proteins. Both nest samples contained four Scgb proteins, submaxillary gland protein 2, ESP6, and cDNA gene 5430402E10 with predicted odor carrier properties.

2.4.3 Protein functions

Of the 273 detected proteins, 68% were annotated in the Gene Ontology (GO) database based on cellular molecular function. Transfer/carrier proteins, which can bind odorants, account for approximately 21% of common proteins; 6% of unique nest proteins; 10% of unique sweat protein; 13% of unique urine proteins protein; and 8% of unique saliva proteins. (Figure 2.3). Signaling proteins, which may act as chemosignals themselves, account for approximately 3% of common proteins; 14% of unique sweat proteins; 13% of unique urine proteins; and 4% of unique saliva proteins (Figure 2.3).

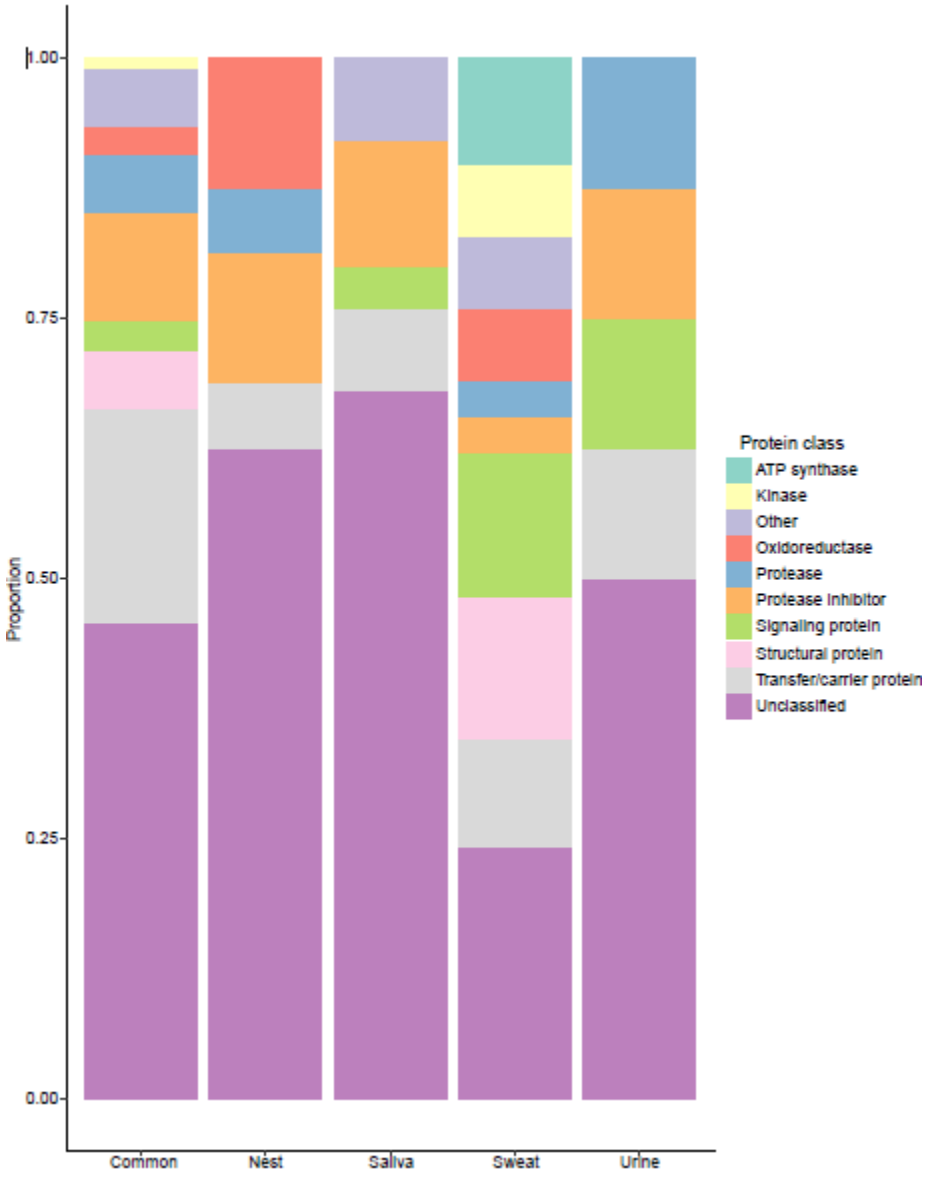

Figure 2.3. Functional classification of common and unique proteins. GO category proportions of proteins found in at least 2 sample types (common) and unique to each sample type. Proteins were only included if their protein IDs were matched in the PANTHER database. Proteins were considered "Unclassified" if the GO search did not provide a listed category.

2.4.4 Most abundant proteins

Based on the proportion of LFQ intensities, six of the top ten proteins in nest samples are members of the MUP family, accounting for just under 50% of total protein abundance in the nest site. Approximately 15% of nest site peptides were matched to Obp1a or predicted gene 14743, which has an estimated carrier protein role (Figure 2.4A). None of the top ten proteins in sweat

samples have a known odorant association role (Figure 2.4B). Seven of the top ten urinary proteins are members of the MUP family accounting for over 90% of total proteins in urine samples (Figure 2.4C). Three of the top ten saliva proteins had odorant related functions (ABP BG27, submaxillary gland protein 3A, and prolactin inducible protein) and account for 13% of total saliva proteins (Figure 2.4D).

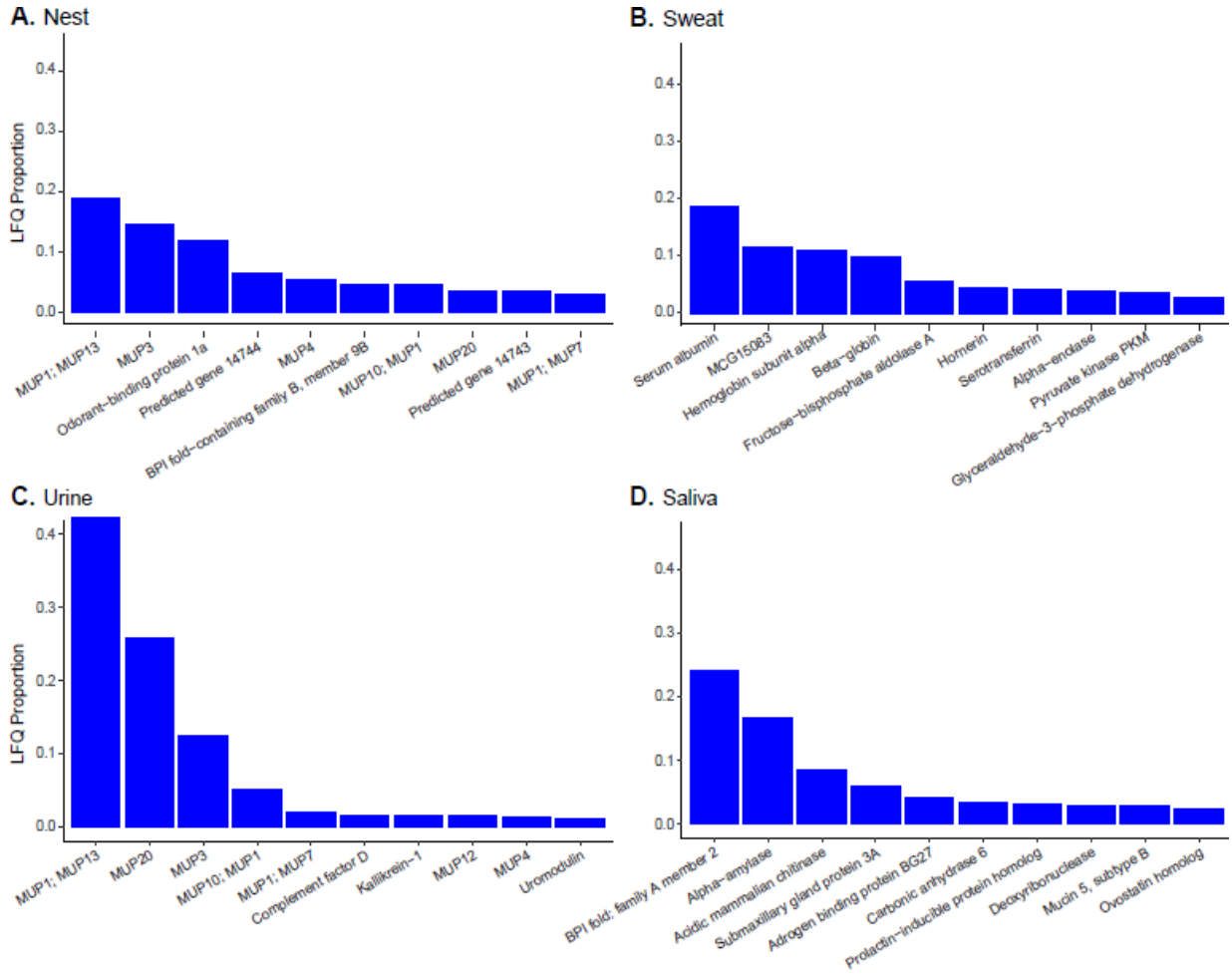

Figure 2.4. Top ten most abundant proteins in each sample type expressed as a proportion of total LFQ intensity across individual samples for (A) nesting material, (B) sweat, (C) urine, and (D) saliva.

2.5 Discussion

Although there is a growing effort to consider how the environment may impact laboratory animal well-being and data reproducibility, the olfactory environment is not given appropriate consideration. In mice, preserving used nesting material has been shown to reduce aggression in

males (Van Loo et al., 2000) and is suggested as part of standard husbandry to preserve odor cues (Weber et al., 2017). However, this is the first report to identify and quantify deposits on the nesting material and other sources to determine the origin of the deposits. Our analyses show that after one week in the mouse cage, nesting material acquires a variety of chemosignal proteins from sweat, saliva, and urine sources. Additionally, nest samples contain unique proteins that may originate from sebaceous glands, other oro-facial glands, or fecal residues. Mice prefer to defecate and urinate away from the nest site (Makowska et al., 2019), but due to the restricted area in a standard mouse cage, urine and feces likely enter the nest due to regular activity. This data provides evidence of urinary proteins in the nest, although we did not record where the mice chose to urinate in relation to the nest site.

Overall, the nest site contains a variety of proteins used by mice for identification. This supports the rationale behind preserving nesting material to maintain familiar scent marks (Weber et al., 2017). To start, the most prevalent proteins in the nest, accounting for approximately half of the total abundance, belong to the MUP family. While MUPs are primarily found in urine, these proteins are also found in sweat and saliva. The diverse MUP ratio between individuals serves as an identification mechanism as mice spend the most time investigating urine marks with a different MUP profile than their own (Hurst et al., 2001). These profiles provide specific information about the signaler such as health and social status (Wyatt, 2010). Even though members of the same inbred strains have little diversity in their MUP profiles (Cheetham et al., 2009), maintaining the high abundance of MUPs through nest transfer is still beneficial for mouse welfare. Instead of being placed into an unmarked, odor-free environment at cage change, nest transfer allows the mice to maintain odor familiarity through the deposits in the preserved material.

Additionally, several ABP and OBP/lipocalin proteins were detected in the nest samples. ABP dimers in the saliva help facilitate mate choice in female mice by providing subspecies identification cues (Laukaitis and Karn, 2012). OBPs are known to transport VOCs and are expressed in several oro-facial glands with the protein product ultimately detected in saliva (Stopková et al., 2016). Since mice typically engage in facial sniffing when initiating social interactions (Latham and Mason, 2004), it has been suggested that these proteins may play a role in chemical communication: the mixture of self and conspecific odor is spread through self-grooming to promote peaceful interactions (Stopka et al., 2016). The presence of OBPs in the nest site may further expand this hypothesis. Through the act of repeated oral nest manipulation, mice

deposit their own OBPs and pick up OBPs from their cage mates. In addition, group sleep in a common nest area may also spread the OBP mixture onto each cage mate's fur, further promoting peaceful social behavior.

Two members of the ESP family were also detected in nesting material: ESP6 and ESP15. ESP genes are clustered near MHC loci in the mouse genome (Kimoto et al., 2007) and are produced primarily by the lacrimal gland (Kimoto et al., 2005). 14 members of the ESP family, including ESP6 and ESP15, are capable of stimulating neurons in the vomeronasal organ (VNO) (Kimoto et al., 2007). Although the direct function of ESP6 and ESP15 are unknown, they may serve as chemosignals since proper sensory activity by the VNO is necessary to express appropriate sex-specific behaviors (Stowers et al., 2002) and many known mouse pheromones function through VNO activation (Stowers and Kuo, 2015). Ultimately, the identification of multiple proteins and potential chemosignals in the nest site is likely a driving factor behind the reduction in male aggression seen when nesting material is preserved at cage change (Van Loo et al., 2000).

Despite the nest's ability to reduce aggression at cage change, one of its most abundant proteins, MUP20 ("darcin"), elicits male aggression at levels comparable to that of whole urine exposure (Chamero et al., 2007). However, MUP20 has been shown to play a crucial role in social learning by female mice. Females pre-exposed to urinary MUP20 form a learned attraction to the source male's VOC profile (Roberts et al., 2010). It is possible that a similar mechanism occurs in male cages where deposited MUP20 within the nest site stimulates learning of cage mate profiles. It is also possible that MUP20 in the nest may be deposited from a variety of secretions. MUP20 is commonly thought of as a urine component that binds VOC pheromones which promote aggression (Robertson et al., 1993). However, our data confirms a previous report of MUP20 being present in saliva (Stopka et al., 2016) and shows that, among several MUP peptides, it is present in sweat as well. MUP20 originating in saliva and sweat may not elicit the same behavioral response as the urinary form since the VOCs it binds are unique to male urine (Novotny et al., 1985). While recombinant MUP20 can elicit aggression on its own (Chamero et al., 2007), perhaps MUP20 in saliva and sweat bind a different ligand that reduces its aggression provoking signal. That answer to that question was beyond the scope of this study's aim.

Pilocarpine was used in this study because sufficient amounts of sweat and saliva could not be collected naturally for analysis. While necessary, it is worth considering the potential impact

of the drug on protein data. Pilocarpine induces fluid release by stimulating M3 muscarinic receptors on the sweat and salivary glands (Landis, 1999; Proctor and Carpenter, 2007). Currently, it is not known how pilocarpine stimulation may influence the secreted gland content, but we acknowledge that these samples may not reflect naturally occurring protein ratios. Additionally, all body fluid samples may have been impacted by each mouse's social status. Sampled mice were chosen based on their dominance ranking, which may have contributed to natural variation between samples. It may also explain variation between protein ratios in the nest compared to other sample types: the nest contains a pooled sample from all mice in the cage, so secretions from dominant and subordinate mice are inter-mixed. Dominant mice are known to produce more MUPs, particularly MUP20, than subordinates (Guo et al., 2015; Lee et al., 2017; Nelson et al., 2015), but it is unknown whether social ranking influences other protein levels.

Overall, our saliva and urine proteomes contained proteins that were also reported in previous studies. In saliva, we detected several ABP analogs, MUPs, ESPs, Kallikerin-1, OBP analogs, prolactin inducible protein homolog, and amylase that match past reports from C57BL/6J and BALB/c mice (Karn and Laukaitis, 2015, 2011; Lamy et al., 2010). In urine, a majority of our detected proteins were members of MUP family, which have been well documented in previous reports (Cheetham et al., 2009; Hurst et al., 2017; Roberts et al., 2018, 2010; Thob et al., 2015). In addition, MUPs have been reported in rat urine, with MUP13 displaying pheromone properties, further supporting their role in olfactory communication across species (Gómez-Baena et al., 2019; Guo et al., 2018).

This initial protein characterization provides a framework for further studies focused on the cage level olfactory environment. Due to the prevalence of identification proteins, it is probable that the nest profile will vary based on strain, sex, as mice age, and with reproductive status. Nest sites from breeder pairs or trios may contain additional signals that strengthen parent-offspring relations. Maintaining familiar odors from the home cage may also prove beneficial when acclimating mice to a new behavioral testing arena. It is also worth examining how the nest contents could change before and after aversive procedures or if the mice are inoculated for an infectious disease study. Situations where the mice become stressed or sick may cause them to produce an aversive signal indicative of danger that should not be preserved in the cage.

More broadly, a recent initiative throughout biomedical science aims to reduce the level of preclinical research that is not reproducible. In a recent survey of the scientific community, 90%

of respondents felt there was either a “slight” or “significant” reproducibility crisis in research data (Baker, 2016). Over 80% of participants also claimed that “selective reporting” and unavailable methods are common factors contributing to the crisis. As an attempt to increase method transparency, the National Centre for the Replacement, Refinement, and Reduction of Animals in Research developed the ARRIVE guidelines for reporting preclinical study procedures (Kilkenny et al., 2010). Item 9 of ARRIVE focuses on animal housing and husbandry in which researchers are instructed to report a wide range of environmental parameters for their study animals. This includes housing environment, lighting conditions, and temperature/humidity ranges throughout the study. However, the ARRIVE guidelines fail to acknowledge the animals’ chemical/olfactory environment and many researchers do not consider how their studies may be affected by odors. Findings from this study bring attention to the diverse olfactory environment found in standard mouse cages.

In summary, we present the first proteome characterization of used nesting material from group housed male mice. It is commonly suggested to preserve used nesting material throughout cage changes to preserve the cage level olfactory environment and this study provides quantitative evidence to support this practice. Used material contains a large assortment of proteins, many of which contain identification information. These identity cues likely play a communication role between cage members. Further research is warranted to explore the role between these complex odor profiles and social behavior.

2.6 Acknowledgements

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CHAPTER 3. COMPOUNDS FROM PLANTAR FOOT SWEAT, NESTING MATERIAL, AND URINE SHOW STRAIN PATTERNS ASSOCIATED WITH AGONISTIC AND AFFILIATIVE BEHAVIOR IN GROUP HOUSED MALE MICE, MUS MUSCULUS

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3.1 Abstract

Excessive home cage aggression often results in severe injury and subsequent premature euthanasia of male laboratory mice. Aggression can be reduced by transferring used nesting material during cage cleaning, which is thought to contain aggression appeasing odors from the plantar sweat glands. However, neither the composition of plantar sweat nor the deposits on used nesting material have been evaluated. The aims of this study were to (1) identify and quantify volatile compounds deposited in the nest site and (2) determine if nest and sweat compounds correlate with social behavior. Home cage aggression and affiliative behavior were evaluated in 3 strains: SJL, C57BL/6N, and A/J. Individual social rank was assessed via the tube test, because ranking may influence compound levels. Sweat and urine from the dominant and subordinate mouse in each cage, plus cage level nest samples were analyzed for volatile compound content using gas chromatography-mass spectrometry. Behavior data and odors from the nest, sweat, and urine were statistically analyzed with separate principal component analyses (PCA). Significant components, from each sample analysis, and strain were run in mixed models to test if odors were associated with behavior. Aggressive and affiliative behaviors were primarily impacted by strain. However, compound PCs were also impacted by strain, showing that strain accounts for any relationship between odors and behavior. C57BL/6N cages displayed the most allo-grooming behavior and had high scores on sweat PC1. SJL cages displayed the most aggression, with high scores on urine PC2 and low scores on nest PC1. These data show that certain compounds in nesting material, urine, and sweat display strain specific patterns which match strain specific behavior patterns. These results provide preliminary information about the connection between

home cage compounds and behavior. Salient compounds will be candidates for future controlled studies to determine their direct effect on mouse social behavior.

3.2 Introduction

Aggression among group housed male mice is one of the most common reasons for premature euthanasia and reduces preclinical research data validity and reproducibility (Kappel et al., 2017; Poole, 1997; Weber et al., 2017). Individual housing appears to be a simple solution, but it comes with its own welfare concerns (Bartolomucci et al., 2003). Mice form complex social structures in the wild (Crowcroft, 1966; Latham and Mason, 2004), which is why group housing for laboratory mice is recommended (National Reseach Council, 2011). Enrichment is commonly suggested to reduce home cage aggression, but results are often inconsistent (Weber et al., 2017). Nonetheless, nesting material is one of the most reliable and recommended types of enrichment, particularly for reducing aggression after cage cleaning (Van Loo et al., 2003; Weber et al., 2017). Routine cage cleaning is a known trigger of escalated aggression in males (Jennings et al., 1998) with time periods of social unrest peaking approximately 15 to 45 minutes afterward (Ambrose and Morton, 2000; Gray and Hurst, 1995). However, this aggression is reduced when a portion of the existing nest is transferred to the new cage (Van Loo et al., 2000). Accordingly, nest transfer has become a widely used practice, but there is no empirical evidence to explain how it decreases aggression.

Although there are minimal data, the prevalent theory explaining these effects focuses on scent cue preservation. The familiar odors within the nesting material may include pheromones, which are commonly produced as volatile organic compounds (VOCs) and play a prominent role in regulating mammalian social interactions (Apps et al., 2015). While pheromones are the most recognized odor signal, odors must meet strict criteria to be considered a pheromone: physiologically relevant concentrations must produce reliable effects in a bioassay (Wyatt, 2017, 2009). Individualized scent profiles can also relay information, and mice rely heavily on both pheromones and individual scent cue mixtures for communication and conspecific recognition (Arakawa et al., 2008; Liberles, 2014; Novotny, 2003; Wyatt, 2017). The disruption of these scent cues can in turn lead to aggressive interactions (Hurst et al., 1993).

While odor signals relay a variety of messages, most of the literature on male, intra-sex, signaling focuses on urine borne signals that are connected to territory marking in wild mice and ultimately promote aggression in the laboratory (Brown, 1985; Chamero et al., 2007; Latham and Mason, 2004; Lee et al., 2017; Novotny et al., 1985, 1984; Stoddart, 1980; Touhara and Vosshall, 2009; Wyatt, 2014a). In contrast, little is known about odor signals that may reduce aggression or promote affiliative behaviors among male mice. In pigs, synthetic androstenone and maternal mammary pheromones effectively reduce aggression in newly mixed groups of prepubescents (Guy et al., 2009; McGlone and Morrow, 1988), but, to the best of our knowledge, compounds with similar effects in mice have not been identified. Affiliative behaviors, for example, are performed to strengthen social bonds between conspecifics, and examples in mice include allo-grooming and group sleep (Brown, 1985). While aggression and affiliative behavior patterns do not always oppose each other (De Waal, 2000), it has been proposed that they can be different context dependent strategies used for resource control. Affiliative behaviors are deemed more beneficial when resources are abundant, such as in a captive enclosure with free food and water access (Pellegrini, 2008). However, almost all work on domestic murine social behavior focuses on encounters with unfamiliar mice in a testing arena. Affiliative patterns between adult males in the home cage have been largely unexplored and will be examined here.

Despite the lack of explicit evidence, it has been suggested that nesting material contains an aggression appeasing odor signal (Van Loo et al., 2000). Specifically, the nest site appears to act as a depository for secretions from the plantar sweat glands which are believed to appease aggression (Van Loo et al., 2000, 2003). However, there is little empirical data describing the properties of plantar sweat. Laboratory mice only have one type of sweat gland, eccrine glands, which are found on their food pads (Crowcroft, 1966). These glands produce an oily substance that is associated with maintaining traction during mobility, marking territory boundaries, and colony member recognition (Brown, 1985; Ropartz, 1977; Taylor et al., 2012). However, the only study to specifically link plantar sweat to a behavioral response demonstrated that the presence of sweat increases locomotion in stranger mice (Ropartz, 1966).

To date, there are no published studies that explore the mechanism behind the reduction in aggression observed in response to used nest material or whether odors exist that can promote affiliative behaviors in mice. Providing nesting material is becoming standard practice for laboratory mice and its transfer during cage cleaning helps reduce aggression although it does not

completely eliminate it. In order to understand what in the nest is specifically effective at altering mouse behavior, we must have better insight into the chemical signals deposited there and where they come from. Once these specific signals have been identified, further research can examine methods to develop compounds that could then be added to mice environments to help reduce aggression. Additionally, there are no reports that quantitatively analyze the VOC contents of murine plantar sweat, which has historically been suggested as the source of nesting material odor deposits. Therefore, the first aim of this experiment was to quantify compounds deposited within the nests of mouse strains known to exhibit different aggression levels and link them to plausible sources. Our working hypothesis was that the compounds present on the nests would exhibit strain specific properties. We predicted that chemical analyses of the nests from historically peaceful mice would contain VOCs in different proportions than those from the nests of historically aggressive males; in particular, they would contain higher levels of VOCs originating in plantar sweat and lower levels of VOCs originating in urine. To do this, we used three strains known for varying aggression levels: SJL (high aggression), C57BL/6 (moderate aggression), and AJ (low aggression). Our second aim was to determine whether these VOC profiles are related to mouse social behavior. Our working hypothesis was that VOC profiles from the nest and sweat correlate with social behavior in group housed males, with the assumption that behavior is affected similarly across strains. We predicted that these odor profiles would be associated with lower rates of aggressive behavior and/or higher rates of affiliative behavior. In contrast, profiles from urine would be associated with higher rates of aggression. Social behavior was taken as a cage level measure, while odor profiles were taken from individuals based on dominance rank in the tube test (Lindzey et al., 1961).

This study served as the first step in a series of projects that aim to identify and validate whether the VOCs identified are true murine pheromones, based on criteria summarized by Wyatt (Wyatt, 2017, 2009). The goal of the current study was solely to compare profiles across experimental groups and identify molecules that align with quantified behavioral measures.

3.3 Methods

3.3.1 Ethics statement

All procedures were approved by Purdue University's Institutional Animal Care and Use Committee (protocol #1707001598) and reporting adhered to the ARRIVE 2.0 guidelines (du Sert et al., 2020). The protocol was not previously registered before conducting the study.

Due to concern over heightened aggression in the cage, we established humane endpoint criteria in which any mouse with wounding greater than $1cm^2$ would be immediately euthanized. Animals were monitored daily for general activity and signs of pain/distress. If any animals developed minor wounding, they were monitored more frequently. No mice reached our endpoint criteria.

3.3.2 Animals

All mice in this study were acquired from Charles River and were free of common known pathogen agents at shipping. More information can be found in ("North American Health Reports by Strain," n.d.). Eight cages each containing five male mice of the following strains were used: SJL/JOrlIcoCrl (SJL)- Wilmington, MA; C57BL/6NCrl (B6)- Kingston, NY; and A/JCr (AJ) – Frederick, MD (N = 24 cages; 120 mice). Per the ARRIVE 2.0 guidelines (du Sert et al., 2020), we are declaring that no strain served as a traditional control due to the study's exploratory nature. Sample size was determined using Mead's resource equation. Due to spatial constraints, the twenty four cages were divided into four equal groups containing two cages per strain. B6 mice were used as they are the most commonly studied inbred mouse and have the widest practical application; SJL males were used as a known high-aggressive strain (Festing, 1998); while AJ mice were used as a known low aggressive strain compared to B6 mice (Southwick and Clark, 1968). Mice arrived at approximately 8 weeks of age and were housed for one week in open top micro-isolator cages, 11.5" x 7.25" x 4.25" (Ancare, Bellmore, NY) with food (Envigo, Teklad 2018, Indianapolis, IN) and reverse osmosis water offered *ad libitum*. Each cage contained aspen wood chip bedding (NEPCO, Warrensburg, NY) and 8.5g of virgin kraft crinkle paper (Enviro-dri, Fibercore, Cleveland, Ohio) for nesting material. Cages were kept under a 12:12 light: dark cycle (lights on at 06:00) with relative humidity ranging between 28-76% and temperature ranging

between 18.8-23.3℃. All mice were weighed at the beginning (mean weight 20.06 ±1.71 g) and end (mean weight: 21.73±1.86g) of the study and ear punched for identification. All animal handling was performed by female researchers and husbandry staff. Male scents can influence stress response in rodents and may alter baseline measurements (Sorge et al., 2014).

Upon arrival, mice were randomly distributed into the cages (5 mice per cage) from the shipping containers using a numerical sequence from RANDOM.org. Cage placement on the two MetroRacks was initially randomized based on a RANDOM.org sequence, and subsequently balanced by strain across two shelves on each rack. Each shelf contained 2 cages and was enclosed by partitions of white foam board (Office Depot, Boca Raton, FL) to remove background noise for video monitoring (see Home Cage Observation below). Light intensity during the day was reduced from 430 lux, in the middle of the room, to an average of 67 lux at each cage location. Each cage was given its own numerical label from 1 to 24 that corresponded to its group and strain. Only the numerical label was present on the cage card to partially blind caregivers to cage identities during routine husbandry and research staff during sample collection/processing, behavior tests, and video coding.

3.3.3 VOC sample collection and processing

3.3.3.1 Nest

Mice were left in their home cage for 7 days after arrival. At the end of the week, 25 strips of crinkle paper were collected for VOC analysis (see below for GC-MS procedure). Samples were taken from both the periphery and center of the nest since mice restructure their nests daily (Jirkof et al., 2013) and it is not known if they are in contact with one area more than another. Some cages did not contain a structured nest, so the area containing dispersed material was divided into quadrants and each quadrant was equally sampled. The weighed sample of crinkle paper was placed in a 10 mL head-space sample vial with a Teflon cap (Gerstel GmbH, Mülheim an der Ruhr, Germany). An acetone (Avantor, Center Valley, PA) washed, straightened, and dried metal paper clip was punched through the vial Teflon seal. A magnetic Gerstel stir bar was attached to the clip above the nest material, 5 µL of 7-tridecanone in methanol (Baker Analyzed, Mallinckrodt Baker Inc., Phillipsburg, NJ) (8 ng/5 µL) was added to the nest material and the vial cap was closed tight. The head-space VOCs were collected at room temperature for 1 hour.

Two exceptions occurred within the AJ strain during nest sample collection. One cage flooded at the end of the third study day. Nest material was soaked and unable to be collected. It was replaced and subsequently collected four days later. A second cage flooded on the sixth study day. The nest from this cage was collected since there was a short proximity to the planned sampling day and enough dry material could be collected for processing. The former data point produced unusual data and was excluded from analysis; however, the latter was included.

3.3.3.2 Sweat

To analyze compounds from mouse sweat, the stir bar surface sampling method (previously used for human skin VOC analyses) was replicated (Penn et al., 2007; Soini et al., 2006). To collect secretions from the plantar sweat glands, mice were anesthetized with compressed isoflurane and each foot was cleaned with ethanol. After air drying, hindfeet and forefeet were given a subcutaneous injection of 50 µL and 20 µL of 1mg/1mL pilocarpine (Sigma- Aldrich, St. Louis, MO) respectively. Previous studies have shown that gland activity is highest approximately 10-20 minutes after injection (Klar et al., 2014; Vilches et al., 2002), so mice were kept under anesthesia for 20 minutes post injection. Sweat was collected on the surface of one forefoot and one hindfoot per mouse using Twister™ polydimethylsiloxane coated stir bars (Gerstel GmbH, Mülheim an der Ruhr, Germany) embedded previously with the internal standard, 7-tridecanone (Sigma- Aldrich, St. Louis, MO) as described previously (Penn et al., 2007). Every five minutes post injection the stir bar was rolled across the surface of the hind and forefeet five times. All collections were performed in the mice's housing room between the 7^{th} and 9^{th} hour of the light cycle. All mice were monitored throughout the procedure for signs of distress (uneven, shallow breaths; pale color of foot tissue).

3.3.3.3 Saliva

Saliva was collected while the mice were anesthetized for sweat collection as the pilocarpine injections also stimulated saliva production. After the mice lost consciousness, the exposed chamber floor was quickly cleaned with ethanol. Saliva samples were collected via pipette from the acrylic chamber floor and transferred into a 1.5 mL centrifuge tube. Saliva samples (25-100 µL) were pipetted into 20 mL glass scintillation vials containing 5.0 mL water (OmniSolv™

LC-MS grade, EMD Millipore Corporation, Billerica, MA), 8 ng of 7-tridecanone as an internal standard and the Twister™ stir bar. The vial was placed in a water bath at 40 ºC for 2.5 hours for static aqueous stir bar extraction. This sampling method was modified from a previously reported study with human saliva (Soini et al., 2010).

3.3.3.4 Urine

Since mice naturally urinate upon handling, each mouse was held over a fresh aluminum foil bowl to collect urine on day 5 of the study week, before behavior testing. Gentle abdominal massage was administered when needed to facilitate collection and samples were transferred via pipette to a 1.5mL centrifuge tube. However, when mice would not urinate during handling, the fluid was collected after the mice acclimated to the plexiglass tube test arena used for the behavioral assay (see Social Ranking section for description).

Urine samples (15-200 µL) were pipetted in a 20 mL glass scintillation vial with the metal foil cap containing 2.0 mL of water (OmniSolv™) (Soini et al., 2009), 8 ng of 7-tridecanone as an internal standard and a Twister™ stir bar. Stir bar extraction was performed for 60 min at room temperature at 850 rpm speed (15-place stir plate Variomag Multipoint HP15, H+P Labortechnic, Oberschleissheim, Germany).

After extraction, all stir bars were washed with OmniSolv™ water, dried with non-lint KimWipes tissue (Kimberly-Clark, Roswell, GA), and placed in a Thermal Desorption Autosampler and Cooled Injection System (TDSA-CIS 4 from Gerstel GmbH) connected to an Agilent 6890N gas chromatograph – 5973iMSD mass spectrometer (Agilent Technologies, Inc., Wilmington, DE).

Since the sampling unit was the cage, sweat and saliva samples were collected from each cage's dominant and subordinate mouse based on results from the tube test (see Social Ranking section for test procedure) as social ranking has been reported to impact pheromone levels (Jemiolo et al., 1985; Lee et al., 2017; Nelson et al., 2015; Novotny et al., 1999). Urine was collected from each mouse, but only samples belonging to each cage's dominant and subordinate were analyzed. All samples were collected at Purdue University and transported to Indiana University for analysis. In total, 24 nest samples, 48 sweat samples, 48 saliva samples, and 42 urine samples were collected. Six mice, each from a different cage of the SJL strain, did not produce urine when stimulated.

Additionally, two sweat samples originating from different cages lost their labels during transport and could not be processed, leaving 46 data points for sweat analysis.

3.3.4 Gas chromatography- mass spectrometry (GC-MS) analysis

Splitless mode was used for thermal desorption sampling (TDS) with a temperature program of 20°C for 0.5 min, then a 60°C/min increase up to 280°C for 8 min. The transfer line temperature was set at 290°C and the cooled injection system (CIS) was cooled using liquid nitrogen to 0°C during the thermal desorption. For the sample introduction into the GC-MS, the CIS was heated at 12°C/s to 280°C and held for 10 min. Solvent vent mode was used for the CIS inlet with a vent pressure of 9.1 psi, a vent flow of 50 mL/min, and a purge flow of 50 mL/min. The gas chromatograph (GC) separation capillary was a DB-5MS (30 m x 0.25 mm, i.d., 0.25 μm film thickness) from Agilent, and the GC carrier gas (helium) head pressure was 9.1 psi at a constant 1.2 mL/min flow mode. The GC oven temperature program started at 40°C for 1 min, then increased at 2°C/ min to 180°C and immediately 10°C/ min to 230°C and held for 6 min (total GC run time 85 min). For the mass spectrometer (MS), positive electron ionization (EI) mode at 70eV was used with a scanning rate of 2.47 scans/s and mass range of 41-350 amu. The mass spectrometric detector (MSD) transfer line temperature was 300°C, the ion source temperature was 230°C, and the quadrupole temperature was set at 150°C.

Compounds were identified or tentatively identified by matching retention times and mass spectra with standard compounds when available (Sigma-Aldrich Chemical Co.) and with spectra through NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library (Version 2.0 a, 2002). Additionally, in-house (Novotny Laboratory) synthesized mouse urinary pheromone compounds and the in-house spectral database were utilized for identifications.

All VOC data was used to calculate odor proportions by dividing each absolute peak value by the sample's total peak area (Whittaker et al., 2018). This was done to determine how behavior is affected by the relative VOC amount perceived by the mice. Due to the low volume of saliva that was collected, the GCMS analysis was unable to provide reliable quantitative values. The saliva VOC profile only served to make qualitative comparisons about nest compound origins. All VOC data was used to calculate odor proportions by dividing each absolute peak value by the sample's total peak area (Whittaker et al., 2018). This was done to determine how behavior is

affected by the relative VOC amount perceived by the mice. Due to the low volume of saliva that was collected, the GCMS analysis was unable to provide reliable quantitative values. The saliva VOC profile only served to make qualitative comparisons about nest compound origins.

3.3.5 Behavioral measures

3.3.5.1 Home cage observations

Cages were continuously recorded for one week from arrival to sample collection with closed circuit television cameras (Sony, Tokyo, Japan) and GeoVision monitoring software (Taipei, Taiwan). Dark cycle recordings used 2 infrared illuminators (Sodial, China) per cage. The following social behaviors were documented: escalated aggression, mediated aggression, allo-groom, group sleep, and social investigation (Table 3.1). Coders were partially blinded to strain due to the difference in coat color between B6 and AJ/SJL. All social interactions were scored using one-zero focal sampling for one minute every five minutes between 12:00AM- 12:00PM on days 1, 2, and 7 of the study.

Since we were interested in compounds deposited on the nest, we were also interested in how mice interacted with the nest. Thus, oral nest manipulation and paw nest manipulation (Table 3.1) were scored using one-zero sampling for one minute every half hour between 12:00AM-12:00PM on days 1, 2, and 7 of the study.

The 12:00AM -12:00PM time frame was chosen because it allows for equal observation across light and dark conditions and the mice experienced the least amount of disturbance during this time frame. Day 1 was monitored to include behaviors that occurred while the mice adjusted to their new cage, before the hierarchy is established; day 2 reflects interactions that occur as the hierarchy is beginning to form; and day 7 reflects the last 24 hours of the study in which the hierarchy is established (Tallent et al., 2018). Day 7 is also a common day for mice to undergo cage cleaning, so the maximum level of secretions in the nesting material represents the amount that many mice are exposed to before their nests are replaced. Ultimately the proportion of active time in which each behavior category occurred was determined for each cage, with the exception of group sleep for which the proportion of all observed time was calculated.

Table 3.1. Ethogram of observed behavior categories. All descriptions were taken from www.mousebehavior.org

Social Behaviors- recorded every 5 minutes using one-zero sampling

Category	Behavior	Description
Mediated Aggression	Resource Theft	A mouse will approach another that is either eating a piece of food or chewing on a piece of bedding. The approaching mouse will then attempt to take the resource from the other's paws or mouth. It may or may not be successful. It is often preceded by facial sniffing and involves one or both mice tugging at the resource.
	Tail Rattling	Fast waving movements of the tail. This behavior may be partially obscured by bedding material, but can be detected by displacement of bedding near a mouse's tail.
	Thrust	The aggressor mouse will first threaten its target cage mate by thrusting its head and fore body towards its cage mate's head or body. The aggressor's paw may come in brief contact with the target, but otherwise no contact is made.
	Mounting	Attempts to mount another animal in the absence of intromission. Palpitations with forepaws and pelvic thrusts may be present.
	Chase	A mouse will chase a fleeing partner, but no biting occurs
	Submissive Upright	A posture where the animal will sit on its haunches in an upright position exposing the belly. The forepaws are off the ground and the mouse may stretch out its forepaws towards the threatening mouse. Mouse can also be laying on its side with one forepaw and one hind paw stretched toward the threatening mouse and its back touching the ground.
	Fleeing	This behavior is characterized by a mouse moving away from the mouse performing an aggressive behavior. Typically fleeing animals will run, but in a confined space may walk or turn first. Also score if the mouse turns away without locomoting. Only score if responding to an aggressive behavior (mediated/escalated) or investigation.
Escalated Aggression	Bite	The aggressor mouse attacks the recipient with open mouth and appears to bite the recipient, or latches onto the recipient by his teeth, or forcefully touches the recipient who responds by jumping or fleeing quickly. This also includes a mouse using its teeth to grab and tug on another's tail. Only score for the mouse that is biting.
	Fighting	A violent behavior displayed by each animal when locked together. Separate behaviors are difficult to distinguish properly due to the fast rolling over and over seen with the animals kicking, biting, and wrestling. The initial victim retaliates towards the attacker. Score for all mice actively involved in the fight.
Group Sleeping	Sleeping that occurs when two or more mice are resting while in contact with the body of another mouse. When in the nest, the animals may not be seen clearly due to camera angles. Only score if the animals are observed going into and staying in a central resting area together once movement ceases for at least 5 seconds. This will typically be in the main nest, but they could remain behind bedding.	
Allo-groom	During grooming, the actor mouths and licks the fur on the recipient's body. The actor will also use its teeth to clean the hair shaft by pulling the fur from the base of the hair shaft upward or outward.	

Table 3.1. Continued

Social investigation	Face sniffing	A mouse sniffing the face of its cage mate
	Ano-genital sniffing	A mouse sniffing the ano-genital region of its cage mate
Nesting Behaviors- recorded every 30 minutes using one-zero sampling		
Paw nesting	Digging	A series of at least 3 fast alternating movements of the forepaws scraping back material. The material will accumulate in a pile under the abdomen of the animal
	Push Dig	The forwards pushing and kicking of bedding material with fast alternating movements of the forepaws. It is accompanied by forward locomotion.
	Sorting-Paw	The placing of specific nesting or bedding material into a particular location, while sitting in the nest. Sorting is done in a deliberate fashion.
	Pulling In	The animal reaches out of the nest and pulls the nesting material in towards the nest. This may also be accomplished, by grasping the material in its mouth and dragging it in to the edge of the nest site. While performing this behavior the animal's hind legs do not leave the nest, and the forelegs are pulled back in each time the animal reaches out of the nest.
	Fluffing	This behavior can be unseen due to insufficient camera angles as it is characterized by the enlargement of the nest from the inside. The walls of the nest appear to jump as the whole nest enlarges. It is assumed that the inside of the nest is being hollowed out by the animal pushing the walls back and up. When visible, fast movement of the forepaws is seen as in push dig. However, no forward locomotion occurs while fluffing.
Oral nesting	Carrying	The animal is mobile while holding pieces of bedding or nesting material in its mouth. The material is transported to a new location in the cage.
	Sorting-Mouth	The placing of specific nesting or bedding material while sitting in the nest, done in a deliberate fashion using the mouth. Animal is not mobile as in "carrying" and does not chew the material is in "fraying".
	Fraying	The animal uses movement of the forepaws to draw material through the mouth. Gnawing movements of the jaw and jerking movements with the head are also seen. Score for oral manipulation/chewing of material. Do not score if the animal is chewing, but material pieces cannot be seen.
Active		Score if the mouse is visible and moving for more than 5 seconds.

3.3.5.2 Nest scores

Daily nest scores were taken around the ninth hour of the light cycle based on Hess et al. (Hess et al., 2008). This time was used as it is when nest scores are typically highest (Jirkof et al., 2013). This scale was used as it provides the most variability for mice that are good nest builders and has been shown to reflect changes based on aggression (Gaskill et al., 2013). Briefly, the nest is divided into a square region and each quarter is given a score from 1-5 based on its complexity with higher scores corresponding to more complex structures. The four quarter scores are then

averaged for the overall nest score of a cage. In situations where more than 1 nest was present in a single cage, the scores from both nests were averaged. Daily values from each cage were used to determine the average score for the study week.

3.3.5.3 Social ranking

On days 5 and 6 of the study, the tube test was run to determine the linearity of each cage's social hierarchy based on Howerton et al. (Howerton et al., 2008). Previously, lower linearity has been reported with higher aggression levels (Howerton et al., 2008). The tube test was run over 2 days due to the time consuming nature of the pairwise tests for all mice within the cage. When conducting the test, strain was blocked by time of day to counteract systematic test order bias. That is, we tested one cage of every strain in each time period (morning (06:30-12:30) and afternoon (13:00- 17:30)).

In brief, the test is conducted using a PVC tube (approx. 2.5cm diameter) connected to two plexiglass containers (approx. 19 cm x 19 cm x 21.5 cm). To acclimate the mice, 24 hours before the trials each mouse was placed in the test arena and given at least five, but no more than ten minutes to acclimate which was defined by the mouse comfortably exploring the areas on each side of the tube. Testing began by placing two mice from the same cage on opposite sides of the tube. They typically entered the tube immediately. The first mouse to place both hindfeet on the floor outside the tube was considered the loser. In a cage of five mice, there were ten different pairwise trials to test. All trials were repeated four times to give forty total trials per cage. The test arena was cleaned with ethanol and allowed to air dry between each trial. Trials were given a cutoff time of two minutes. Each mouse received a dominance score (V_{ij}) determined by the number of trials won by mouse *i* when competing against mouse *j*. V_{ij} scores were used to calculate the hierarchy linearity of the cage based on Landau's h (Landau, 1951).

$$h = \frac{12}{N^3 - N} \sum_{i=1}^{N} \left[V_i - \left(\frac{N-1}{2} \right) \right]^2$$

Where N = the number of mice per cage and V_i is the summation of V_{ij} for each mouse *i* on its opponent mouse *j*. Scores near 1 correspond to a near complete hierarchy while scores near 0 signify the lack of a hierarchy. Each mouse's rank was also calculated by determining the number

of trials won over all trials in which he participated. These scores were used to determine the dominant and subordinate mice used for sweat and saliva sampling.

3.3.6 Data availability

All raw GC-MS and behavior calculations are available online at https://doi.org/10.1371/journal.pone.0251416 under "Supporting information."

3.3.7 Statistical analysis

3.3.7.1 Sample VOC profiles

Before formal analysis, all VOC data were visualized using a Venn diagram to summarize similar and unique compounds across sample types. R Studio (version 3.4.3) and the *VennDiagram* package were used to create the visualization.

3.3.7.2 Strain and VOC profiles

Individual nest (N = 23), sweat (N = 46), and urine (N = 42) samples were separately visualized in two dimensions using non-metric multidimensional scaling (NMDS) to examine similarity based on VOC proportions across strain. Sweat and urine data were also examined for similarity between two levels of social rank. Factor differences were tested using the Adonis test since the datasets did not meet multivariate normality. Beta dispersion assumption was checked post hoc. Since cages were run in four groups over time, the batch number was also included as a blocking factor. NMDS, Adonis test, and assumption check were run in R Studio (version 3.4.3) using *vegan*, *tidyverse*, *ggplot2*, and *mvnormtest* packages.

Additionally, since mice were sampled based on ranking in the tube test, we wanted to confirm differences in two known urinary pheromones, β-farnesene and 2-sec-butyl-thiazoline (SBT) between social rank. Both pheromones have been previously reported to vary based on social rank (Harvey et al., 1989). Proportions of β-farnesene and SBT were analyzed using restricted maximum likelihood mixed models with strain, rank, and their interaction as fixed effects, and batch number as a random factor. Cage nested within strain was also included as a

random factor to account for repeated sampling from the same cage. The models were run in JMP Pro (version 14.0.0), and assumptions were checked post hoc.

VOC profiles and social interactions

Cage level proportion data for each sample type (nest, sweat, urine) and social behavior were run in separate Principal Component Analyses (PCA) with values scaled to a mean of 0 and standard deviation of 1. The broken stick model (BSM) was used for principle component (PC) retention with the following exception: for the behavior PCA, BSM showed that only PC1 was significant. However, behavior PC2 explained a large portion of the variance, 29.67%, and had an eigenvalue of 1.48, therefore it was kept for further analysis. The following numbers in parentheses represent the number of retained PCs for each dataset: nest (2), sweat (1), urine (3), and behavior (2). Varimax rotation was used on the nest, sweat, and urine PCAs to maximize variable separation across PCs.

Mixed models were used to determine how nest, sweat, and urine odors affect behavior. Strain, and PCs from the nest, sweat, and urine data were used as independent variables, while PCs from the behavior data were tested separately as dependent variables. The cage average weekly nest score and Landau's H were included as covariates. Batch number was used a random factor. Non-significant variables were manually excluded from the models and those with the lowest AIC value were kept for interpretation. Since this study used two models to assess whether VOCs impact behavior, p values were adjusted using the sequential Bonferroni procedure to correct for multiple comparisons (Eichstaedt et al., 2013). All further analyses examining strain effects on VOC PCs and individual VOCs were also run as mixed models. Individual VOC models had compound specific hypotheses and therefore a multiple comparisons correction was not performed. Individual VOCs were only tested in a mixed model if their PC of origin showed strong correlation with behavior based on Pearson's r. Normality and homogeneity of variance were tested post hoc by visually examining the residual Q-Q plot and spread of the residual by predicted plots for each model(Gosselin, 2019). PCAs were run in R Studio (version 3.4.3) using *FactoMineR, factoextra,* and *tidyverse* packages. JMP Pro (version 14.0.0) was used for the mixed models and assumption check (Gosselin, 2019).

Data from one AJ nest was excluded due to flooding, making group sizes for the nest dataset unbalanced for NMDS, Adonis test, and PCA (AJ: n= 7, B6 and SJL: n = 8).

Behavior across study days

To validate historical differences in strain social behavior and explore differences in strain nesting behavior, cage level behavior proportions from each day of observation (1, 2, 7) were tested in a series of REML mixed models with strain, day and the interaction as fixed effects, and batch number and cage nested within strain as random factors (N= 72, 3 observations from 24 cages). Post hoc Tukey tests were used to assess factor level differences. Assumptions of normality and homogeneity of variance were tested by visual examination of the residual Q-Q plot using JMP Pro (version 14.0.0) and Levene's test using R Studio (version 3.4.3) respectively. A $\log_{10}$ transformation was used on social investigation data, and square root transformations were used on the mediated aggression and allo-groom data. Data for escalated aggression was extremely skewed and transformation was unsuccessful to meet model assumptions. Therefore, count data per day were calculated and analyzed using a generalized linear mixed model (GLIM) with a negative binomial distribution. Custom tests corrected for multiple comparisons were used to identify specific factor differences.

3.4 Results

Cages containing five male mice of SJL/JOrlIcoCrl (SJL), C57BL/6NCrl (B6), or A/JCr (AJ) strain were kept for one week (n=8 cages per strain; N=24 total cages). At the end of the week, samples of used nesting material were taken from each cage. Samples of sweat, saliva, and urine were also collected from each cage's dominant and subordinate mouse as determined by the tube test. All samples were analyzed using gas chromatography- mass spectrometry (GC-MS) and proportions of each sample's VOCs were evaluated. However, saliva samples were only sufficient enough for qualitative assessments. One AJ nest sample was excluded from analyses due to a flooded cage during the study (leaving N=23); two sweat samples (one B6 and one AJ) were excluded due to missing labels (leaving N=46); and six SJL mice did not urinate when stimulated (leaving N=42). See Methods for further details.

Video data from days 1, 2, and 7 were collected and analyzed for social interactions (mediated aggression, escalated aggression, social investigation, allo-grooming, and group sleep) and nesting behaviors (paw nesting and oral nesting). Full behavioral descriptions can be found in the methods. Ultimately, we calculated the proportion of time that each behavior was observed. Unless otherwise indicated, behavior proportions represent values for all three days observed. An overview of the sample size used in each analysis is provided (Appendix C, Table C.1).

3.4.1 Sample VOC profiles

To address aim 1, we identified or tentatively identified 32 compounds across all sample types (Table 3.2). Among those, 53% were found in at least 2 sample types; 6% were unique to nest samples; 22% were unique to sweat; 16% were unique to saliva; and 3% were unique to urine (Figure 3.1). Subsequent analyses excluded saliva samples due to low sample volumes (see Gas Chromatography- Mass Spectrometry Analysis in Methods). As indicated in Table 3.2, nesting material and urine samples shared many previously identified mouse urinary compounds. In turn, sweat samples showed several cyclic ketone compounds also found in the nesting material, which were not detected in urine samples.

Table 3.2. List of identified compounds across sample type in order of ascending run time.

Compound	SIC m/z	Nesting Material	Sweat	Urine	Saliva
		Rt (min)	Rt (min)	Rt (min)	Rt (min)
acetic acid[1]	60	3.44	3.44	3.44	3.44
5,5-dimethyl-2-ethyl-4,5-dihydrofuran[2, #]	126	5.56		5.56	
2-furanmethanol[1]	98	7.83	7.83	7.83	8.05
Z-5,5-dimethyl-2-ethylidenetetrahydrofuran[2, #]	126	7.98		7.98	
E-5,5-dimethyl-2-ethylidenetetrahydrofuran[2, #]	126	9.38		9.38	
*1,2-cyclopentadione	98	10.85		10.82	11.08
6-hydroxy-6-methyl-3-heptanone[2, #]	127	11.47		11.47	
3-methyl-(2(H)-furanone[1]	98		13.03		
**a ketone (m/z 55, 84, 114)	114				14.87
2-isopropylthiazoline[2, #]	114	15.57		15.57	
methylcyclopentenolone[1]	112	16.1	16.1		
limonene[1]	68				16.21
*2-hydroxybenzaldehyde	122	17.17			
dehydrobrevicomin[2, #]	111	17.6		17.6	
3,4-dimethyl-1,2-cyclopentadione[1]	111		19.15		
o-toluidine[1]	107		19.86	19.18	
2-sec-butylthiazoline[2, #]	115	21.2		21.2	21.16
nonanal[1]	98				21.52
3-ethyl-2-hydroxy-2-cyclopenten-1-one (ethylcyclopentenolone)[1]	126		22.02		
*n-formylmorpholine	115		22.66		
*5-ethylthiazolidine	117	26.64	24.65	24.64	24.75
3,5-diethyl- 2-hydroxycyclopent-2-en-1-one[2]	126		29.69		
indole[1]	117		33.82		
**m/z 126 compound	111				34.28
**m/z 152 compound	70	41.38			
geranylacetone[1]	69	43.93	43.92		43.92
β-farnesene[2, #]	69	44.26		44.26	
α-farnesene[2, #]	69			50.65	
methyldihydrojasmonate[1]	69				55.54
hexadecanol[1]	55		67.92	67.92	
hexadecanoic acid[1]	60	72.12	72.12	72.12	
octadecanoic acid[1]	60	76.41	76.41	76.41	

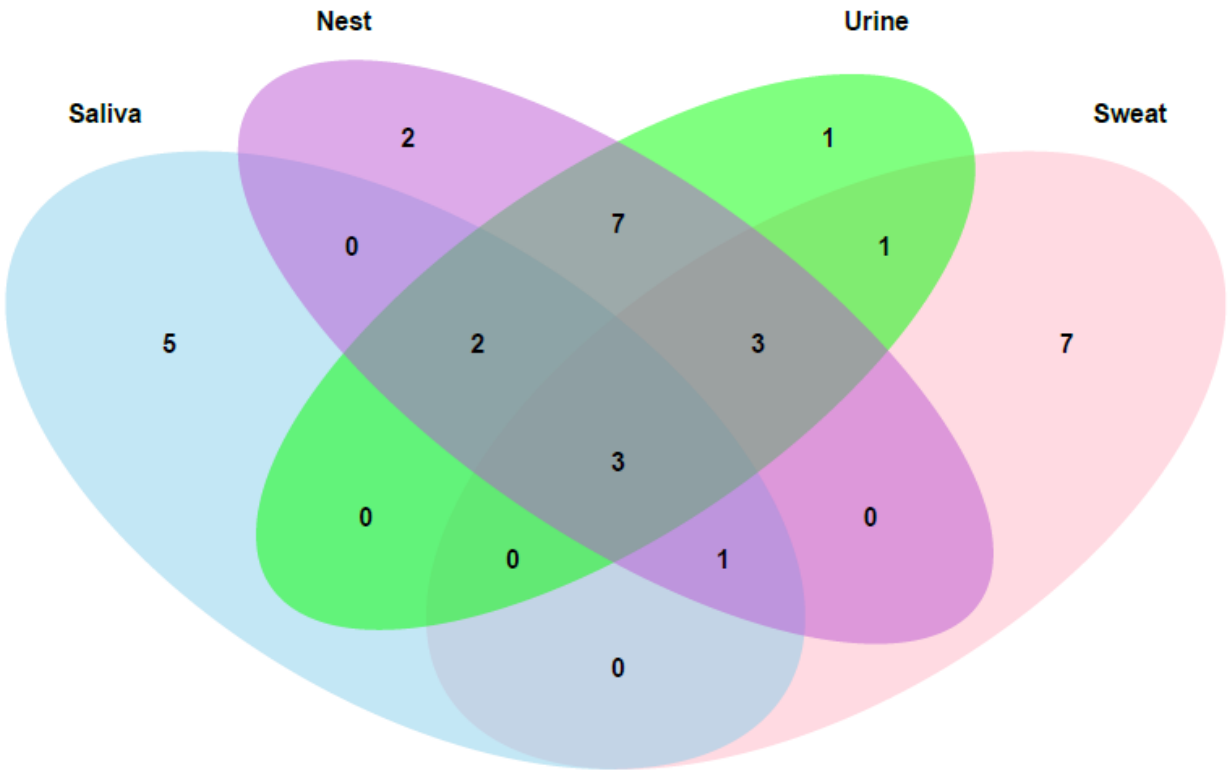

Figure 3.1. Venn diagram of the number of volatile organic compounds detected in each sample type.

3.4.2 Strain and VOC profiles

Visual examination of sample profiles using non-metric multidimensional scaling (NMDS) showed sample separation based on strain for nesting material, sweat, and urine VOC profiles (Figure 3.2). Social (dominant versus subordinate) ranking effects were not distinguishable in the sweat and urine samples (Figure 3.2B + 3.2C).

Analyses using the Adonis test showed that strain significantly impacted VOC proportions in nesting material, sweat, and urine (p values<0.01; Appendix C, Table C.2). Social rank did not significantly influence VOC proportions in sweat or urine (p values> 0.05; Appendix C, Table C.2).

Additionally, proportions of two urinary pheromones (β-farnesene and 2-sec-butyl-thiazoline (SBT)) were analyzed based on strain and social rank. Higher quantities of both pheromones have been reported in dominant compared to subordinate urine (Harvey et al., 1989), so we used Restricted Maximum Likelihood mixed models to confirm rankings from the tube test. Here, proportions of neither of these pheromones differed by social rank (p values > 0.05; Appendix C, Table C.3), although AJ and B6 mice produced more SBT than SJL mice (Tukey: p< 0.05). Even though we assigned a "dominant" or "subordinate" label to the sampled mice, dominance rank was based solely on the tube test, and may not reflect in-cage behavior. Since dominance rank was not a significant source of variation between sweat and urine samples, the two samples from each cage were averaged together to give single cage mean values for subsequent analyses. However, in cases where only one sample was collected from a cage (see Methods for additional information), that sample alone was used for analysis (Appendix C, Table C.1).

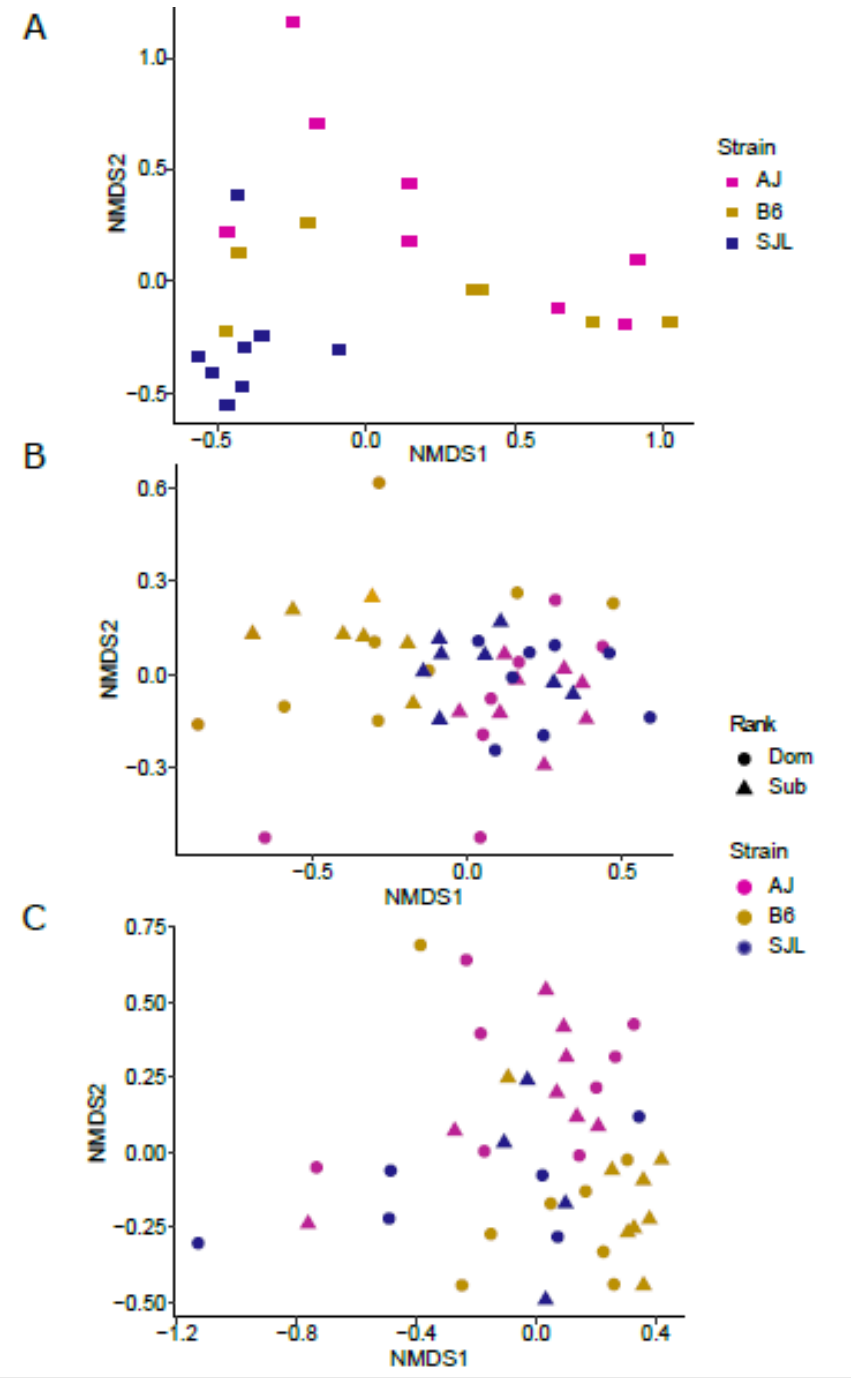

Figure 3.2. Volatile organic compound profiles of (A) nesting material, (B) plantar sweat, and (C) urine showed strain specific patterns. Non-metric multidimensional scaling using Bray-Curtis dissimilarity matrices for (A) used nesting material (stress = 0.095, N=23), (B) plantar sweat (stress = 0.113, N=46), and (C) urine (stress = 0.162, N=42) showed sample separation corresponding to strain. Multivariate analyses using the Adonis test showed a significant difference in profiles between strains for all sample types: nest, p=0.006; sweat, p=0.001; and urine, p=0.001. In contrast, (B) sweat and (C) urine samples did not show separation based on social ranking and Adonis tests did not show significant profile differences

3.4.3 VOC profiles and social interactions

Separate principal component analyses (PCA) were run for each sample type and the behavior data. Strong PC loadings (absolute value ≥ 0.300) considered important are indicated in gray highlighted cells and bold black numbers in Table 3.3. Influential PCA components from each data set were kept for mixed models. Aggressive behaviors and social investigation had high positive loadings on PC1 while allo-grooming had a strong negative loading. On PC2, group sleep and allo-grooming behaviors had high loadings. Loading values for all influential sweat, nest, urine, and behavior PCs are listed in Table 3.3.

To address aim 2, two mixed models were run and p values were corrected using the sequential Bonferroni procedure (Eichstaedt et al., 2013): one for each behavior PC. All significant VOC PCs and strain were included as independent variables, as well as two covariate measures: average cage nest complexity score, and dominance linearity as measured by Landau's H. Please refer to the methods for further description. All non-significant fixed effects were dropped from the final models for parsimony.

The only significant effect on behavior PC1 was strain (Table 3.4). Tukey tests showed that SJL mice had the highest scores, followed by AJ, and then B6 mice (Table 3.4). Strain also significantly impacted behavior PC2 (Table 3.4): AJ mice had lower scores than B6 and SJL mice (Table 3.4). Urine PC3 had a positive effect on behavior PC2 ($F_{1,18.55}$= 5.73, p_{adj}=0.027; η^2= 0.278). Compounds with high loading on urine PC3 were β-farnesene, 5-ethylthiazolidine (tentative identification), hexadecanol, and 2-isopropylthiazole related to the inter-male aggression promoting pheromone SBT (Novotny et al., 1985) (Table 3.3).

Since the behavior PCs were primarily impacted by strain, historical social behavior patterns were confirmed in the featured strains to determine if they vary across study days. As expected, AJ, B6, and SJL mice displayed different levels of each social behavior: escalated aggression; mediated aggression; social investigation; allo-grooming; and group sleep (Table 3.5). Study day only impacted escalated aggression while the day*strain interaction was not significant for any behavior category.

After correcting for multiple comparisons, post-hoc custom tests showed that there was less escalated aggression on day 7 than 1 (GLIM: $\chi(1) = 5.88$, p=0.015). SJL mice displayed more escalated aggression than AJ mice (GLIM: $\chi(1) = 7.95$, p<0.005), while post hoc Tukey tests showed SJL displayed the most mediated aggression (Tukey: p<0.05) and social investigation

(Tukey: $p<0.05$). B6 and AJ mice displayed similar levels of all three behaviors (p values>0.05). B6 mice displayed the highest level of allo-grooming (Tukey: $p<0.05$) while SJL and AJ mice displayed similar levels ($p>0.05$). B6 also displayed more group sleep than AJ mice (Tukey: $p<0.05$), but SJL mice were similar to both strains (p values>0.05). All strain patterns are depicted in Figure 3.3.

Table 3.3. Loading values for all principal components (PCs) retained for mixed models.

Sweat VOCs	Sweat PC1	Nest VOCs	Nest PC1	Nest PC2	Urine VOCs	Urine PC1	Urine PC2	Urine PC3	Behaviors	Behavior PC1	Behavior PC2
acetic acid	-0.2744	acetic acid	0.2644	0.2307	acetic acid	**-0.3421**	-0.1522	0.1843	Mediated aggression	**0.9248**	0.2948
hexadecanoic acid	-0.2521	hexadecanoic acid	0.2013	**-0.3177**	hexadecanoic acid	0.2033	**0.3590**	-0.0798	Escalated aggression	**0.9538**	0.1987
octadecanoic acid	-0.2592	octadecanoic acid	0.2174	**-0.3018**	octadecanoic acid	0.1254	**0.4337**	-0.0655	Allo-Groom	**-0.6467**	**0.6776**
2-furanmethanol	-0.2429	2-furanmethanol	0.2499	-0.2432	2-furanmethanol	**-0.3654**	-0.1987	0.1066	Social Invest.	**0.9341**	0.2073
5-ethyl thiazolidine*	0.1102	5-ethyl thiazolidine*	-0.0171	-0.2847	5-ethyl thiazolidine*	-0.0252	0.1604	**-0.5360**	Group Sleep	-0.2355	**0.9246**
hexadecanol	-0.1217	hexadecanol	0.1919	-0.2664	hexadecanol	0.1329	0.0381	**0.3545**			
geranylacetone	-0.2798	geranylacetone	**0.3334**	0.1998	α-farnesene	0.0874	0.1535	0.2034			
3-methyl-2(H)-furanone	0.2838	β-farnesene	-0.0437	-0.0627	β-farnesene	0.0144	0.1638	**0.3407**			
o-toluidine	0.2145	1,2-cyclopentadione	**0.3004**	-0.0751	1 2-cyclopentadione	**-0.4024**	-0.1653	0.0867			
3,4-dimethyl-1,2-cyclo pentanedione	**0.3692**	dehydrobrevicomin	0.0732	**0.3475**	dehydrobrevicomin	0.0034	**0.3647**	0.2565			
N-formyl morpholine*	-0.1708	2-isopropylthiazole	0.1520	**0.3934**	2-isopropylthiazole	-0.0710	0.2397	**0.3703**			
indole	0.2068	2-sec-butyl thiazoline	0.1465	**0.3691**	2-sec-butyl thiazoline	-0.0745	-0.1605	0.2979			
ethylcyclo pentenolone	**0.3266**	5,5-dimethyl-2-ethyl-4,5-dihydrofuran	-0.2729	0.1583	5 5-dimethyl-2-ethyl-4 5-dihydrofuran	**0.3689**	-0.2395	0.1111			
3,5-diethyl- 2-hydroxycyclop ent-2-en-1-one	**0.3362**	Z-5,5-dimethyl-2-ethylidene tetrahydrofuran	**-0.3078**	-0.0796	Z-5 5-dimethyl-2-ethylidene tetrahydrofuran	**0.3652**	-0.1590	0.1483			
methylcyclo pentenolone	0.2737	E-5,5-dimethyl-2-ethylidene tetrahydrofuran	**-0.3228**	-0.0493	E-5 5-dimethyl-2-ethylidene tetrahydrofuran	**0.3407**	-0.1464	0.1601			
		6-hydroxy-6-methyl-3-heptanone	-0.1421	0.2035	6-hydroxy-6-methyl-3-heptanone	**0.3013**	**-0.3145**	-0.0300			
		MW 152 compound**	**0.3653**	0.0016	o-toluidine	0.1308	-0.3097	-0.1152			
		2-hydroxy benzaldehyde	0.2490	0.0823							

Table 3.4. Strain patterns on VOC profile and behavior based on mixed models.

Dependent Variable	Strain Main Effect	Tukey Differences
Behavior PC1	$F_{2,18}$= 256.62, **p_{adj}<0.001**	SJL > AJ > B6
Behavior PC2	$F_{2,17.06}$= 23.75, **p_{adj}<0.001**	(B6 = SJL) > AJ
Nest PC1	$F_{2,18.14}$= 6.10, **p_{adj}=0.036**	(AJ = B6) > SJL
Nest PC2	$F_{2,17.52}$= 0.85, p_{adj}=0.886	---
Sweat PC1	$F_{2,18}$= 19.61, **p_{adj}<0.001**	B6 > (AJ = SJL)
Urine PC1	$F_{2,18}$= 7.97, **p_{adj}=0.015**	B6 > AJ; SJL = B6; SJL = AJ
Urine PC2	$F_{2,18}$= 20.05, **p_{adj}<0.001**	SJL > AJ > B6
Urine PC3	$F_{2,18}$= 0.02, p_{adj}=0.983	---
Landau's H	$F_{2,18}$= 1.49, p_{adj}=0.753	---
Nest Complexity Score	$F_{2,18}$= 148.74, **p_{adj}<0.001**	AJ > B6 > SJL

p_{adj} represents adjusted p values based on the Bonferroni sequential method. Significant p values for main effects are listed in **bold**. Specific differences between mouse strains was determined using post-hoc Tukey tests ($p<0.05$). '---' indicates that a post-hoc test was not conducted due to the main effect not being significant.

Table 3.5. Effects of strain and day on behaviors of interest based on mixed models.

	Strain	Strain comparison	Day	Day comparison
Escalated Aggression [a]	$\chi(2) = 8.06$, **p=0.018**	SJL > AJ; B6 = SJL; B6 = AJ	$\chi(2) = 7.31$, **p=0.026**	Day 1 > Day 7; Day 2 = Day 1; Day 2 = Day 7
Mediated Aggression [b]	$F_{2,59.98}$= 26.09, **p<0.001**	SJL > (AJ = B6)	$F_{2,42}$= 0.73, p=0.486	---
Social Investigation [b]	$F_{2,50.86}$= 19.71, **p<0.001**	SJL > (AJ = B6)	$F_{2,42}$= 0.03, p=0.973	---
Allo-grooming [b]	$F_{2,52.43}$= 43.91, **p<0.001**	B6 > (AJ = SJL)	$F_{2,42}$= 0.59, p=0.557	---
Group Sleep [b]	$F_{2,57.85}$= 5.56, **p=0.006**	B6 > AJ; SJL = B6; SJL = AJ	$F_{2,42}$= 1.60, p=0.213	---
Nesting- paw [b]	$F_{2,55.26}$= 3.21, **p=0.048**	AJ > B6; SJL = B6; SJL = AJ	$F_{2,42}$= 2.01, p=0.147	---
Nesting- mouth [b]	$F_{2,39.31}$= 4.48, **p=0.018**	AJ > SJL; B6 = AJ; B6 = SJL	$F_{2,42}$= 0.41, p=0.663	---

[a] analyzed with generalized linear mixed model and Bonferroni corrected contrasts ($p<0.017$); [b] analyzed with general linear mixed model and post-hoc Tukey test ($p<0.05$); Significant p values are listed in **bold**. '---' indicates that a post-hoc test was not conducted due the insignificant main effect. The strain*day interaction was tested and not significant in any model.

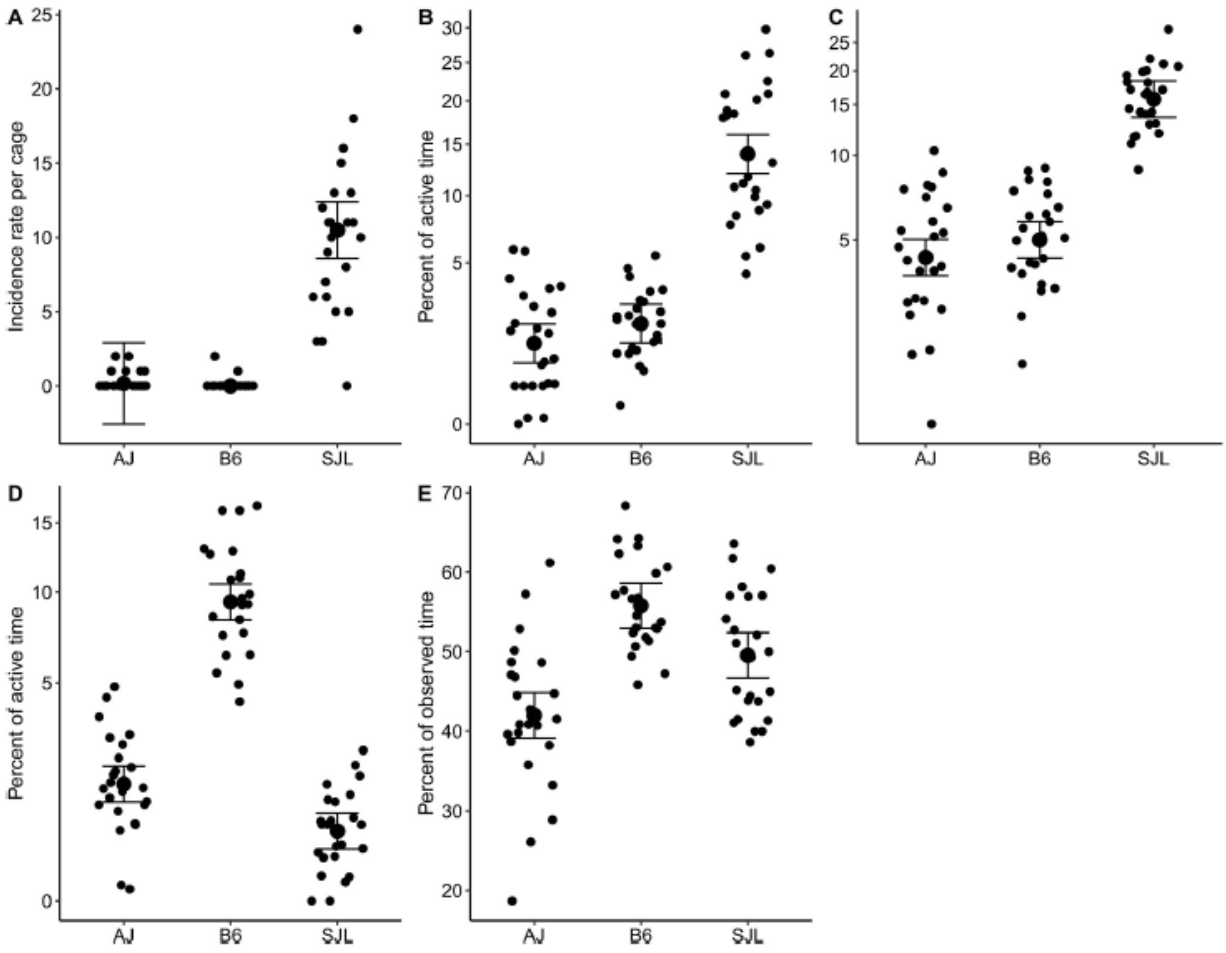

Figure 3.3. Aggressive and affiliative behavior patterns varied according to strain. SJL mice had (A) the highest rate of escalated aggressive behaviors (occurrences per day; $p=0.018$). They also spent the highest percent of active time performing (B) mediated aggression ($p<0.001$) and (C) social investigation ($p<0.001$) behaviors. B6 mice spent the highest percent of active time (D) allo-grooming ($p<0.001$) and highest percent of observed time in (E) group sleep ($p=0.006$). All data are presented as strain LSM +/- SE with the scatter of the individual data points' residual differences from the LSM (N = 72, 3 observations from 24 cages). Y axes are shown on a square root back transformed scale in B and D, and on a $\log_{10}$ back transformed scale in C.

Because strain had such an overwhelming effect on behavior, each of the tested VOC PCs and covariates were run in a mixed model to determine the impact of strain. VOC and genetic effects can both influence behavior, either independently or in conjunction with one another, which is why mixed models were used to examine whether strain influenced VOC PCs. Overall, strain had a significant effect on sweat PC1, nest PC1, urine PC1, and urine PC2 (Table 3.4). Post hoc Tukey tests showed SJL cages had lower nest PC1 scores and higher urine PC2 scores than B6 and AJ. B6 cages had higher scores on sweat PC1 and lower scores on urine PC2 than SJL and AJ. They also had higher scores on urine PC1 than AJ mice. AJ cages had lower scores on urine PC1 than B6, similar scores to B6 on nest PC1, and similar scores to SJL on sweat PC1. On urine PC2, AJ mice had higher scores than B6 and lower scores than SJL (Table 3.4). Strain did not affect urine PC3 or nest PC2. In terms of covariate measures, strain significantly impacted average nest complexity score, but did not impact Landau's H. (Table 3.4). AJ mice built the most complex nests, followed by B6, and SJL (Tukey: $p<0.05$).

In this study, the strain pattern of sweat PC1 matches that of allo-grooming, while patterns of nest PC1 and urine PC2 match that of aggression. Therefore, VOCs with high loading on these PCs were chosen for further analysis. Scores on sweat PC1 were positively correlated with both allo-grooming (Pearson's $r= 0.66$, 95% CI: 0.35-0.84, $p<0.001$) and group sleep (Pearson's $r= 0.52$, 95% CI: 0.15-0.76, $p=0.011$). The following compounds had high positive loading on sweat PC1: 3,4-dimethyl-1,2-cyclopentanedione, ethylcyclopentenolone, and a newly identified compound, 3,5-diethyl- 2-hydroxycyclopent-2-en-1-one (Table 3.3; Figure 3.4C-E; Appendix C, Figure C.1). A verified structure (Figure 3.4E) is related to ethylcyclopentenolone (Figure 3.4D). Of these, 3,4-dimethyl-1,2-cyclopentanedione and 3,5-diethyl- 2-hydroxycyclopent-2-en-1-one varied by strain and were correlated with allo-grooming; 3,5-diethyl- 2-hydroxycyclopent-2-en-1-one only was correlated with group sleep (Table 3.6).

Scores on nest PC1 were negatively correlated with both escalated (Pearson's $r= -0.56$, 95% CI: -0.79- -0.20, $p=0.005$) and mediated aggression (Pearson's $r= -0.49$, 95% CI: -0.75- -0.10, $p=0.018$). Compounds with high positive loading on nest PC1 were geranylacetone, 1,2-cyclopentadione, and another unknown compound (Table 3.3; Figure 3.5). We will refer to this unknown compound as MW 152 based on its assumed molecular weight. Currently the identity of MW 152 has not been determined. Two dehydration products of 6-hydroxy-6-methyl-3-heptanone had high negative loading on nest PC1 (Table 3.3; Figure 3.5). Since positively loading compounds

would be associated with less aggression, only they were analyzed. Geranylacetone was negatively correlated with both mediated and escalated aggression and varied by strain (Table 3.6). MW 152 was negatively correlated with escalated aggression and was not impacted by strain (Table 3.6).

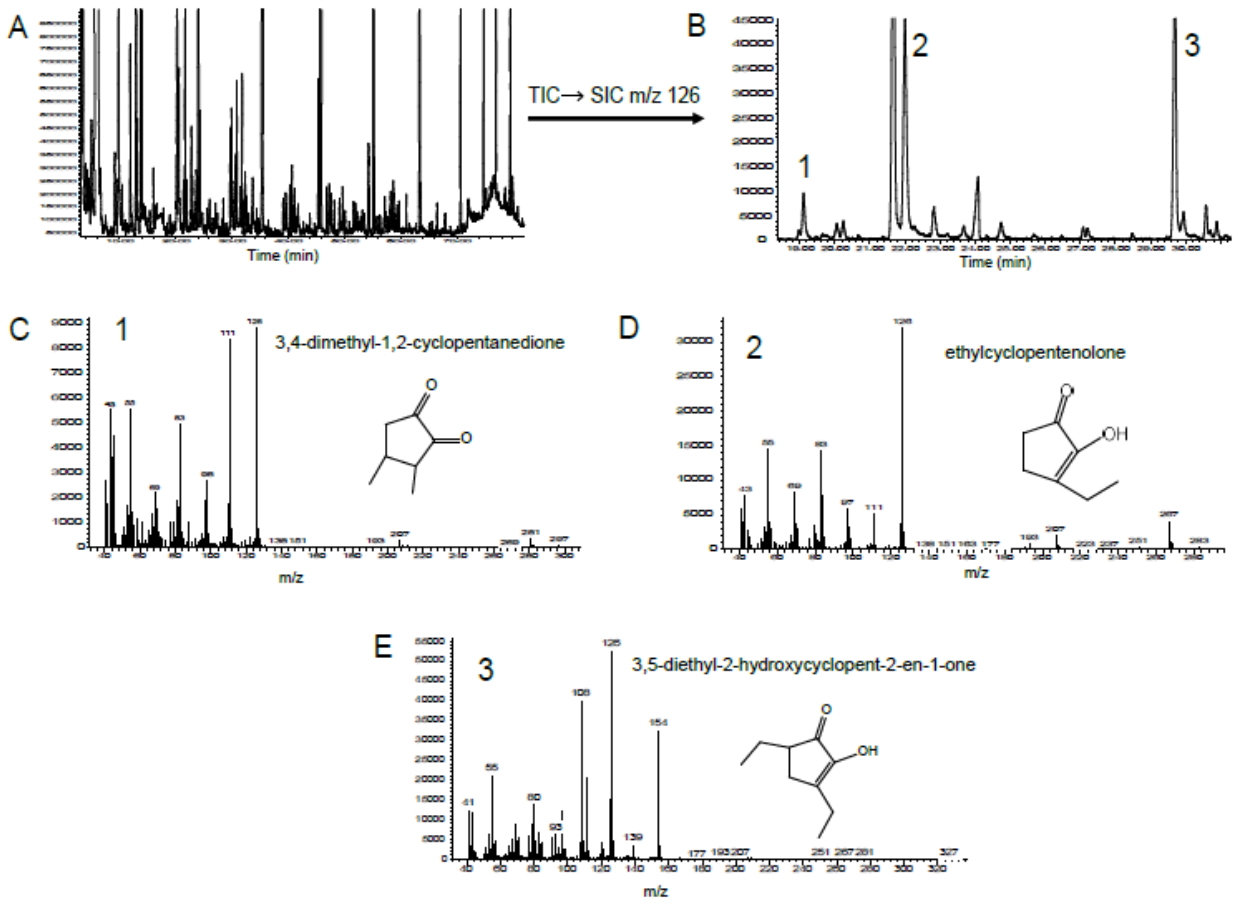

Figure 3.4. High loading compounds on sweat PC1 and their mass spectra (EI 70 eV). (A) Total ion chromatogram (TIC); (B) Post-run extracted m/z 126 single ion current chromatogram (SIC); (C) Compound 1, 3,4-dimethyl-1,2-cyclopentadione from SIC at retention time 16.1 min; (D) Compound 2, ethylcyclopentenolone from SIC at retention time 22.02 min; (E) 3,5-diethyl- 2-hydroxycyclopent-2-en-1-one from SIC at retention time 29.69 min.

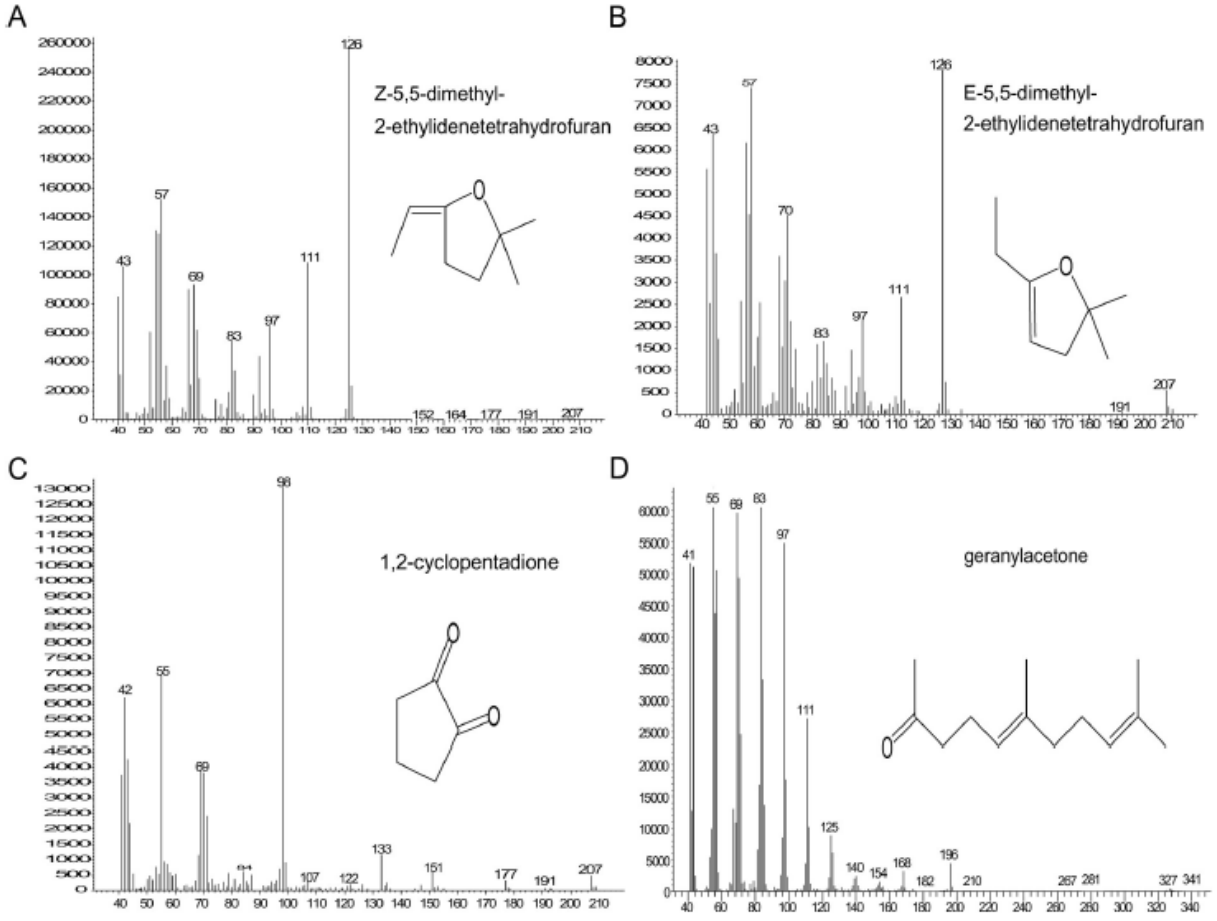

Figure 3.5. High loading compounds on nest PC1 and their mass spectra (EI 70 eV). (A) Z-5,5-dimethyl-2-ethylidenetetrahydrofuran at retention time 7.98 min; (B) E-5,5-dimethyl-2-ethylidenetetrahydrofuran at retention time 9.38 min; (C) 1,2-cyclopentadione at retention time 10.85 min; (D) geranylacetone at retention time 43.93 min

Scores on urine PC2 were positively correlated with both escalated (Pearson's r= 0.63, 95% CI: 0.31-0.82, p<0.001) and mediated aggression (Pearson's r= 0.59, 95% CI: 0.24-0.80, p=0.002). Compounds with high positive loading on urine PC2 were hexadecenoic acid, octadecanoic acid, and aggression-related dehydrobrevicomin (Touhara and Vosshall, 2009), while testosterone dependent 6-hydroxy-6-methyl-3-heptanone (Novotny et al., 1984) had high negative loading (Table 3.3). Negatively loading compounds would be associated with less aggression, so only 6-hydroxy-6-methyl-3-heptanone was further analyzed. It was correlated with allo-grooming and varied by strain (Table 3.6).

Table 3.6. Relationship between behavior, strain, and high loading VOCs from sweat PC1, nest PC1, and urine PC2.

VOC	Odor PC	Behavior correlation	Strain	Strain comparison
3,4-dimethyl-1,2-cyclopentanedione	Sweat PC1	**Allo-grooming**: Pearson's r= 0.58, 95% CI: 0.23- 0.80, **p=0.003**	$F_{2,18}$=14.66 **P<0.001**	B6 > (SJL = AJ)
ethylcyclopentenolone	Sweat PC1	NS	$F_{2,18}$=1.07 P=0.364	---
3,5-diethyl- 2-hydroxycyclopent-2-en-1-one	Sweat PC1	**Allo-grooming**: Pearson's r= 0.62, 95% CI: 0.29- 0.82, **p=0.001** **Group sleep**: Pearson's r= 0.54, 95% CI: 0.17- 0.77, **p=0.007**	$F_{2,18}$=8.27 **P=0.003**	B6 > (SJL = AJ)
geranylacetone	Nest PC1	**Escalated aggression**: Pearson's r= -0.52, 95% CI: -0.77- -0.13, **p=0.011** **Mediated aggression**: Pearson's r= -0.43, 95% CI: -0.72- -0.02, **p=0.04**	$F_{2,17}$=4.85 **P=0.022**	SJL < AJ; B6 = AJ; B6 = SJL
1,2- cyclopentadione	Nest PC1	NS	$F_{2,17}$=0.87 P=0.435	---
MW 152	Nest PC1	**Escalated aggression**: Pearson's r= -0.41, 95% CI: -0.71- -0.001, **p=0.05**	$F_{2,17}$=2.76 P=0.091	---
6-hydroxy-6-methyl-3-heptanone	Urine PC2	**Allo-grooming**: Pearson's r= 0.60, 95% CI: 0.25- 0.81, **p=0.002**	$F_{2,18}$=19.48 **P<0.001**	B6 > (SJL = AJ)

Significant p values are listed in **bold**. 'NS' indicates no significant correlations detected. '---' indicates that a post-hoc test was not conducted due the insignificant main effect.

Since urine PC3 significantly impacted behavior PC2, it was also compared to each behavior. Urine PC3 did not correlate with any individual behaviors or show strong strain variation (Table 3.4), so high loading compounds were not examined further.

In summary, SJL mice displayed substantially more aggressive behavior and social investigation. They also had the highest scores on urine PC2 and the lowest on nest PC1. B6 mice displayed the most allo-grooming, had the highest scores on sweat PC1, and the lowest on urine PC2. AJ mice displayed minimal social behavior, performed the most nesting behavior, and had the highest nest complexity scores (Figure 3.6).

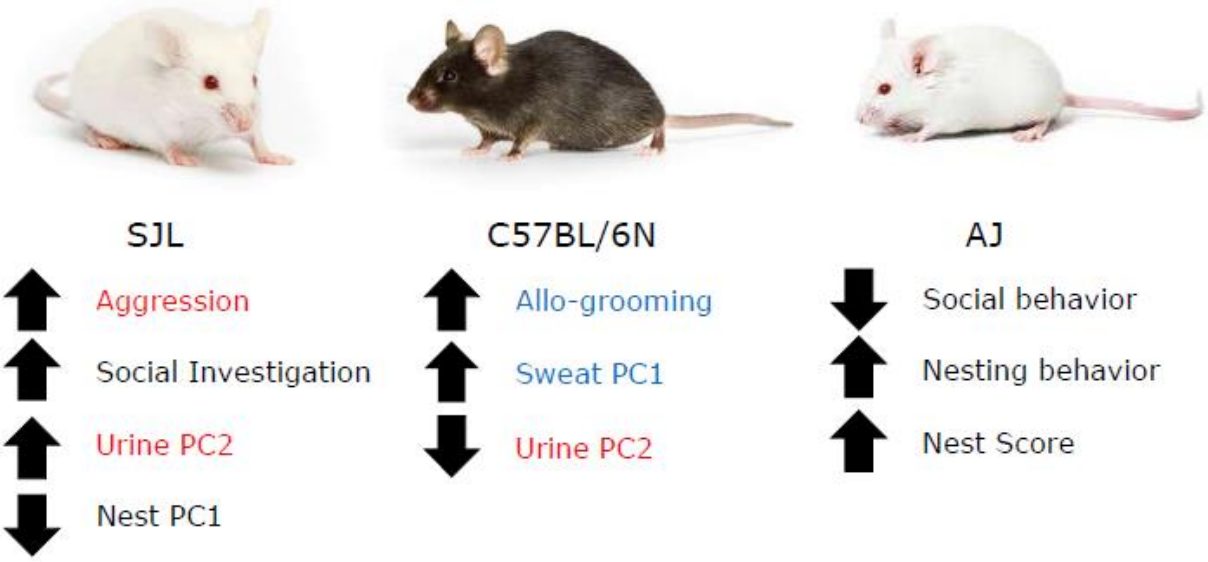

Figure 3.6. Summary diagram of observed strain patterns. Variables that were associated with aggressive behavior are listed in red, while those associated with an affiliative behavior are listed in blue.

3.4.4 Strain and nesting behavior

A side objective of this study was to explore how nest manipulation behaviors varied across the three strains, since the main focus examined secreted chemical contents on nesting material resulting from manipulation with the paws or mouth. Separate mixed models were run for manipulation performed with the paws and mouth. Behaviors performed with the paws were expected to influence compounds originating in the sweat while behaviors performed with the mouth would have more impact on compounds from the saliva. Nesting done with the paws and mouth were significantly influenced by strain (Table 3.5). Post hoc Tukey tests showed that AJ mice performed more nesting with their paws than B6 (Tukey: $p<0.05$), while SJL were similar to

both (p values>0.05). AJ mice performed more nesting with their mouth than SJL (Tukey: $p<0.05$), while B6 were similar to both (p values>0.05).

3.5 Discussion

To our knowledge, this experiment is the first to report the VOC profiles of used nesting material and foot plantar gland sweat in male laboratory mice (aim 1). It is also the first to examine the relationship between these profiles and social behavior (aim 2). It has been shown that preserving used nesting material can reduce aggression at cage change (Van Loo et al., 2000), but the theory that nesting material holds aggression reducing plantar sweat has remained speculation until now.

3.5.1 Observed behavior

The behavior PCA, PC1 showed that mediated aggression, escalated aggression, and social investigation were strongly correlated across all cages. In contrast, allo-grooming was negatively associated with the latter three behaviors on PC1, and positively associated with group sleep behavior on PC2. However, all of these patterns were strongly explained by strain. On behavior PC1 (high aggression and low allo-grooming), SJL had the highest scores followed by AJ and then B6. This reflects the greater amount of aggression and social investigation performed by SJL and the greater amount of allo-grooming performed by B6. On the other hand, behavior PC2 scores (high allo-grooming and group sleep) reflect the higher amount of group sleep performed by B6 and SJL mice than AJs.

Several of these strain patterns were unexpected. First, SJL mice are known for excessive inter-male aggression (Festing, 1998), but they also displayed the most social investigation behavior. Our coding scheme was not detailed enough to make conclusions about the direct behavioral sequence, but anecdotally, social investigatory sniffing tended to precede aggressive interactions. Initially the ethogram did not include a separate category for social investigation, but it was added after observing this pattern after the first few cages. This calls into question the underlying motivations of sniffing behavior, as it is traditionally considered to be neutral or exploratory (Grant and Mackintosh, 1963; “Mouse Ethogram,” n.d.; van Abeelen, 1963). However, these data make the actor mouse’s intentions less clear.

Second, B6 males are frequently the subject of caretaker complaints about aggression. Here they displayed minimal aggression which is consistent with previous work (Bisazza, 1981; Lidster et al., 2019), but we anticipated conflict in some cages in order to demonstrate a more linear relationship between VOCs and observed aggression. Thirdly, AJ cages displayed minimal social interactions, aside from group sleep. They are known for minimal levels of inter-male aggression (Festing, 1998), so we mistakenly presumed that this would equate to higher rates of affiliative behavior. Generally, aggressive and affiliative behaviors are performed more by species that are sociable, like mice (Crowcroft, 1966). However, AJ have previously demonstrated low sociability to stranger mice (Moy et al., 2007, 2004), so these data extend this pattern to behavior towards familiar cage mates.

We purposefully designed this experiment to incorporate multiple inbred mouse strains in order to ensure that a wide range of specific behaviors were observed. However, we did not expect to find such limited variation within these strains. Thus, strain unfortunately acts as a confounding factor for subsequent interpretations.

3.5.2 VOC patterns that match behavior

Overall, we found that several VOCs in urine, sweat, and nesting material aligned with strain specific patterns of social behavior in the home cage. While VOCs did not directly account for a significant amount of variation in aggressive behavior, it is possible that they may be one of the many factors that contribute to inherent strain differences in behavior. Along with relatively high aggression levels, SJL mice displayed low scores on nest PC1, and high scores on urine PC2. Scores on each of these respective PCs were negatively and positively correlated with aggression. Therefore, VOCs with a positive loading on nest PC1 and a negative loading on urine PC2 showed potential for an aggression appeasement role.

Geranylacetone was the only VOC from the nest to be both negatively correlated with aggression and have a strain specific pattern. It was produced less in SJL mice than AJ, but quantities in B6 were similar to both other strains. It was also present in sweat and saliva samples and has previously been detected in hamster ventral glands (Rendon et al., 2016). This gland is typically used for territory marking (Wynne-Edwards et al., 1992) and there is some evidence that secretions are capable of changing in response to individual social interactions (Rendon et al.,

2016). Perhaps proportions from the nest samples related to aggression due to a dilution effect from being in the environment. Odor signals are often effective at small concentrations, so the values seen here from pure body fluids may be too high to relate to behavior. Additionally, quantities of geranylacetone showed the same strain pattern as nest complexity score. Scores were lowest in SJL, which supports previous research showing that nest score decreases with the number of wounded mice in a cage (Gaskill et al., 2013). As mice engage in more aggressive interactions that include rapid fighting or chasing, any existing nest structure is likely to be destroyed during escape attempts. As stress and pain levels rise in the cage, motivation to restructure or maintain a complex nest decreases (Gaskill et al., 2013).

MW 152 was negatively correlated with escalated aggression and was the only compound not detected in any of this study's body fluid samples. Furthermore, it was not present in control (unused) nest samples, so it is possible that it originated from another body gland, fur oils, or fecal residues. Although precautions were taken to minimize contamination with fecal residue (e.g., cleaning the surface of the foot and the anesthesia chamber), it is possible that fecal odors could have contaminated the samples and future work could examine how the fecal VOC profile may impact behavior. At this time, a verified structure for MW 152 has not been determined.

The only VOC with a high negative loading on urine PC2 was 6-hydroxy-6-methyl-3-heptanone, a MUP ligand that accelerates puberty in female mice (Novotny et al., 1999). Although it did not directly relate to aggression, it was positively correlated with allo-grooming and was produced more by B6 mice than SJL and AJ. This result was unexpected since male mouse pheromones from urine have been shown to promote aggression between males (Novotny et al., 1990, 1985). However, to our knowledge, 6-hydroxy-6-methyl-3-heptanone has not been directly tested for effects in males. Based on this data, it may have a role promoting affiliative behavior.

Although this study aimed to find aggression reducing compounds, the relationship between sweat and social behavior was central to the study hypothesis. B6 mice had the highest scores on sweat PC1 and displayed the most allo-grooming. Of the VOCs with a high positive loading, 3,4-dimethyl-1,2-cyclopentanedione and 3,5-diethyl- 2-hydroxycyclopent-2-en-1-one both were correlated with allo-grooming and were produced in higher quantities by B6 mice than AJ and SJL. 3,5-diethyl- 2-hydroxycyclopent-2-en-1-one was also correlated with group sleep. To the best of our knowledge, 3,4-dimethyl-1,2-cyclopentanedione does not have a known behavioral role, but shows potential for improving mouse welfare. It would be a worthy candidate for future

behavioral testing to explore its potential role in mouse communication, along with the newly discovered 3,5-diethyl- 2-hydroxycyclopent-2-en-1-one . To our knowledge, these kinds of cyclopentanone derivatives are unique to mouse plantar sweat and, based on our data, may play a role in promoting affiliative behaviors.

3.5.3 Dominance hierarchy

Surprisingly, dominance linearity in the tube test as measured by Landau's H did not account for significant differences in behavior. This result is the opposite of what we had expected. A previous study showed that increasing values of Landau's H correlated with lower levels of aggression, suggesting that certainty in social rank reduces escalated interactions (Howerton et al., 2008). One main difference between that study and this was that the former used outbred CD-1 mice, while inbred strains were used here. This may reflect a strain impact on the relationship between dominance linearity in the tube test and aggression. Additionally, the previous study measured aggression and linearity during multiple time periods and across changes in cage enrichment. Our study focused on a one-week time period and kept housing conditions stable. Even though mice were acclimated to the arena before testing, it has been argued that there is a learned component to tube testing, such that more than one tube testing session is required for mice to display valid rankings outside the home cage (Wang et al., 2014). However, a previous assessment of stable male groups found the tube test produced inconsistent rankings over 3 weeks' time, with the most stable relationships occurring between the second and third trials (Varholick et al., 2018). This finding was published while our experiment was in progress; consequently, the approach used here does not take these new findings into consideration. It was also suggested that competitive learning in the tube test may be specific to that arena and not reflect home cage behavior (Varholick et al., 2018). Considering both the contrasting relationship between dominance linearity and aggression, and the lack of variation in β-farnesene and SBT between dominant and subordinate urine, it is likely that the tube test, at least as it was carried out here, may not be a valid indicator of individual in-cage social rank. That being said, the lack of a relationship between rank stability in the tube arena and aggression in the cage may still be meaningful. Further research will be valuable in explaining differences between tube test social rank and in cage social rank.

3.5.4 Limitations and future research

In this study, we were concerned about obtaining a sufficient quantity of sweat for analysis and utilized pilocarpine injections to increase sample volume. We do acknowledge that using pilocarpine to induce plantar sweat secretion may have unknown effects on VOC ratios. Pilocarpine functions by stimulating M3 muscarinic receptors on exocrine glands, such as the sweat glands (Landis, 1999). Currently, there is little evidence to determine how the increased gland activity impacts VOC content, but it is possible the compounds were diluted in the larger sample volume. Work in humans shows that sweat induced by pilocarpine is generally similar in content to sweat induced by exercise, although the latter contains more compounds indicative of a more demanding metabolic state (Delgado-Povedano et al., 2018). However, mice do not produce sweat to thermoregulate, and to the best of our knowledge, there are no direct VOC comparisons of fluids collected without stimulation versus pilocarpine. Additionally, individual variation in responses to the pilocarpine treatment could have impacted the data. Pilocarpine is a common treatment for dry mouth in humans, but efficacy can depend on the individual (Fox et al., 1991). At this time, factors that impact pilocarpine success have not been identified, and it was not possible to quantify the volume of collected sweat based on the sampling method.

A second limitation worth noting is that this study only focused on VOC profiles. It is possible that protein signals could have impacted these data. Urinary MUP20 (“darcin”) in particular is a pheromone that promotes aggression between males, but also is necessary for social learning to occur (Chamero et al., 2007; Roberts et al., 2010). Darcin is expressed more in mice of the C57 lineage (Kwak et al., 2012), so it is possible that it caused B6 mice to become familiar with cage mates more quickly than other strains and as a result perform more affiliative behavior. That being said, production itself cannot predict aggression since AJ and SJL mice both produce low levels of darcin (Cheetham et al., 2009), but more complex compound interactions have yet to be explored. This study also did not address the effect on behavior of individual differences in odor perception. Many odor signals, especially pheromones, are detected by the vomeronasal organ (VNO) (Bradbury and Vehrencamp, 2011). Gene expression in the VNO, particularly those encoding chemoreceptors, show great variation between strains and could be a major contributor to variability in behavior (Duyck et al., 2017). While strain specific expression was the focus of this study, we cannot assume that sensitivity follows the same pattern. For example, even though

darcin is produced more by the C57 line, BALB/c males (Castle lineage) are still reactive and display the expected scent marking response when exposed to it (Kaur et al., 2014).

Another point of consideration in this study was the amount of time mice in each cage spent performing nesting related behaviors, as this is likely to impact the relative amount of VOC deposits in the nest. AJ mice performed the most nesting done with the paws and mouth, but their scores on nest PC1 were similar to those of B6. Of the high loading compounds on nest PC1, geranylactone was detected in both sweat and saliva samples, and 1,2-cyclopentadione (tentative) was detected in saliva. However, all of the VOCs in the nest samples traced to sweat or saliva were also detected in urine. Since the VOCs in the nest deposits are produced in multiple body fluids, it is difficult to conclude how time spent nesting directly impacted the nest VOC profile. This is especially true since our saliva samples were not sufficient for quantitative analyses. Anecdotally, AJ mice produced the lowest volume of saliva, so the increased time spent nesting may be necessary for compounds levels on the nest to be similar to B6.

3.5.5 Conclusion

Overall, this study found that, in the home cage, odor profiles from sweat, nesting material, and urine, show strain specific patterns that align with affiliative and aggressive behavior. These findings warrant future studies that directly test the influence of compounds found in sweat, urine, and nesting material on expression of social behaviors, to hopefully put the field one step closer to promoting socio-positive behaviors and improving laboratory mouse welfare.

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CHAPTER 4. WHO'S THE BOSS? ASSESSING CONVERGENT VALIDITY OF AGGRESSION BASED DOMINANCE MEASURES IN MALE LABORATORY MICE, MUS MUSCULUS

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4.1 Abstract

Aggression among group housed male mice continues to challenge laboratory animal researchers because mitigation strategies are generally applied at the cage level without a good understanding of how it affects the dominance hierarchy. Aggression within a group is typically displayed by the dominant mouse targeting lower ranking subordinates; thus, the strategies for preventing aggression may be more successful if applied specifically to the dominant mouse. Unfortunately, dominance rank is often not assessed because of time intensive observations or tests. Several correlates of dominance status have been identified, but none have been directly compared to home cage behavior in standard housing. This study assessed the convergent validity of three dominance correlates (urinary darcin, tube test score, preputial gland to body length ratio) with wound severity and rankings based on home cage behavior, using factor analysis. Discriminant validity with open field measures was assessed to determine if tube test scores are independent of anxiety. Cages were equally split between SJL and albino C57BL/6 strains and group sizes of 3 or 5 (N=24). Home cage behavior was observed during the first week, and dominance measures were recorded over the second. After controlling for strain and group size, darcin and preputial ratio had strong loadings on the same factor, which was a significant predictor of home cage ranking showing strong convergent validity. Tube test scores were not significantly impacted by open field data, showing discriminant validity. Social network analysis revealed that despotic power structures were prevalent, aggressors were typically more active and rested away from cage mates, and the amount of social investigation and aggression performed by an individual were highly correlated. Data from this study show that darcin and preputial ratio are representative of home cage aggression and provide further insight into individual behavior patterns in group housed male mice.

4.2 Introduction

Excessive aggression in male mice is a leading welfare problem in the animal laboratory which can impact data validity and numbers of animals used in experiments. Many solutions offered to mitigate excessive aggression have been proposed, but inconsistencies occur between studies (Van Loo et al., 2003; Weber et al., 2017). This may not be surprising because most aggression studies only measure behavior at the cage level, not at the individual level. According to Rowell (1974), the term dominance is used to indicate the outcome between individuals during competition over resource or during a negative interaction, with a reasonable degree of predictability. While dominance is most often associated with aggression, in primates dominance status is in fact best predicted by the number of retreats performed by a subordinate, regardless of whether an aggressive act preceded it (Michel and Moore, 1994; Rowell, 1974). In mice, dominance and aggression can be one and the same, as the mouse who attacks most also receives the most overall submissions (Mondragón et al., 1987; Williamson et al., 2016b). In general, aggression is only one component of dominance, but it is the behavior of concern in a vivarium. Thus, individual ranking should be considered when trying to reduce aggression in the home cage. In order to evaluate ranking and the hierarchy in the cage, valid measures of dominance are necessary. This will help researchers understand the motivations behind excessive aggression.

Past behavioral analyses show that male mice form complex social hierarchies, with most groups displaying a linear or despotic power structure (Curley, 2016; Mondragón et al., 1987; Poshivalov, 1980; So et al., 2015; Ulrich, 1938; Williamson et al., 2017, 2016a, 2016b). However, in depth behavioral observations, like those done with social network analysis, are time intensive, making them impractical for quick evaluation. A dominance measure that requires less time to quantify, and one that can be validated based on relationships developed within the cage, would be a more realistic option. While less time intensive measures of dominance exist, they have only been compared to behavior in resident-intruder tests or complex group competitions and may not reflect behavior in a typical laboratory cage (Weber et al., 2017).

One commonly used measure of dominance is the tube test (Lindzey et al., 1961; Messeri et al., 1975). In brief, pairwise trials are conducted between cage mates in an arena composed of two Plexiglas chambers connected by a PVC tube. Contestants are placed at each end of the tube, locomote to the center, and the less dominant one will back out upon encountering the opponent. The tube test is meant to replicate competitive situations without exposing the mice to direct

conflict. As reviewed by Wang et al., 2014, stable tube test scores correlate with agonistic behavior, urine marking, and resource possession; however, there may also be a learning element involved, requiring mice to undergo repeated trials for stable results (Bernstein, 1981; Wilson, 1968). Data from Varholick et al., 2018 supports a learned component to tube test outcomes, where mice kept in long-term familiar groups displayed considerable rank variation over three trials, suggesting that scores are affected by the duration of the test and the test environment. Indeed, less than half of male mice competing in the tube test maintained a consistent ranking over three trials, and many groups displayed a dynamic, unstable relationship (Varholick et al., 2019). Currently, no studies have compared time dependent tube test ranks to other dominance correlates. These types of comparisons can assess convergent validity (how well similar measures reflect the same construct (Streiner et al., 2015)) and discriminant validity (dissimilarity between measures that reflect different constructs (Streiner et al., 2015)) of dominance correlates, to identify which measures accurately portray home cage interactions.

In addition to exploring the convergent validity of tube test scores with other dominance measures, this study aims to compare tube test scores with measures of anxiety from the open field maze (OFM). This question arose from past work, where tube test ranking did not predict levels of two urinary pheromones that are known to differ between dominant and subordinate mice (Barabas et al., 2021). Therefore, it is possible that the tube test may reflect other behavior that is not necessarily associated with dominance *per se*, such as anxiety or perhaps general locomotor activity. The tube test is conducted outside of the home cage, and it is possible that anxiety may cause mice to remain in the tube out of thigmotactic comfort, and not dominance over an opponent. Further, models of chronic social defeat have been shown to be related to higher levels of anxiety in various assays (Kinsey et al., 2007; Patki et al., 2013). Thus, it is possible that victimized mice remain in the tube out of security. Recently, a systematic review showed that measures of anxiety are not significantly different between dominant and subordinate mice (Varholick et al., 2021). However, the high level of study heterogeneity found in this review could mask an effect from social rank. The tube test was only used in 35% of included studies, so a direct link between those scores and anxiety could have been lost (Varholick et al., 2021). Additionally, the tube test could be subjected to effects of general activity: it is possible that a mouse could win simply by being inactive and waiting for an opponent to retreat (Zhou et al., 2018). Past work has shown that general activity measured in the OFM does not relate to tube test rank, but mice competed in daily

tube test trials for a week and likely became familiar with the expectations in that arena (Wang et al., 2011). It is unknown if locomotion plays a role for mice who may not be as familiar with the tube test arena. Assessing discriminant validity should help provide an answer to whether measures from the tube test are associated with anxiety or locomotion.

While behavioral tests can be beneficial, other measures may be more accurate at indicating dominance in the home cage as they do not require an external testing arena and therefore are not subjected to the same confounding environmental factors. One such indicator of aggression is the Pelt Aggression Lesion Scale (PALS). This method evaluates wound severity and is a validated indicator of wounding, specifically due to aggression (Gaskill et al., 2016). However, it is unknown how PALS relates to individual behavior and has only been used to assess substantial wounding in black mice who have pigment follicles that burst with injury. Another measure of dominance is the ratio of preputial gland weight to body length, which increases in males with less wounding and in those who display more attack behavior (Bronson and Marsden, 1973; Harvey et al., 1989). While potentially useful, mice used in previous studies were housed in isolation between weaning and the study period, calling into question the social competency of these test subjects. Even if socially competent, it is unknown whether this pattern holds true for mice housed in stable groups. Further, some research found conflicting evidence of this association where no relationship was found between the preputial glands and social status. However, these analyses were based solely on gland weight, not the relation to body size (Benton et al., 1980; Tanabe and Kimura, 1995). A final physiological measure is urinary levels of MUP20 (darcin) which has been connected to social rank, with mice who display more attack behavior, win more conflicts, and possess a more desirable nest site producing higher levels than opponents or other enclosure occupants (Guo et al., 2015; Lee et al., 2017; Nelson et al., 2015). Again, these studies were not done in stable groups of mice but were either based on resident-intruder trials or complex competition arenas where distinct territories could be formed.

Additionally, there is much to learn about how dominance measures and aggression may relate to other behaviors within the home cage. Anecdotal observations from our lab have shown that mice who attack most often also sleep away from cage mates and build separate nests, which aligns with historical observations of wild *Mus musculus* (Crowcroft, 1966). To our knowledge, the only formal assessment of the relationship between resting location and aggression found that mice who attack more spend more time resting away from cage mates (Mondragón et al., 1987).

While the Mondragón et al. (1987) study provides support for our own observations, the sample period from their study only consisted of two hours per day and may have missed occurrences of other mice resting on their own. Another behavior that might provide insight into the social dynamic within the cage is allo-grooming, which has rarely been studied. Previous research suggests that allo-grooming is most often performed between subordinate mice (Mondragón et al., 1987); however, more recent work found that an individual's place in a grooming network does not relate to their place in aggression networks (So et al., 2015). Other behaviors of interest may be specific only to subordinate mice. Various primate species respond submissively to those above them in rank and this pattern extends to various mouse strains (Mondragón et al., 1987; Rowell, 1974; Williamson et al., 2016b). However, several strains of inbred mice are known for excessive inter-male aggression (i.e. SJL (Festing, 1998)), and it is worth exploring if persistent fighting is due to a lack of appropriate submissive behavior by low ranking mice. Contrasting claims also exist regarding social investigation behavior (i.e. sniffing). It is often used as a measure of sociability towards stranger mice and has been considered a neutral exploratory behavior (Grant and Mackintosh, 1963; Mackintosh, 1981; Van Oortmerssen, 1971), but recent work has shown it to be predictive of aggression (So et al., 2015) and at the group level, it correlates with aggressive behavior (Barabas et al., 2021).

This study aimed to assess the convergent validity of three dominance measures (tube test score, preputial gland to body length ratio, and urinary darcin) with PALS score and home cage dominance ranking based on an aggression focused social network analysis (SNA). We considered measures to have strong convergent validity if they loaded strongly on the same axis of a factor analysis and were significant predictors of home cage dominance in a linear model. This study also aimed to test the discriminant validity of tube test scores with two measures of anxiety and one of locomotion in a novel environment. This would be indicated by a lack of significance in a linear mixed model. Additionally, this study sought to address four aims focused on home cage interactions in an aggression focused SNA: 1) assess how strain and group size may influence power distribution of male mice housed in standard shoebox cages; 2) examine how individual attack behavior relates to socio-positive behaviors and time spent in proximity to other cage mates; 3) determine if victim mice respond appropriately to aggression; and 4) conduct a formal analysis on how social investigation behavior correlates with submissive and aggressive behaviors.

4.3 Methods

4.3.1 Ethics statement

All animal use was approved by Purdue University's Institutional Animal Care and Use Committee under protocol #1707001598 (not previously submitted as a Registered Report).

Due to concern over excessive home cage aggression, humane endpoint criteria required any mouse with wounding greater than $1cm^2$ to be immediately euthanized. Animals were monitored daily for wounding, general activity, and signs of pain/distress. Four cages reached our criteria during the study (see Appendix D, Table D.1 for more information).

4.3.2 Animals

This study used a 2x2 factorial design based on strain and group size. *A-priori* sample size was determined using Mead's Rule (Mead, 1988). In total, 48 SJL/JOrlcoCrl (SJL) and 48 B6N-*Tyr*$^{c\text{-}Brd}$/BrdCrCrl (albino B6) specific pathogen-free mice were acquired from Charles River (Wilmington, MA) and housed in groups of three or five, N=24 cages. Albino B6 were chosen over pigmented B6 in order to ensure researchers and care staff could not distinguish strains based on coat color. Five is a common group size in a typical shoebox cage but less aggression has been observed in groups of three (Jirkof et al., 2020; Van Loo et al., 2001). Treatments were replicated in time with 3 batches of cages each time, due to spatial constraints. Each batch contained n=2 cages per strain x group size combination. Mice arrived at approximately 8 weeks of age and were housed for two weeks in open top micro-isolator cages (Ancare, Bellmore, NY) with customized lids (Alternative Design, Siloam Springs, AR) and external water bottles for overhead viewing (Appendix D, Figure D.1). Food (Envigo, Teklad 2016, Indianapolis, IN) and reverse osmosis water were offered *ad libitum*. Cages contained aspen wood chip bedding (NEPCO, Warrensburg, NY) and 8.5g of virgin kraft crinkle paper (Enviro-Dri, Cleveland, Ohio) for nesting material. Cages were kept under a 12:12 light: dark cycle (lights on at 06:00) with relative humidity ranging 24-64% and temperature ranging 17.8-23.3℃. Cages were changed weekly, with the exception of two cages (one albino B6 group of 5 and one SJL group of 5) in batch 1 that were changed on study day 4 and 5 respectively, due to excessive condensation on the cage walls and lid.

A numerical sequence from RANDOM.org was initially used to place cages on a rack shelf. Strain and group size treatments were ultimately balanced across rack shelves and the relative distance to the room's door. Two cages occupied each shelf and were surrounded by white foam board (Office Depot, Boca Raton, FL) as done previously to block background movement during video recordings (Barabas et al., 2021). Each cage was given its own letter label from A to X representing its group size and strain. Only these labels were visible in order to blind caregivers and research staff to strain treatment during sample collection, behavior tests, and video coding. It was only possible to be blind to group size when analyzing data from individual mice.
In the following sections, procedures are listed in the order in which mice experienced them.

4.3.3 Home cage behavior

All mice were individually marked with a fur marker (Stoelting, Wood Dale, IL) and continuously monitored with overhead and side view infrared closed circuit television (CCTV) cameras (Sony, Tokyo, Japan; HDview, Los Angeles, CA) and GeoVision monitoring software (Taipei, Taiwan). Data were analyzed on days 2 and 7 of the study period to capture early interactions during acclimation to the new cage and interactions at the end of the week, when mice were more familiar with each other (Barabas et al., 2021; Tallent et al., 2018). Each 24-hour period, from the two days, was watched using all occurrence sampling for one minute every five minutes. Individual occurrences of the following interaction types were recorded: escalated aggression, mediated aggression, submissive behavior, allo-grooming, and social investigation (Table 4.1). For each interaction, both the actor and recipient mouse were recorded as well as the time stamp. In the morning before each observation period, individual markings were retraced using permanent marker (Sharpie, Oak Brook, IL) as the fur marker was not visible under infrared lighting.

On day 2 and 7, time budget and location data were also recorded for each mouse using instantaneous scan sampling every five minutes. The following behaviors were included in the time budget: active, group sleep, and solitary sleep (Table 4.1). From these data, we calculated the proportion of observations each mouse spent performing each behavior. For the location data, a 4 x 2 transparent grid was overlaid on the video screen and the square where each mouse was observed was recorded to assess whether mice were alone or together. When active, mice were recorded in the square that contained their head; when resting, mice were recorded in the square

that contained more than half of their body. However, when mice were observed resting in a central nest site and that site spanned multiple squares, all mice were documented in the square containing the center of the nest. Location data were used to determine the proportion of observations where mice were observed alone. For all behavior observations, inter-rater reliability was assessed with Cohen's Kappa coefficient based on previous criteria (Martin and Bateson, 2007). Social behavior and time budget reliability were acceptable at 0.71 and 0.76, respectively. Location reliability was excellent at 0.93. A maximum of two observers coded each behavior category (A.J.B. and a trained undergraduate assistant). Two 24-hour periods were used for reliability, equating approximately 5% of the total video. The first period was randomly selected from the cages of five SJL mice, as it was assumed that they would contain the most aggression. The second period was randomly chosen from cages of three albino B6 to counterbalance strain and group size. While two 24-periods were used for the official reliability calculation, the total amount of training video varied across each student coder. The student who coded location data reviewed approximately 8% of the entire dataset as this was relatively simple data to record (the mice's location was limited in this housing). The students who coded the time budget and interactions respectively each reviewed approximately 13% of the dataset between practice and reliability.

Table 4.1. Ethogram of observed behavior categories. Descriptions taken from www.mousebehavior.org

Social Behaviors- actor and recipient recorded every 5 minutes using all occurrence sampling; the mouse who performed a submissive behavior first was considered the loser of each interaction

Category	Behavior	Description
Mediated Aggression	Resource Theft	A mouse will approach another that is either eating a piece of food or chewing on a piece of bedding. The approaching mouse will then attempt to take the resource from the other's paws or mouth. It may or may not be successful. It is often preceded by facial sniffing and involves one or both mice tugging at the resource.
	Tail Rattling	Fast waving movements of the tail. This behavior may be partially obscured by bedding material, but can be detected by displacement of bedding near a mouse's tail.
	Thrust	The aggressor mouse will first threaten its target cage mate by thrusting its head and fore body towards its cage mate's head or body. The aggressor's paw may come in brief contact with the target, but otherwise no contact is made.
	Mounting	Attempts to mount another animal in the absence of intromission. Palpitations with forepaws and pelvic thrusts may be present.
	Chase	A mouse will chase a fleeing partner, but no biting occurs

Table 4.1 continued

Escalated Aggression	Bite	The aggressor mouse attacks the recipient with open mouth and appears to bite the recipient, or latches onto the recipient by his teeth. The recipient responds by jumping or fleeing quickly. Aggressor mouse may rush or leap at the victim. This includes any rough and tumble actions and any mouse using its teeth to grab and tug on another's tail. Only score for the mouse that is biting.
	Fighting	Displayed by two or more animals when locked together. Separate behaviors are difficult to distinguish properly due to the fast rolling over and over seen with the animals kicking, biting, and wrestling. The initial victim retaliates towards the attacker. Score for all mice actively involved in the fight.
Submissive	Submissive Upright	A posture where the animal will sit on its haunches in an upright position exposing the belly. The forepaws are off the ground and the mouse may stretch out its forepaws towards the threatening mouse. Mouse can also be laying on its side with one forepaw and one hind paw stretched toward the threatening mouse and its back touching the ground.
	Fleeing	This behavior is characterized by a mouse moving away from the mouse performing an aggressive or investigative behavior. It can also be done by a mouse when it is approached by another. Typically fleeing animals will run, but in a confined space may walk or turn first. Also score if the mouse turns away without locomoting.
Allo-groom	During grooming, the actor mouths and licks the fur on the recipient's body. The actor will also use its teeth to clean the hair shaft by pulling the fur from the base of the hair shaft upward or outward.	
Social investigation	Sniffing directed towards another mouse (face, ano-genital, or body trunk). Only score this behavior if the actor's nose is seen directly oriented at or is close to touching another mouse. This will typically involve a slight head bob. Only score if the sniff lasts at least 1 second.	
Time Budget- recorded every 5 minutes using instantaneous scans		
Active	Score if the mouse is alert and conscious. This includes locomoting around cage, eating/drinking, interacting with cage mates, self-grooming, sniffing the cage/air, or passively sitting in the cage.	
Group Sleep	Sleeping that occurs when two or more mice are resting while in contact with the body of another mouse. When in the nest, the animals may not be seen clearly due to camera angles. If there is no movement in the nest, it is assumed the animals are sleeping. This will typically be in the main nest, but if no nest exists, they could remain behind the same pile of bedding.	
Solitary Sleep	Score if the mouse is seen resting in a location away from a central rest area	

Note: While observing video from day 7 in the first batch of mice (6 cages), individual identities could not be seen in infrared lighting due to inadequate markings. Video data from this time period were omitted from all analyses.

4.3.4 Urinary darcin

On study day 7, all mice were individually placed in empty cage bottoms with a wire floor grid to collect fresh urine. Only 70% of mice urinated while on the wire grids. For those that did not produce urine, sample collection was attempted in the OFM or while acclimating to the tube test arena (see methods below). In total, urine was collected from 85% of mice in this study (90% of SJL-5; 87% SJL-3; 76% albino B6-5; 92% albino B6-3). After collection, urine was stored in a -80℃ freezer until analysis at the Purdue Proteomics Facility (West Lafayette, IN).

Sample preparation followed previous methods (Aryal et al., 2018; Barabas et al., 2019). Briefly, proteins were precipitated using 4x the sample volume of acetone and denatured with 40 µL of 8M urea. Bicinchoninic acid assay was used to calculate total protein amount in each sample. 50 µg protein (equivalent volume) was reduced using 10mM dithiothreitol at 37 °C for 1 hour followed by alkylation using alkylating reagent (195 uL acetonitrile, 1 µL triethylphosphine and 4 µL of Iodoethanol) and incubated for 1 hour at 37 °C. After reduction and alkylation, samples were dried in a vacuum centrifuge. The trypsin/LysC mix was dissolved in 400 µL of 50 mM ammonium bicarbonate, and 80 µL was added to each sample for digestion. Digestion was performed at high pressure using a Barocycler (50 °C; 60 cycles: 50 seconds at 20 kPSI and 10 seconds at 1 ATM). Digested peptides were desalted using MicroSpin columns (C18 silica; The Nest Group), and dried in a vacuum concentrator at room temperature. Dried clean peptides were resuspended in 97% purified water, 3% ACN, and 0.1% FA at a final concentration of 1 µg/µL

Samples were analyzed by reverse-phase LC-ESI-MS/MS system using the Dionex UltiMate 3000 RSLC nano System coupled to the Q-Exactive High Field Hybrid Quadrupole Orbitrap Spectrometer (Thermo Fisher Scientific, Waltham, MA) as previously described (Aryal et al., 2018). Peptides were loaded onto a trap column (300 µm ID×5mm) packed with 5 µm 100Å PepMap C18 medium, and then separated on a reverse phase column (50-cm long×75 µm ID) packed with 2 µm 100Å PepMap C18 silica (Thermo Fisher Scientific, Waltham, MA) at a flow rate of 200nL/min. The column temperature was maintained at 50 °C. The positive ion mode was used for all the MS measurements, with 120min LC gradient and standard data-dependent mode50. MS data were acquired with a Top20 data-dependent MS/MS scan method. Instrument calibration was done using calibration mix solution (Thermo Fisher Scientific, Waltham, MA) at the start of each batch run and then after every 72hours. Instrument performance was also evaluated routinely using Hele cell digest (Thermo Fisher).

LC-MS/MS data were analyzed using MaxQuant software (version 1.6.3.3) against the UniProtKB *Mus musculus* genome (85,159 sequences as of Feb. 2020, www.unitprot.org). Default settings were used unless otherwise stated. The following parameters edits were made for this search: 10 ppm precursor mass tolerance; trypsin/Lys-C enzyme specificity; variable modification was oxidation of methionine (M); fixed modification was iodoethanol of cysteine (C); false discovery rate (FDR) of 0.02; peptide spectral match (PSM) and protein identification was set to 0.01. Label free quantitation (LFQ) was selected. All quantifications were calculated by MaxQuant. After the search, peptides with MS/MS counts under 2 were removed from the dataset. Standardized LFQ values for MUP20/darcin were used for subsequent analyses.

4.3.5 Open field maze

Open field maze (OFM) procedures were based on previous methods (Seibenhener and Wooten, 2015). Briefly, mice were tested individually in one of two 60 x 60 cm OFM arenas on study day 8. Arenas were cleaned with ethanol and allowed to air dry before the first and between subsequent trials. Mice were handled using plastic tubes (3 7/8" long x 2" inside diameter; 1/8" wall; BioServ, Flemington, NJ) as traditional tail handling can alter anxiety measures (Gouveia and Hurst, 2017). Due to time constraints, half the mice were randomly assigned to morning (07:00-09:00) or afternoon (15:00-17:00) testing, balanced across treatments. All trials were ten minutes long and recorded with CCTV cameras (Sony, Tokyo, Japan) for analysis using Ethovision software (Noldus, Wageningen, Netherlands). Grid squares (10 cm^2) were superimposed over the test arena, and the total distance traveled in cm and percent of time spent outside of the outer edge were calculated. The number of fecal boli were also tallied on testing day.

4.3.6 Tube tests

Like in OFM methods, half of the mice were acclimated and tested in the morning and half in the afternoon. Mice kept their same testing time assignment throughout the study. Briefly, the arena consisted of two plexiglass holding areas (approx. 19 cm x 19 cm x 21.5 cm) connected by a PVC tube (approx. 2.5cm inner diameter). On study day 9, mice were individually acclimated to the arena. Each mouse was given at least five minutes to comfortably explore, but no more than ten minutes. Gentle nudges were given when needed for all mice to cross the tube. On study days

10, 11, and 12, each cage underwent a round of tube testing based on previous methods (Howerton et al., 2008). Each cage competed in three total rounds of tube testing. Mice from each cage competed in pairwise trials, with one mouse starting at each end of the tube. Upon entering, a timer was set for two minutes. Trials ended when the first mouse backed out of the tube and placed both hindfeet on the holding area floor. If no winner emerged by the end of two minutes, then it was considered a loss for both mice. Each pairing was replicated four times, yielding 40 total trials in cages of five mice and 12 trials in cages of three mice per round. The arena was cleaned with ethanol and allowed to dry between trials. Each mouse received a dominance score based on the number of trials won out of the number competed.

4.3.7 Preputial glands

On study day 13, mice were euthanized by prolonged exposure to CO_2. Preputial glands were isolated, cleaned of connective tissue, and weighed in mg using an analytical balance (Ohaus, Parsippany, NJ). Each mouse's body length (tip of nose to base of tail) was also recorded in mm using calipers to calculate the preputial gland to body length ratio.

4.3.8 PALS score

The pelt aggression lesion scale (PALS) (Gaskill et al., 2016) was used to evaluate the final amount of wounding on each mouse. Currently PALS has only been validated to distinguish aggression-related wounding from ulcerative dermatitis and has not been directly linked to behavior. Additionally, while PALS is able to detect the presence of burst pigment follicles in black mice due to previous fighting (Gaskill et al., 2016), white mice do not possess this pigment. The ability to assess aggression history using PALS may be limited in white mice and will be explored in this study.

After preputial gland removal, pelts were removed from the carcass through gentle manipulation. The limbs were stretched and pinned so the pelt formed a rectangle and a subcutis image was taken of each pelt (Sony, Tokyo, Japan). A 9 x 9 grid was placed over each image and stretched from base of neck to base of tail. Each grid space was scored in terms of % visible area impacted and wound severity. Wound severity was assessed on a 0-4 scale with the following descriptions: 0) no visible damage; 1) five or fewer bites (double puncture sites); 2) more than five

bite wounds with non-coalescing discoloration OR coalescing discoloration on less than 25% of the square; 3) coalescing discoloration on at least 25% of the square OR full thickness wounding covering less than 25% of the square; 4) full thickness wounding covering more than 25% of the square. Each grid space was given a score based on the following equation (Gaskill et al., 2016): PALSgridScore = SeverityScore x AreaScore x 0.25

Anterior, mid, and posterior regions were given an average score based on the three grid scores in each region. All analyses were done using the average posterior scores for each mouse, as it is most predictive of aggression related wounding (Gaskill et al., 2016).

4.3.9 Data availability

All raw data are available online at https://www.frontiersin.org/articles/10.3389/fvets.2021.695948/full#supplementary-material . Raw urinary protein files are available from the Mass Spectrometry Interactive Virtual Environment (MassIVE) repository (file ID: MSV000086740).

4.3.10 Statistics

Analysis note: N=24 cages were set up, but four cages of albino B6 (one group of five and three groups of three) were prematurely euthanized due to extreme aggression (Appendix D, Table D.1). Behavior data on day 2 were collected from one cage of albino B6-3 before euthanasia, and were included in SNA models. Additionally, a cage of SJL, group of three, was excluded due to dehydration from a faulty water sipper. Day 2 data from an SJL cage of five could not be observed due to camera malfunction. In total, there were N=20 cages for SNA analyses and 19 for measure validation. Based on Mead's equation and the law of diminishing returns (Gaskill and Garner, 2020; Mead, 1988), this sample size was large enough for sufficient error degrees of freedom in cage level models. Appendix D, Table D.2 provides details of experimental units used in each model described below.

4.3.10.1 Aggression network analysis

Analyses of aggression (referred to as aggression network analysis) were conducted based on previous methods for SNA. Occurrences of mediated and escalated aggression were combined

into directed frequency sociomatrices for each cage (Croft et al., 2008). Each row and column corresponds to each individual within a cage, with actor and recipient mice represented by matrix rows and columns, respectively. Each value within a matrix tallies the number of times each "actor i" won an attack over each "recipient j". In this study, all observed contests were won by the mouse who initiated them, so these values represent both the number of fights initiated and fights won. For each contest, the first mouse who fled or performed a submissive upright posture was considered the loser. Directed binary sociomatrices were also calculated from each cage's frequency matrix to yield presence/absence data. This indicates whether each "actor i" was ever observed attacking each "recipient j".

The following global hierarchy measures were calculated using data from the binary sociomatrices: **Density**- the proportion of all possible interactions that occurred within a cage (Croft et al., 2008); **Directional Consistency (DC)**- a proportion of interactions that occurred from the most frequent direction to the least frequent direction within each dyad. DC scores closer to 1 indicate unidirectional interactions and scores closer to 0 indicate interactions that are more equally reciprocated. A measure of hierarchy linearity was not done as the interactions in this dataset were so skewed in favor of the alpha male that ranks between other cage mates were not stable enough to calculate a measure such as Landau's H or triangle transitivity (data not shown).

Individual social hierarchy ranking was calculated from the frequency sociomatrices using the **Glicko Rating System** (Glickman, 1999). In brief, individuals lose points for every social defeat and win points for every victory. However, the number of points won/lost is dependent on the score difference between the opponents. E.g., if an actor defeats a recipient that has a much lower rating than itself, the actor will receive fewer points than if defeating a recipient with a rating that is close to its own. Rating certainty is also calculated based on the number of contests each individual engages in and the time since the last contest. There is more rank certainty in individuals who compete more frequently. For further explanation and evaluation of the Glicko System, please refer to So et al. (2015). Since Glicko ratings have a default value of 2200, the net change in score was calculated for each mouse in order to better account for the variation in interaction frequency between cages and for scores to be more intuitive (i.e., victim mice have negative scores). Additionally, **individual out-strength** (the number of times the individual performed a behavior) and **in-strength** (the number of times the individual was the recipient of a behavior) were calculated for each animal for aggression, submission, allo-grooming, and social investigation

(Farine and Whitehead, 2015). All hierarchy and SNA measures were organized and calculated using R Studio (version 3.6.1) with the following packages: compete (Curley et al., 2015), sna (Butts, 2008), and PlayerRatings (Stephenson and Sonas, 2014).

General linear models (GLM), general linear mixed models (GLMM), or generalized linear mixed models (GLIMM) were used to address the following aims:

1) examine how power is distributed in the cage based on aggression density and DC (GLM);
2) assess how individual change in Glicko score relates to time budget, proportion of time observed in proximity to a cage mate, and allo-grooming in- and out-strength (GLMM). Time budget data were condensed using Principal Component Analysis (PCA). Only components with eigenvalues over 1.0 were analyzed in a GLMM.
3) Evaluate the relationship between aggression in-strength and submission out-strength (i.e., do attack victims respond appropriately with submission; GLMM); and
4) explore how displays of social investigation correlate with those of aggression and how likely recipients are to respond with submission (GLIMM).

All model assumptions were checked post-hoc and transformations were made when needed. In all models, strain, group size, and the interaction are included as fixed effects. All data were originally analyzed with day and all 2- and 3- way interactions as fixed effects. Non-significant interactions were dropped from all models. If day was not a significant factor, data were summarized for the study week and reanalyzed. Since each cage only contained one strain and group size, each factor was nested within cage and included as random effects for models addressing aims 2-4. It was also included in aim 1 models that included day as a fixed effect. For models addressing aims 2-4, mouse nested within cage was included as a random effect if the model tested the effect of day. Models for aims 1-3 were run in JMP Pro (version 14.0.0) with post hoc Tukey tests where applicable. For aim 4, correlation was assessed in JMP Pro using Pearson's correlation coefficient and a logistic regression model of each social investigation occurrence was run in SAS using PROC GENMOD with Bonferroni corrected post hoc contrasts (alpha = 0.05/6 comparisons = $P<0.0083$). An occurrence was given a 1 if submission occurred within five seconds of the social investigation, otherwise it was assigned a 0. For aim 4, only cages that had behavior observations from both study days were analyzed (Appendix D, Table D.2). Data

organization and filtering for aim 4 were done in R studio using the tidyverse package. All figures were made in R Studio using ggplot2 and cowplot packages.

4.3.10.2 Dominance measure validation

Dominant and subordinate mice from each cage were determined using the change in Glicko score from interactions over both days of video. However, since the subordinate's behavior can be more indicative of dominance than aggression (Rowell, 1974), Glicko scores were recalculated for each cage to reflect submissive behavior (Glick-Sub). These scores reflect all submission performed in response aggression, social investigation, or approach behavior. The mice in each cage with the highest and lowest Glicko-Sub scores were considered the respective dominant (mice who received the most submissions) and subordinate (mice who received the least submissions). The original scores from the aggression network analysis (Glicko-Agg), which specifically distinguishes aggressor from victim mice, were compared to other measures of focus to determine if they reflect both dominance and aggression. Darcin, fecal boli count in OFM, proportion of time in the center of the OFM, scores from three rounds of tube tests, PALS, and preputial data were only analyzed from these designated mice (38 total). However, two mice did not produce urine, causing missing values for darcin, and were excluded from the convergent validity factor analysis (N=36 mice; Appendix D, Table D.1 + D.2).

An exploratory factor analysis was done to determine if darcin, scores from three rounds of tube tests, PALS, and preputial data have convergent validity with the net change in both Glicko scores. First, all measures were standardized and run in GLMMs to isolate effects from strain and group size. Using previous methods (Miller et al., 2006), the residuals from the darcin, tube test, PALS, and preputial models were run in a factor analysis of correlations using JMP Pro. Maximum likelihood was used as the factoring method and prior communality was based on the squared multiple correlations. Varimax rotation was used on the loadings to improve factor interpretation. Loading threshold was set at 0.45 as done previously, since it is a mid-range value between what is used by behaviorists and biostatisticians (Miller et al., 2006). This analysis maintained the 5:1 subject to variable ratio for factor analysis (36 subjects/6 variables = 6). Scores from the resulting factors were tested in GLMs for direct effects on the change in Glicko-Sub and Glicko-Agg.

To assess discriminant validity, first scores from three rounds of tube tests were condensed in a principal component analysis. Only the first axis had an eigenvalue over 1 and represented all three scores (loading values over 0.90; Appendix D, Table D.3). The scores from this axis (tube test PC) were analyzed using a GLMM to test effects of fecal boli count in OFM, proportion of time in the center of the OFM, total distanced moved in the OFM, strain, and group size. Batch number and time of testing were included as blocking factors, but neither factor was significant, so they were dropped from the final model. Cage nested within strain and group size was included as a random effect.

4.4 Results

4.4.1 Aggression network analysis

4.4.1.1 Aim 1: How strain and group size affect power distribution

Aggression density was only significantly impacted by group size (GLM: $F_{1,14} = 17.43$,$\eta_p^2 = 0.55$, $P<0.001$), with cages of three showing higher density than cages of five (Figure 4.1A). However, all density values were low (Inter quartile range (IQR): 0.26- 0.48). Aggression DC was not significantly impacted by strain or group size (GLM strain: $F_{1,14}= 0.17$, $P= 0.69$; group size: $F_{1,14}=0.22$, $P =0.65$) and was generally high across cages (IQR: 0.72 – 0.90).

4.4.1.2 Aim 2: Influence of time budget, cage mate proximity, and allo-grooming on Glicko-Agg score

PCA of time budget data yielded one significant component (Budget PC), with all behaviors loading strongly. Time spent active and performing solitary sleep had high positive loadings while time spent in group sleep had a high negative loading (Table 4.2). Scores from Budget PC had a positive relationship with the change in Glicko-Agg score (GLMM: $F_{1,69.15} = 24.46$, $\eta_p^2 = 0.26$, $P<0.001$; Figure 4.1B) while the proportion of time observed alone, based on location data, had a negative relationship (GLMM: $F_{1,72.39} = 5.02$, $\eta_p^2 = 0.06$, $P=0.028$; Figure 4.1C). As time alone increased, the change in Glicko-Agg scores decreased. Neither allo-grooming in-strength nor out-strength had a significant effect on the change in Glicko-Agg score (GLMM: $F_{1,45.03} = 1.12$, $P=0.296$; $F_{1,71.23} = 0.81$, $P=0.371$).

4.4.1.3 Aim 3: Relationship between submission out-strength and aggression in-strength

Submission out-strength was significantly impacted by the strain*aggression in-strength interaction (GLMM: $F_{1,120} = 7.21$, $\eta_p^2 = 0.06$, $P<0.001$) as well as the day*aggression in-strength interaction (GLMM: $F_{1,124.1} = 34.83$, $\eta_p^2 = 0.22$, $P<0.001$): albino B6 mice and mice on day 7 performed more submissions relative to the attacks they received (Figure 4.1D + 4.1E).

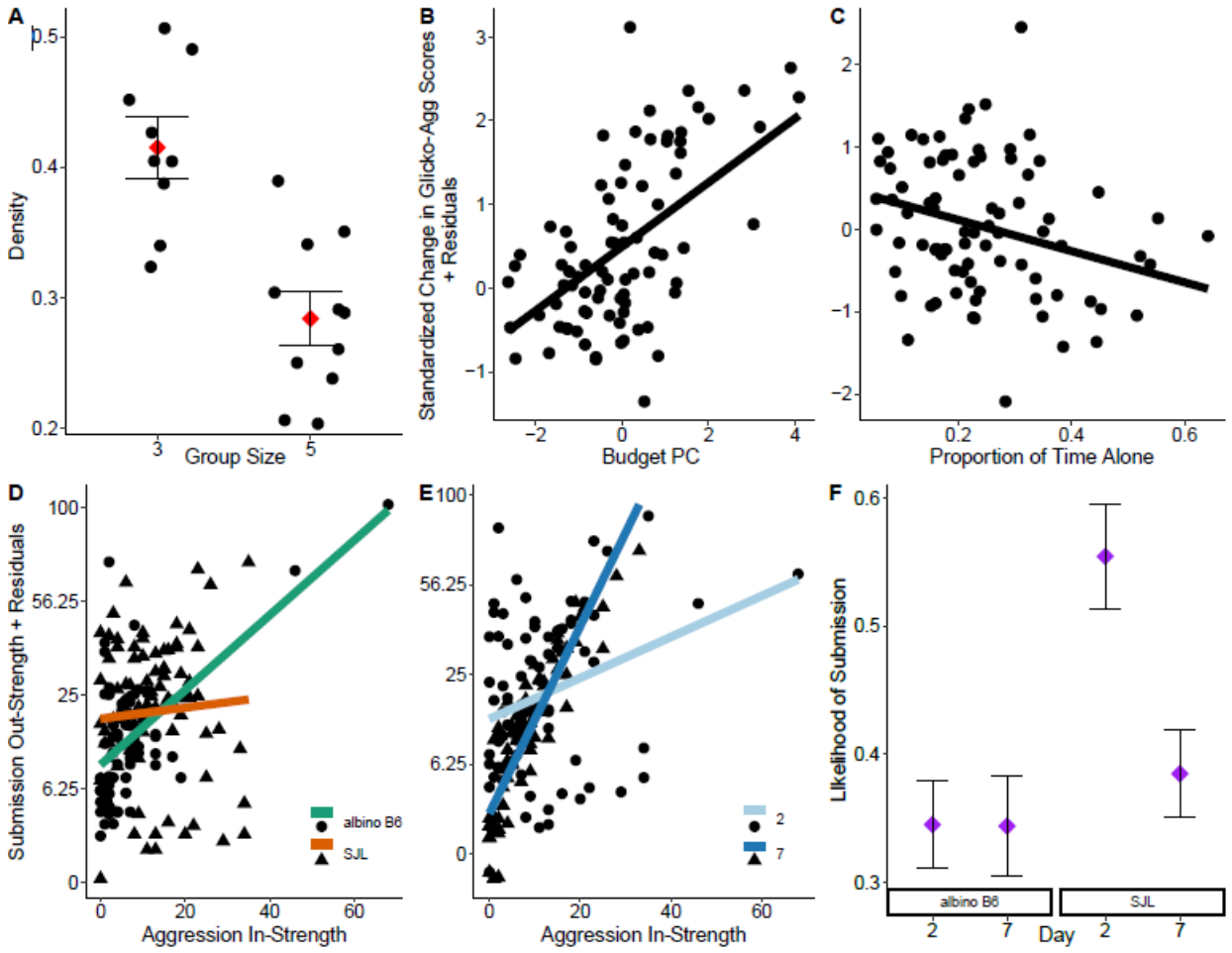

Figure 4.1. Social network analyses of group housed albino B6 and SJL male mice. (A) Group size significantly impacted aggression density (adj. R^2 = 0.57, N = 20). Change in individual Glicko-Agg score was impacted by (B) Budget PC1 and (C) the proportion of time observed alone (adj. R^2 = 0.12, N = 82). Interactions of (D) strain*aggression in-strength and (E) day*aggression in-strength significantly influenced individual submission out-strength (adj. R^2 = 0.39, N = 156). Y axes in D and E are shown on a square root back transformed scale. (F) Binary logistic regression revealed a significant strain*day interaction on the likelihood that social investigation is followed by submission (N= 2192). Data in A and F are presented as factor level LSM ± SE. Data in A are presented over the scatter of individual residual points.

4.4.1.4 Aim 4: How social investigation relates to aggression and submission

There was a high correlation between social investigation out-strength and aggression out-strength (Pearson's R = 0.79, P<0.001, 95% CI: 0.72- 0.84). Logistic regression was used to assess the likelihood of submission occurring within five seconds of a social investigation. There was a significant strain*day interaction on this likelihood (GLIMM: χ^2_1 = 5.76, P=0.016). The

probability of submission after social investigation was highest in SJL mice on study day 2 (Tukey: P's< 0.002, Figure 4.1F).

Table 4.2. Loading values from Principal Component Analysis of time budget behaviors. Only the first component was interpreted based on eigenvalue analysis. Scores from this component were used in a GLMM.

	Budget PC
Solitary Sleep	0.75601
Group Sleep	-0.97370
Active	0.69162
Eigenvalue	2.00
Total variance explained (%)	66.60

4.4.2 Dominance measure validation

Strain had a significant effect on the following dominance measures: preputial gland ratio (GLMM: $F_{1,13} = 9.17$, $\eta^2 = 0.41$, P=0.009); darcin (GLMM: $F_{1,12} = 55.53$, $\eta^2 = 0.82$, P<0.001); and PALS score (GLMM: $F_{1,13} = 38.58$, $\eta^2 = 0.75$, P<0.001). Preputial ratios and darcin levels were higher in albino B6 mice while PALS scores were higher in SJL mice. A strain * group size interaction impacted tube test scores from round 1 (GLMM: $F_{1,13} = 7.12$, P=0.019), but post hoc Tukey tests showed no significant differences. Further, strain impacted Glicko-Sub score (GLMM: $F_{1,13} = 5.45$, $\eta^2 = 0.30$, P=0.036). Please refer to Appendix D, Table D.4 for strain*group size least square means. The random factor, CageID, was significant in PALS (P=0.029), Glicko-Sub (P=0.003), and Glicko-Agg (P=0.002) models. Correlation values for all variables are presented in Appendix D, Table D.5. Notably, Glicko-Agg and Glicko-Sub scores were highly correlated (Pearson's R= 0.97, P<0.001, 95% CI: 0.95- 0.99).

For convergent validity, eigenvalue analysis showed that two factors were sufficient to interpret the dataset. The first factor accounted for over 42% of total variation and reflected scores from all three rounds of tube testing and PALS score. The second factor accounted for over 20% of total variation and reflected urinary darcin, and preputial gland ratio (Table 4.3;Figure 4.2). Factor two was a significant predictor of both Glicko-Sub score (GLM: $F_{1,35} = 15.70$, $\eta^2 = 0.31$, P<0.001) and Glicko-Agg score (GLM: $F_{1,35} = 20.86$, $\eta^2 = 0.37$, P<0.001).

Table 4.3. Loading values from factor analysis to assess convergent validity of measure residuals. Values below the loading threshold of 0.45 are presented in grey. Factors with eigenvalues over 1 are shown.

	Factor 1	Factor 2
Preputial gland: body length ratio	-0.152076	0.719176
Urinary darcin	-0.239981	0.734438
Average posterior PALS score	0.467036	-0.176451
Tube test- round 1	0.777146	-0.197103
Tube test- round 2	0.989930	-0.141560
Tube test- round 3	0.804816	-0.296798
Eigenvalue	2.53	1.23
Total variance explained (%)	42.18	20.58

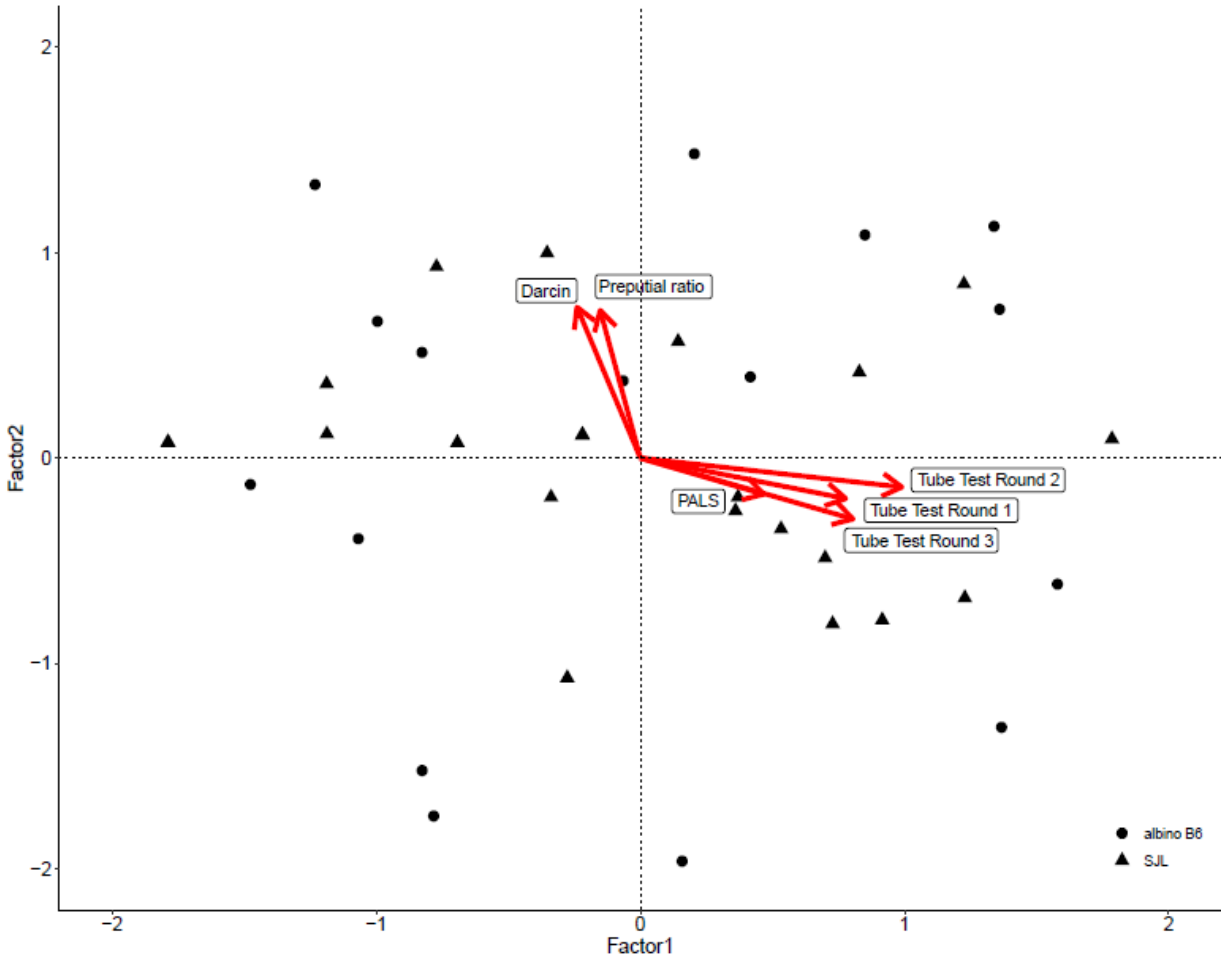

Figure 4.2. Biplot of factor analysis used to assess convergent validity. Individual data point scores are plotted along Factor1 and Factor2, with shape based on strain. Variable loadings for each factor are depicted by red arrows

For discriminant validity, neither the number of fecal boli (GLMM: $F_{1,25.93} = 0.80$, P=0.381), proportion of time in the center of the OFM (GLMM: $F_{1,19.34} = 0.04$, P=0.851), nor total distance traveled (GLMM: $F_{1,15.42} = 1.82$, P=0.196) were significant predictors of the tube test PC.

4.5 Discussion

The main aim of this study was to assess the convergent validity of dominance measures with home cage rankings based on SNA in group housed male mice. Additionally, the discriminant validity of the tube test was assessed in relation to measures of anxiety and locomotion from the OFM. Although dominance in some situations is best predicted by subordinate behavior instead of aggressive behavior (Rowell, 1974), Glicko scores in our study calculated from both aggression and submission data were highly correlated. For the cages used in this study, aggression was likely a good indicator of dominance. Additionally, both scores were predicted by the same factor representing urinary darcin and preputial gland: body length ratio. This suggests that both measures show convergent validity with home cage behavior. This extends the patterns found in previous work on males reared in isolation or tested in complex competition arenas (Bronson and Marsden, 1973; Guo et al., 2015; Harvey et al., 1989; Lee et al., 2017; Nelson et al., 2015). The correlation between preputial gland ratio, urinary darcin, and home cage aggression is likely testosterone mediated since all three are testosterone dependent (Beeman, 1947; Brown and Williams, 1972; Guo et al., 2015). In fact, testosterone treated females are more aggressive and their urine can trigger intense attacks towards castrated males and normal females, which supports an olfactory based mechanism behind aggression (Lee and Griffo, 1974; Svare and Gandelman, 1974). However, it is likely dependent on more complex, tissue specific levels of testosterone and receptor density since circulating levels have been shown to not predict individual wounding or aggression levels (Selmanoff et al., 1977). This solidifies the utility of the preputial gland ratio and urinary darcin to indicate individual dominance ranking, keeping in mind that both measures were strain dependent. As shown here and previously, mice of the C57 lineage produce more darcin than mice of Castle or Swiss lineages (Cheetham et al., 2009; Kwak et al., 2012; Roberts et al., 2010). Further, albino B6 mice also had larger preputial gland ratios than SJL mice. To the best of our knowledge, strain effects on gland ratio have not been previously examined. However, based

on the η^2 for each model, strain had a stronger effect on darcin than preputial gland ratio. This may explain why darcin accounted for so much less variability in Glicko score than preputial ratio.

On the other hand, the average posterior PALS score loaded on a factor that did not predict either aggression-based or submission-based Glicko score. As discussed below, the cages here primarily displayed despotic hierarchies, but the level of wounding varied across cages. For cages that display aggression with more forceful biting, the level of wounding could be a powerful indicator of dominance. However, it will not be as predictive for cages that primarily display mediated forms of aggression like mounting and chasing and it would not predict social rank based on submission in interactions that do not involve physical contact. Additionally, the relationship may not be as clear for more linear relationships where there is conflict between mid-ranking mice. Since this is the first direct comparison between PALS score and behavior, further work will have to examine its value in different social structures. Additionally, this relationship could have been impacted by the mice's pigmentation. PALS' predictive ability has been validated in black mice where burst pigment follicles indicate mild aggression (Gaskill et al., 2016). Since the white mice used here do not have these follicles, only more severe wounding could be documented. This limits PALS' predictive ability in cages of white mice that display more mediated aggression. However, these findings support the robustness of using darcin and preputial gland ratio as they correspond with dominance behavior, regardless of how much vascular damage may be present.

Scores across three rounds of tube tests also loaded on an axis that did not predict Glicko score. This result was surprising since previous work has found tube test rankings to correlate with agonistic behavior both in the home cage and in an unfamiliar setting (Howerton et al., 2008; Wang et al., 2011), so we expected that tube test scores would at least predict Glicko-Agg scores. In one case, the difference could be due to previous assurance of rank stability in the tube test, which was not done here (Wang et al., 2011). Additionally, these data could be a product of their respective environments: aggressive behavior used to calculate Glicko scores was recorded in the home cage while tube test scores were from a specialized arena. It is possible that the relationship here reflects the tendency for some subordinate mice to regain confidence when away from their attacker (Williamson et al., 2017). However, it has also been shown that many hierarchies based on the tube test produce unclear ranks over time, which could indicate that dominance ranks have a transient nature (Varholick et al., 2019), or it could reflect another trait all together. The data reported here support the latter option since scores from all rounds showed high correlation and

loaded on the same factor. Interestingly, posterior PALS score had a weak relationship with the same factor as tube test scores. This is the first known comparison between tube test scores and wounding, but since most aggression related wounding is located in a posterior region, it would be advantageous for mice who are already injured to remain in the tube to prevent further attacks from behind. In relation to OFM measures, tube test scores displayed good discriminant validity, implying that general locomotion and anxiety in a novel environment do not influence tube test performance. The lack of relationship with distance moved confirms past work (Wang et al., 2011). In terms of anxiety, mice experienced the OFM and acclimation to the tube test arena before testing, so it is possible that they displayed less anxiety each time they left the cage. Interestingly, in both factor analyses, scores from all three tube test rounds loaded strongly on the same factor. Previously, it has been shown that tube test scores are more consistent between the second and third round, suggesting that mice must be repeatedly tested for stable scores (Fan et al., 2019; Varholick et al., 2018). These conflicting results may be reflective of strain or environmental conditions: the former studies used pigmented C57BL/6 mice tested in facilities outside the Unites States. Facility to facility environmental differences are known to influence behavioral data across several strains of mice (Crabbe et al., 1999).

Taken together, measuring urinary darcin or the preputial gland: body length ratio would be a more practical alternative for researchers than time intensive home cage observations. However, both measures have their draw backs: preputial gland ratio comes with the challenge of being an end of life measure while darcin is more impacted by strain variation. If it is feasible to only determine social rank at the end of the study, then preputial ratio is suggested. Otherwise, urinary darcin may be more advantageous depending on strain.

This project also aimed to better understand how individual aggression patterns relate to other home cage behaviors through aggression focused SNA. While previous SNA work has provided valuable insight on mouse social dynamics, it was either based on limited, live person sampling that may only reflect behavior at certain times or used large vivarium housing that may not accurately represent the conditions most laboratory mice experience in a typical shoebox cage.

For group level measures, our data revealed that aggression density is primarily low, and DC is high in these two strains of male laboratory mice. This indicates that key mice within each cage consistently perform aggression and the attacks are not typically reciprocated. This matches previous work which found that male mice often display despotic power structures (Mondragón et

al., 1987; Poshivalov, 1980; Ulrich, 1938; Williamson et al., 2016b). Past reports show that linear hierarchies are also common (Curley, 2016; Mondragón et al., 1987; So et al., 2015; Ulrich, 1938; Williamson et al., 2017, 2016b, 2016a), however the interactions in this dataset were so skewed in favor of the alpha male that ranks between other cage mates were not stable enough to calculate a traditional linearity measure such as Landau's H or triangle transitivity (data not shown). The only significant treatment effect in this experiment indicates that group size influenced aggression density: cages of 3 had higher density than cages of 5. Although data were analyzed as a proportion in order to account for more mice and potential interactions in groups of 5, this difference may still be due to the fact that fewer mice in a cage inherently reduces the number of potential interactions, so a single pair-wise interaction will have a larger impact on density.

In terms of the individual, Glicko-Agg scores were only impacted by time budget, as represented by the Budget PC and the proportion of time observed alone in the cage. PCA of time budget behaviors (active, group sleep, and solitary sleep) revealed that mice who were more active spent more time sleeping alone. These same mice who were more active and performed more solitary sleep, had a higher change in Glicko-Agg score over the study week. To the best of our knowledge, how aggression relates to activity in the home cage has not been formally studied, but this pattern is consistent with previous work using a resident intruder paradigm. Mice that undergo social defeat daily and then are housed separately from their attacker, using a cage partition, show reduced activity, and display characteristics of depression (Bartolomucci et al., 2003; Dadomo et al., 2011; Hammels et al., 2015). However, this could also represent a higher motivation to patrol territory in dominant, aggressor mice, who are known to claim territory through scent marks more than subordinates (Arakawa et al., 2008). These results also confirm anecdotal observations and past work that more aggressive mice rest away from cage mates (Mondragón et al., 1987). However, this contrasts with the negative relationship seen between the proportion of time observed alone and the change in Glicko-Agg score. This is likely because the time observed alone accounts for both active and inactive periods. Mice who are frequently targeted by an aggressor have been shown to actively avoid them, particularly when there is a despotic dynamic (Curley, 2016), so it is possible that the pattern seen here is representative of active times when subordinate mice are fleeing from their aggressor. Additionally, the amount of allo-grooming performed and received by these inbred strains did not relate to the change in Glicko score, which agrees with past work on outbred mice showing little correlation between position in a grooming network and

the position of individuals in networks derived from other behaviors (So et al., 2015). However, this does not necessarily mean that allo-grooming is solely motivated by affiliation in laboratory mice. If it did, we would expect a negative relationship between change in Glicko score and the amount of grooming performed. It has been suggested that allo-grooming may serve a dual purpose by providing emotional support between subordinate mice and acting as reconciliation when done by dominants after aggression (So et al., 2015). The latter has been frequently observed in primates (De Waal and Ren, 1988; De Waal, 2000; Ren et al., 1991); however assessing the direct sequence pattern of allo-grooming was beyond the scope of this study and would be a worthwhile future topic.

The Glicko-Agg score model, and those mentioned above for density and DC, used combined data from the two days over the course of the study week, since study day did not have an impact on these measures. This suggests that, in albino B6 and SJL mice, dominant males emerge by the end of the second housing day and mice maintain their social rank, at least over the first week of housing. Previous work with CD-1 mice showed a similar pattern, however a subset of those observed groups took over two weeks to stabilize ranks, which may be a product of that strain (Williamson et al., 2016b).

In general, the amount of submission the mice performed was positively related to the number of attacks they received, aligning with past work on outbred mice (Williamson et al., 2016b). The interaction of day* aggression in-strength showed that submission rate was best explained by aggression on study day 7. Additionally, the interaction of strain* aggression in-strength showed that albino B6 mice performed more submission in relation to the number of times they were attacked. In fact, the fitted line relating aggression in-strength and submission out-strength for SJL mice only has a slightly positive slope, implying that this relationship was primarily seen in albino B6 mice. However, this is not to suggest that SJL mice do not submit when attacked, only that their submission rate cannot solely be explained by attacks. The likelihood that a mouse would submit after social investigation was higher for SJL mice on study day 2, which likely impacted the relationship depicted by both interactions in this model. One point of consideration is that SJL mice had higher PALS scores than albino B6, so even though the number of attacks did not vary across days, those from SJL mice presumably caused more physical damage. However, a downfall of PALS is that it is a cumulative, end of life measure, so it cannot differentiate between a recent, gentler attack and one that was more severe and partially healed.

Still, it is likely that more damage was caused by attacks at the start of the study since male mice are less tolerant of each other when they are unfamiliar (Crowcroft, 1966; Mackintosh, 1970). This may have triggered subordinate SJLs to perform more submission on day 2 in response to sniffing to prevent the interaction from escalating into an attack, since there was high correlation between the number of times an individual attacked and sniffed a cage mate. This high correlation confirms previous work (Barabas et al., 2021; Lee et al., 2019; So et al., 2015) and suggests that the motivation for social investigation may not always be neutral, as previously considered (Grant and Mackintosh, 1963; Mackintosh, 1981; Van Oortmerssen, 1971).

4.5.1 Conclusion

In summary, this study showed that urinary darcin and preputial gland: body length ratio have good convergent validity with home cage aggression, both mediated and escalated behaviors, and would be a practical alternative to home cage observations for identifying individual dominance rank. However, both are subject to strain variation and preputial ratio must be done as an end of life measure. Additionally, tube test scores have good discriminant validity with measures of locomotion and anxiety from the OFM. Finally, these data confirm that despotic power structures are prevalent in male social groups of inbred laboratory mice, aggressors are often more active and rest away from other cage mates, and that social investigation behaviors can be linked to aggression. This information provides more understanding of mouse home cage behavior and can be utilized to help develop aggression mitigation strategies.

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CHAPTER 5. ASSESSING THE EFFECT OF COMPOUNDS FROM PLANTAR FOOT SWEAT, NESTING MATERIAL, AND URINE ON SOCIAL BEHAVIOR IN MALE MICE, *MUS MUSCULUS*

5.1 Abstract

Home cage aggression causes poor welfare in male laboratory mice and reduces data quality. One of the few proven strategies to reduce aggression involves preserving used nesting material at cage change. Volatile organic compounds from the nesting material and several body fluids not only correlate with less home cage aggression, but with more affiliative allo-grooming behavior. To date, these compounds have not been tested for a direct influence on male mouse social behavior. This study aimed to determine if 4 previously identified volatile compounds impact home cage interactions. A factorial design was used with cages equally split between C57BL/6N and SJL male mice (N=40). Treatments were randomly assigned across cages and administered by spraying each respective compound solution on the cage's nesting material. Treatments were refreshed after study day 3 and during cage change on day 7. Home cage social behavior was observed throughout the study week and immediately after cage change. Several hours after cage change, feces were collected from individual mice to measure corticosterone metabolites as an index of social stress. Wound severity was also assessed after euthanasia. Measures were analyzed with mixed models. Volatile treatments did not impact most study measures. For behavior, SJL mice performed more aggression and submission and B6 mice performed more allo-grooming. Wound severity was highest in the posterior region of both strains, and the middle back region of B6 mice. Posterior wounding also increased with more observed aggression. Corticosterone metabolites were higher in B6 mice and in mice with more wounding treated with 3,4-dimethyl-1,2-cyclopentanedione. This data confirms previous strain patterns in social behavior and further validates wound assessment as a measure of escalated aggression. The lack of observed treatment effects could be due to limitations in the compound administration procedure and/or the previous correlation study, which is further discussed.

5.2 Introduction

Aggression among group housed male laboratory mice continues to challenge researchers despite its negative impacts on animal welfare and research data quality (Poole, 1997; Weber et al., 2017). Although aggression is a complex social situation caused by a variety of factors (Kappel et al., 2017; Theil et al., 2020; Weber et al., 2017), it is often suggested that odor signals could appease conflict since they are a natural form of communication for many mammalian species (Apps et al., 2015; Liberles, 2014; Wyatt, 2017). For mice specifically, aggression can be triggered by scent cue disruption (Hurst et al., 1993). For example, the routine cage cleaning that mice experience can often cause bouts of violent, escalated aggression that peak approximately 15-45 minutes afterward (Ambrose and Morton, 2000; Gray and Hurst, 1995). One of the few proven remedies for aggression related to cage change is transferring used nesting material into the new cage (Van Loo et al., 2000), and for decades it has been speculated that this mechanism is due to odor signals preserved in the material. Only recently has it been shown that used nesting material does in fact contain a variety of proteins used by mice for identification purposes (Barabas et al., 2019), so the practice of transferring used nesting material is supported by an ethologically relevant form of communication.

Specifically, it has been suggested that mice deposit pheromones in nesting material that appease aggression among familiar conspecifics. Pheromones are a subcategory of odor signals that must meet specific criteria for classification. For instance, an odor signal must produce reliable effects in a bioassay at physiologically relevant concentrations to be considered a pheromone (Wyatt, 2017, 2009). In mice, the only known pheromones that impact same sex social behavior are those produced in urine that promote inter-male aggression (Chamero et al., 2007; Kaur et al., 2014; Novotny et al., 1985, 1984). In general, research on mammalian odor signals is dominated by urinary compounds that promote aggression (Barabas et al., 2021a). However, preliminary work has shown that geranylacetone detected in used nesting material has a negative correlation with home cage aggression (Barabas et al., 2021c). This compound has also been found in murine saliva and plantar sweat (Barabas et al., 2021c) and the ventral gland of hamsters, which is typically used for marking territory (Rendon et al., 2016; Wynne-Edwards et al., 1992). To the best of our knowledge, it has not been tested for a direct behavioral role in mice.

While minimizing home cage aggression would improve the welfare of laboratory mice, it is only the bare minimum that could be done for the animals' social environment. Promoting

positive affect and pleasurable emotional states is one key component of good overall welfare (Fraser et al., 1997). Since mice are naturally a social species (Latham and Mason, 2004), their welfare would be greatly enhanced if socio-positive/affiliative behaviors could be promoted in captivity. However, it has also been suggested that affiliative behaviors can play a context dependent role in resource control, proving more beneficial in situations with abundant resources, such as the laboratory (Pellegrini, 2008). Unfortunately, there is a lack of fundamental knowledge on how specific odors directly impact affiliative behaviors: in a scoping review focused on how odor signals impact mammalian social behavior, less than 2% of behavioral measures were affiliative (Barabas et al., 2021a). For mice, most work on captive social behavior focuses on aggression between unfamiliar males, leaving affiliation in the home cage overlooked. A key murine affiliative behavior is allo-grooming, which is often done to strengthen social bonds (Brown, 1985). Preliminary work found that three volatile organic compounds (VOC) correlate with allo-grooming in group housed male mice: 3,4-dimethyl-1,2-cyclopentanedione, 3,5-diethyl-2-hydroxycyclopent-2-en-1-one, and 6-hydroxy-6-methyl-3-heptanone (Barabas et al., 2021c). The two cyclopentanone compounds have never been tested for a direct animal behavior role and appear to be unique to murine plantar sweat glands (Barabas et al., 2021c). Plantar sweat does not have a confirmed role in terms of social interactions, but it has been associated with territory marking and colony member recognition (Brown, 1985; Ropartz, 1977). On the other hand, 6-hydroxy-6-methyl-3-heptanone is found in male mouse urine and is known to accelerate puberty in female mice (Novotny et al., 1999a). However, it has never been tested for a role between male mice.

This study served as a follow up to previous work demonstrating a correlation between four VOCs and social behavior in group housed male mice (Barabas et al., 2021c). All four VOCs show potential to be murine pheromones, but must undergo more stringent testing to be considered so (Wyatt, 2017, 2009). Therefore, the goal of this study was to examine the direct role of geranylacetone, 3,4-dimethyl-1,2-cyclopentanedione, 3,5-diethyl-2-hydroxycyclopent-2-en-1-one, and 6-hydroxy-6-methyl-3-heptanone on murine social behavior. We hypothesized that all four compounds could act as murine pheromones and alter social behavior. We had two predictions: first, geranylacetone would reduce aggression in the home cage; second, 3,4-dimethyl-1,2-cyclopentanedione, 3,5-diethyl-2-hydroxycyclopent-2-en-1-one, and 6-hydroxy-6-methyl-3-heptanone would increase allo-grooming among familiar male mice. In addition to social behavior,

subcutis wounding was examined as a secondary aggression measure and fecal corticosterone metabolites were assessed as an index of social stress.

5.3 Methods

5.3.1 Ethics statement

Animal procedures were approved by Purdue University's Institutional Animal Care and Use Committee (protocol # 1707001598). Humane endpoint criteria were established for cages displaying excessive aggression. Any mouse with wounding greater than $1cm^2$ would be immediately euthanized. Cages were monitored daily for wounding, signs of pain/distress, and general activity. Welfare checks occurred within two hours of the mice's active period to identify any wounding as quickly as possible. No cages met these criteria.

5.3.2 Treatment preparation

Three of the four compounds were obtained from commercial vendors and were stored according to manufacturer recommendations: geranylacetone and 3,4-dimethyl-1,2-cyclopentadione (Sigma- Aldrich, St. Louis, MO); 6-hyroxy-6-methyl-3-heptanone (Chemspace, Monmouth Junction, NJ). 3,5-diethyl-2-hydroxycyclopent-2-en-1-one was synthesized at Indiana University (Bloomington, IN) using previously described methods (Barabas et al., 2021c) and was kept in a -80°C freezer when not in use. Test solutions of each compound were formed based on natural concentrations that correlate with either lower levels of aggression or higher levels of affiliative behavior (Barabas et al., 2021c). The maximum compound weight previously detected in a single sweat or urine sample was adjusted to represent five mice per cage and used to calculate the concentrations for this study. The final concentrations are reported in Appendix E, Table E.1. However, we acknowledge that it is unknown if levels of 3,4-dimethyl-1,2-cyclopentadione and 3,5-diethyl-2-hydroxycyclopent-2-en-1-one are natural since pilocarpine was previously used to stimulate sweat production and it is unknown how compound values were affected (Barabas et al., 2021c). Stock solutions were made with ethanol (Thermo Fisher Scientific, Waltham, MA), and were further diluted to natural concentrations in a 3% polyethylene glycol (PEG; Sigma- Aldrich),

acetone (Thermo Fisher Scientific) solution. All ethanol stocks were stored at -80°C and acetone test solutions were stored in a refrigerator.

In order to determine how long treatments would be detectable in the cage, test solutions were administered to empty mouse cages containing chow, water, aspen bedding, and crinkle paper nesting material. Samples from the cages with the test solution were compared to samples from cages with a control solution (3% PEG in acetone only) to detect increased levels of the test compounds in the cage headspace. Test and control cages were sampled in adjacent, positive pressure rooms. First, 100µL of the solutions were applied to a square of clean medical gauze placed in a metal tea ball (Shuo, Novi, MI) that rested on top of the wire food hopper. Samples of the cage headspace were collected using Twister^{TM} polydimethylsiloxane coated stir bars (Gerstel USA, Linthicum, MD) on days 1, 3, 5, and 7 after treatment application. One stir bar was placed at each end of each cage in a metal tea ball and suspended from the wire food hopper for eight hours on each collection day. Stir bars were analyzed using gas chromatography- mass spectrometry (see below, "Gas chromatography- mass spectrometry").

Using natural concentrations, the test compounds were not elevated in the cage headspace compared to the control. Therefore, the compound concentrations were increased by 5x (Appendix E, Table E.1), and the procedure was repeated. The 5x concentration was sufficient to see increased levels of 6-hydroxy-6-methyl-3-heptanone in the headspace on collection days 1 and 3. The other three test compounds were not detectable in the headspace on any collection day. However, geranylacetone is a liquid at room temperature while 3,4-dimethyl-1,2-cyclopentadione and 3,5-diethyl-2-hydroxycyclopent-2-en-1-one are solids at room temperature, so the compounds likely retained these physical forms on the medical gauze instead of diffusing into the headspace.

Consequently, the administration route was changed, and the solutions were applied to the nesting material, so the mice could be in direct contact with the compounds (see below, "Treatment administration). Extractions from the treated nesting material were not tested as the processing chemicals in the material would have likely masked the compounds of focus. However, the treated nesting material's headspace was analyzed (see below, "Gas chromatography- mass spectrometry") and increased levels of 6-hyroxy-6-methyl-3-heptanone were detected on days 1 and 3 after treatment. For application consistency, all the test solutions were given to the mice at 5x natural concentrations and refreshed after 3 days.

5.3.3 Gas chromatography- mass spectrometry

All sample processing and analysis took place at the Indiana University Mass Spectrometry Facility (Bloomington, IN). Samples of nesting material were stored in Ziploc bags and refrigerated at 4°C. Samples were analyzed on the same day they were received. The procedure was started within an hour of receipt from Purdue University. Approximately 0.58 g of each nesting material sample was placed into a clean 20 mL headspace vial. A previously conditioned and cleaned Twister™ PDMS coated stir bar (10 x 0.5 mm, Gerstel USA, Linthicum, MD) suspended in a glass headspace vial adapter (Gerstel USA) and the vial was sealed with a new screw cap containing a PTFE-silicone septum (Restek Corp, Bellafonte, PA). The vials were left at room temperature for 1 hour.

All Twister™ stir bars (both those that were suspended in the test cages and those that were in vials with the nesting material) were placed in standard 7" desorption tubes and desorbed using Gerstel TDSA2 autosampler feeding a TDU 3 thermal desorption unit (Gerstel). Each Twister™ was flushed with 52 mL/min of He and was heated at 60 C/min to 270°C and held at 270°C for five minutes. The gas stream was directed into a Gerstel CIS-4 programmable temperature vaporizer inlet held at -80°C throughout the desorption process. The condensed sample molecules were introduced into an Agilent 7890B gas chromatograph (GC) by heating the CIS4 at 12°C/sec to 270°C and holding at 270°C for five minutes. The GC was set to solvent vent mode, and 23.573 psi was held in the inlet for 1.2 minutes. The GC column was a 30 m long, 250 µm inner diameter Agilent DB-5ms column with a 0.25 µm thick stationary phase. The oven was held at 40°C for one minute and then ramped at 2°C/min to 180°C followed by a ramp at 10°C/min to 270°C and held at that temperature for six minutes. The total cycle time was 86 minutes. An Agilent G7250B quadrupole-time-of-flight mass spectrometer served as the detector using a 70 eV electron ionization source. Mass spectra were recorded from m/z 41-400 at 5 scans/sec. Individual extracted ion chromatograms for each of the compounds were extracted using version 10.0 of Agilent Qualitative Analysis for GC-TOF.

5.3.4 Animals and housing

A factorial design was used based on the five solutions (four VOC test solutions and 3% PEG, acetone control) and two mouse strains. One hundred male mice of each of the

SJL/JOrlIcoCrl (SJL- Wilmington, MA) and C57BL/6NCrl (B6- Raleigh, NC and Kingston, NY) strains from Charles River were used (200 mice total). These strains were chosen based on correlation data from previous work (Barabas et al., 2021c). Mice arrived at 8 weeks of age and were housed in open top cages (11.5" x 7.25" x 4.25"; Ancare, Bellmore, NY) in groups of five for a one-week study period (N=40 cages). This sample size was determined *a priori* using Mead's resource equation (Mead, 1988). All cages contained aspen bedding (Envigo, Indianapolis, IN), 8g of crinkle paper nesting material (Enviro-dri, Fibercore, Cleveland, Ohio), and *ad libitum* food (Envigo, Teklad 2018) and water. A 12:12 light cycle was used throughout the study (lights on at 6:00). All mice were ear punched for identification and randomly allocated into cages upon arrival using a sequence from RANDOM.org. All mice were weighed at arrival and the end of the study. On average, mice were 21.70 ± 1.86g at arrival and 22.00 ± 2.26g at sacrifice.

Odor treatments cannot be administered in the same room due to cross contamination risk. Therefore, two rooms, each in a different building, were used in an incomplete block design: each solution was tested in each room, but the same solution was never tested concurrently in both rooms. Both facilities were located on Purdue University's West Lafayette, IN campus. Rooms in different facilities were intentionally chosen to examine if the treatments could overcome potential behavioral variation across facilities (Crabbe et al., 1999). Major parameter differences between the facilities are outlined in Table 5.1. Since only two rooms were used at one time, mice arrived in five batches of forty, equally split between strains (40 mice/5 mice per cage; n=8 cages per batch; 4 cages per room).

Table 5.1. Outline of parameter differences between housing rooms in different facilities.

	Facility A	**Facility B**
Temperature high interquartile range	22.22 – 22.78 °C	23.33 – 23.89 °C
Temperature low interquartile range	21.11 – 21.67 °C	20.56 – 21.11 °C
Humidity high interquartile range	43.5 – 50%	51 – 57%
Humidity low interquartile range	30 – 40%	30 – 43.5%
Air changes per hour	9.5	20.1
Water	Reverse osmosis	Tap water
Species on the same floor	Mice and pigs	Mice and rats
Care staff sex	Female only	Male and female

5.3.5 Treatment administration

Treatment order for each room was randomly assigned using a RANDOM.org list generator (Appendix E, Table E.2). Wash out periods between treatments lasted at least one week. Treatment solutions were administered using an opaque 5mL glass spray bottle (Your Oil Tools, Hooksett, NH). Approximately 120µL of each treatment were applied to the 8g of nesting material before the mice were allocated to their cages. Based on personal consultation with the company, each spray pump distributes approximately 60µL of solution (2 sprays/treatment). After treatment administration, empty cages sat for at least ten minutes to allow the acetone to evaporate, leaving PEG bound to any test compounds on the nesting material.

Based on headspace levels of 6-hydroxy-6-methyl-3-heptanone (see above, "Treatment preparation"), treatments were refreshed on day 3 of the study. Each cage received an additional 120µL of their assigned treatment applied to 1g of fresh nesting material. Each additional gram of treated nesting material sat for ten minutes in the housing room before being distributed to the mice. On study day 7, cages were cleaned with new bottoms, clean aspen, and 8g of fresh nesting material containing 120µL of the respective treatment. Like previous administrations, ten minutes passed between treatment application and transferring mice to the new cages.

5.3.6 Home cage behavior

Mouse cages were placed on wire metro racks, in video booths made of white foam board (Office Depot, Boca Raton, FL) to reduce background movement as done previously (Barabas et al., 2021c). Two shelves on each rack were used, and each shelf contained two cages, one of each strain. Video data was continuously recorded using infrared closed-circuit television cameras (HDview, Los Angeles, CA) and GeoVision surveillance software (Taipei, Taiwan). Social behavior was scored during the dark phase (18:00-6:00) using the following categories: escalated aggression, mediated aggression, submissive, and allo-grooming (Table 5.2). Data was collected using one-zero focal sampling every five minutes the first night after arrival (night 1), the night before the treatment refresher (night 3), the night after the treatment refresher (night 4), and the final night (night 7). Further, behavior was recorded for one hour after cage change (occurring approximately between 8:30-9:30 on day 7) as aggression can peak 15-45 minutes after cage change (Ambrose and Morton, 2000; Gray and Hurst, 1995). Two observers coded video (AJB

and a trained undergraduate assistant). Cages were randomly assigned a numerical label to blind observers to treatment, and they were viewed in a random order. It was not possible to blind observers to strain due the differing coat colors between B6 and SJL mice. Ten 12-hour periods of video were used for training representing 6.5% of the total video watched. Formal interrater reliability was calculated before coding began using Cronbach's alpha and was based on four observations periods (two per strain). Initial reliability scores are as follows: 0.97 (general activity), 0.93 (mediated aggression), 0.81 (escalated aggression), and 0.83 (allo-grooming). After coding was complete, reliability was assessed again using the last three observation periods viewed in the study. Final reliability scores are as follows: 0.97 (general activity), 0.81 (mediated aggression), 0.70 (escalated aggression), and 0.87 (allo-grooming). To replicate the methods used to identify the VOC and behavior correlations (Barabas et al., 2021c), all behaviors categorized as mediated aggression and submissive were initially coded as mediated aggression. However, in order to distinguish reactions to aggression from mediated behaviors, a single observer (AJB) recoded any instances of observed aggression to specify if submissive behaviors were performed. Hence, there is no reliability measure for submissive behaviors. From the video data, the proportion of active time in which each behavior category was observed was calculated per night per cage, as well as after cage change. These behavior measures are considered the primary outcome for this study.

Table 5.2. Ethogram of behaviors observed during the study. Definitions were taken from www.mousebehavior.org

Category	Behavior	Description
Mediated Aggression	Resource Theft	A mouse will approach another that is either eating a piece of food or chewing on a piece of bedding. The approaching mouse will then attempt to take the resource from the other's paws or mouth. It may or may not be successful. It is usually preceded by a social investigation and typically involves both mice tugging at the resource.
	Tail Rattling	The fast waving movements of the tail. This behavior may be partially obscured by bedding material, but can be detected by displacement of bedding near a mouse's tail.
	Thrust	The aggressor mouse will first threaten its target cage mate by thrusting its head and fore body towards its cage mate's head or body. The aggressor's paw may come in brief contact with the target, but otherwise no contact is made.
	Mounting	Attempts to mount another animal in the absence of intromission. Palpitations with forepaws and pelvic thrusts may be present.

Table 5.1 Continued

	Chase	A mouse will chase a fleeing partner, but no biting occurs
Submissive	Submissive Upright	A posture where the animal, will sit on its haunches in an upright position exposing the belly. The forepaws are off the ground and may stretch out its forepaws towards the threatening mouse. Mouse can also be laying on its side with one forepaw and one hind paw stretched toward the threatening mouse and its back touching the ground.
	Fleeing	This behavior is characterized by a mouse moving away from the mouse performing an aggressive behavior. Typically fleeing animals will run, but in a confined space may walk or turn first. Also score if the mouse turns away without locomoting. Only score if responding to an aggressive behavior (mediated/escalated).
Escalated Aggression	Bite	The aggressor mouse attacks the recipient with open mouth and appears to bite the recipient, or latches onto the recipient by his teeth, or forcefully touches the recipient who responds by jumping or fleeing quickly. Aggressor mouse may rush or leap at the victim. However, it also includes a mouse using its teeth to grab and tug on another's tail. Only score for the mouse that is biting, not the victims.
	Fighting	A behavior displayed by each animal when locked together. Separate behaviors are difficult to distinguish properly due to the fast rolling over and over seen with the animals kicking, biting, and wrestling. The initial victim retaliates towards the attacker and does not submit appropriately. Score for all mice actively involved in the fight.
Allo-grooming	In this interaction, an actor mouse frequently uses its forepaws for stability when grooming the recipient. During grooming, the actor mouths and licks the fur on the recipient's body. The actor will also use its teeth to clean the hair shaft by pulling the fur from the base of the hair shaft upward or outward.	
Active	Score if the mouse is visible and moving for more than 5 seconds.	

5.3.7 Fecal corticosterone metabolites

On day 7, fecal samples were collected by individually housing the mice in cages with a shallow layer of aspen bedding for two hours. Fecal corticosterone metabolites (FCM) increase approximately 8-10 hours after a spike in corticosterone if it occurs during a period when mice are mostly inactive (Touma et al., 2003). This analysis method is capable of detecting corticosterone spikes approximately 10 hours after injection procedures and 8-10 hours after an ACTH challenge, as well as corticosterone reductions starting 8 hours after dexamethasone administration (Touma et al., 2004). Further, since collection is non-invasive, data are not influenced by procedure related

stress, which is a risk of plasma collection. Previous data from this lab has shown that aggression counts peak in the last two hours of the dark, active period (unpublished data). Sample collections began between 13:30-14:00 to capture these final hours of the mice's active period, with most of the lag time occurring during the inactive period. Collecting during a limited time range also ensured that daily glucocorticoid fluctuations would not influence the data.

Afterwards, feces were gathered with metal forceps, placed in 1.5mL Eppendorf tubes, and stored in a -80°C freezer until processing. Samples were only analyzed from each cage's dominant and subordinate mouse, as glucocorticoids are elevated in animals undergoing repeated social defeat (Avitsur et al., 2001; Becker et al., 2008; Ely and Henry, 1978; Kinsey et al., 2007; Patki et al., 2013). Dominant and subordinate mice were determined by their preputial gland weight: body length ratio as this has been shown to align with individual conflict win/defeat patterns within a cage (Barabas et al., 2021b). Glands were weighed in mg with an analytical balance (Ohaus, Parsippany, NJ) and body lengths were taken in mm with digital calipers. Since this measure is obtained after euthanasia, feces were collected from all mice, but only analyzed from the mice with the highest and lowest preputial gland ratio per cage. If any of those mice did not produce enough feces for analysis (at least 20mg dry weight), they were excluded. Across cages, 90% of dominant mice and 92% of subordinate mice produced enough feces for analysis, leaving N=71 samples.

FCMs were obtained using a previously described method (Touma et al., 2003). Briefly, samples were dried at 80°C for two hours, dry mass weights were obtained, and each sample was crushed to a powder. A 20 - 50 mg (depending upon availability) aliquot of each dry sample was weighted. Steroids were extracted by adding 1mL of 80% methanol to the 50 mg of dry feces, or an aliquot in case of samples with less weight). Then samples were vortexed by hand for three 30 second periods and centrifuged for ten minutes at 2500 g. A portion (0.5 mL) of each methanolic supernatant was placed in a new Eppendorf tube and dried at 70°C for two hours. Dried extracts were shipped to the University of Veterinary Medicine, Vienna (Vienna, Austria) for enzyme immunoassays. After redissolving them in 80% methanol and diluting (1:20) with assay buffer, an aliquot was analyzed (in duplicate) in a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (details see: Touma et al., 2003), which has been successfully validated for use in mice (Touma et al., 2004).

5.3.8 Wounding

After fecal collection, mice were euthanized with prolonged CO_2 and carcasses were frozen. Wounding was assessed using the Pelt Aggression Lesion Scale (PALS; Gaskill et al., 2016). Briefly, pelts were gently separated from the carcasses and pinned to a dissection board at each limb. Photos of the subcutis were taken (Sony, Tokyo, Japan) and then evaluated using a 9 x 9 grid, which were overlaid on each pelt image. The grid was stretched from the base of the neck to the base of the tail. Each grid square was evaluated on a 0-4 scale in terms of percent of subcutis visible and wound severity. This scale has been previously described (Gaskill et al., 2016), but higher scores represent more visibility and severe damage. Each square was scored with the following equation:

PALS Grid Score = Severity Score x Visibility Score x 0.25.

The average anterior, middle, and posterior region scores were calculated using the three squares closest to the base of the neck, three in the center column of the grid, and three closest to the base of the tail, respectively. Posterior scores can distinguish aggression related wounding from ulcerative dermatitis (Gaskill et al., 2016), but this study served to validate these scores with behavior. For each mouse, PALS were averaged per region, then region averages summed across all the mice in the cage. This provided an overall level of wounding in each body region in a particular cage.

5.3.9 Data availability

All raw behavior, FCM, and wounding data are available in Appendix E.

5.3.10 Statistics

Missing data note: for behavior data, video from four cages on night one was excluded due to technical failure. These data points were balanced across strain, but were all from the same treatment (3,4-dimethyl-1,2-cyclopentadione). Further, one mouse from a cage of SJL treated with the control solution was found dead the morning of treatment refreshment (day 3), so video was only analyzed from night 1 and 3. This mouse did not contain wounding that met the humane endpoint criteria, so the cause of death is likely unrelated to aggression. Escalated aggression levels in this cage from days 1 and 3 were between the 60-75 quantile of values observed in the study

and the sum of posterior wounding in the cage was between the 50-55 quantile. Feces were not collected from this cage, but wound scores were included in the analysis. Ultimately, repeated measure behavior models contained N=154/160 observations, cage change behavior models contain N=39/40 observations, the wounding model contained N=120 observation (3 pelt region sums x 40 cages), and the FCM model contained N=71/80 observations.

All measures were analyzed with general linear mixed models. Strain, treatment, and the interaction were tested as fixed effects. Repeated measure behavior data also included study day as a fixed effect, as well as any 2-way interactions. The wounding model included pelt region and total proportion of escalated aggression performed in the cage as fixed effects and any 2-way interactions. The FCM model included dominance status and individual posterior PALS score along with any 2-way interactions. Any non-significant interactions were dropped from the final models due to a lack of orthogonal data. Facility was tested as a block and cageID nested in strain and treatment was tested as a random effect. Batch number served as a blocking factor, and would typically be tested as a fixed effect. However, since the study was designed using incomplete blocks, the analyses would not run with batch as a fixed effect. It has been argued that blocking factors can be considered random if treatments are randomly assigned to incomplete blocks (Dixon, 2016), which they were here. Any non-significant covariates or blocking factors were dropped from the final models. Model assumptions were evaluated post-hoc by examining the predicted by residual and normal Q-Q plots and transformations were made as needed. An exception was made for allo-grooming in the post cage change period. This behavior did not occur often during the observation period, so a Poisson regression was used to analyze behavior counts. Significant main effects were further analyzed with post hoc Tukey or student's t-tests. All analyses were done in JMP Pro (version 16.1.0). Significant P values from the behavior models were adjusted with the sequential Bonferroni correction to account for the multiple models assessing social behavior (Eichstaedt et al., 2013).

5.4 Results

5.4.1 Home cage behavior

Active period- repeated measures

Volatile treatment did not affect any active period behavior (see Table 5.3). All social behavior categories were significantly impacted by strain, while mediated aggression and allo-grooming were also impacted by study day (P values <0.001). SJL mice performed more escalated, mediated, and submissive behavior than B6 mice (Figure 5.1A, 5.1B, 5.1D). However, B6 mice performed more allo-grooming than SJLs (Figure 5.1E). Mediated aggression and allo-grooming were performed less on study day 1 compared to days 3, 4, and 7 (Tukey: P<0.05, Figure 5.1C, 5.1F).

For models where treatment was not significant, the effect size and least significant number (LSN) needed for a significant outcome with 80% power are reported in Table 5.4.

Table 5.3. Fixed effects and model R_{adj}^2 for each behavior measured across the study week (N=154).

	Strain	Treatment	Strain* Treatment	Day	Model R_{adj}^2
Escalated aggression	**$F_{1,28.09}$=114.04, P_{adj}<0.001**	$F_{4,28.12}$=0.89, P=0.484	$F_{4,28.06}$=1.36, P=0.274	$F_{3,110}$=0.09, P=0.967	0.73
Mediated aggression	**$F_{1,27.41}$=48.89, P_{adj} <0.001**	$F_{4,27.42}$=0.65, P=0.632	$F_{4,27.34}$=0.99, P=0.429	**$F_{3,109.8}$=7.65, P_{adj} <0.001**	0.47
Submission	**$F_{1,29.28}$=212.21, P_{adj} <0.001**	$F_{4,29.31}$=0.77, P=0.553	$F_{4,29.28}$=0.64, P=0.636	$F_{3,110.8}$=0.87, P=0.457	0.92
Allo-grooming	**$F_{1,29.73}$=56.18, P_{adj} <0.001**	$F_{4,29.76}$=0.28, P=0.887	$F_{4,29.73}$=0.51, P=0.731	**$F_{3,111.1}$=8.65, P_{adj} <0.001**	0.84

Significant effects are shown in bold; P_{adj} represents P values adjusted using the sequential Bonferroni correction

Table 5.4. Effect size (η_p^2) and least significant number (LSN) needed for a significant effect of treatment on each measure analyzed using mixed models.

	η_p^2	LSN
Escalated aggression- repeated	0.112	1041
Mediated aggression- repeated	0.087	656
Submission- repeated	0.095	1341
Allo-grooming- repeated	0.037	1098
Escalated aggression- cage change	0.104	173
Mediated aggression- cage change	0.045	386
Submission- cage change	0.129	261
Wounding	0.177	928
Fecal corticosterone metabolites	0.364	---

"---" indicates LSN not calculated as a significant effect was found

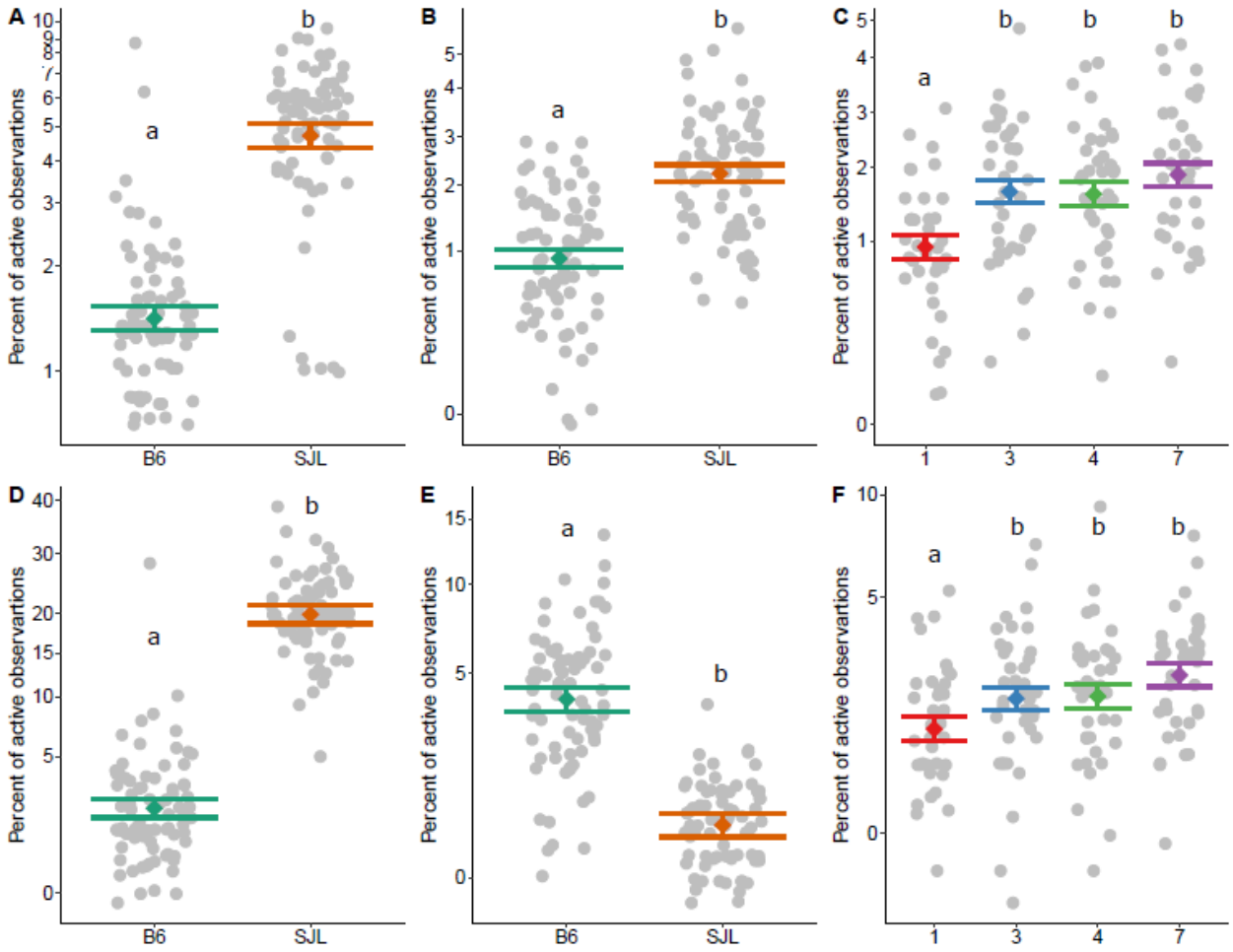

Figure 5.1. Social behavior was affected by strain and study day. SJL displayed more (A) escalated (P_{adj}<0.001) and (B) mediated aggression (P_{adj}<0.001). (C) Mediated aggression was also performed less on the first study day (P_{adj}<0.001). (D) SJL mice performed more submissive behavior (P_{adj}<0.001). (E) B6 mice performed more allo-grooming than SJL mice (P_{adj}<0.001).). (F) Allo-grooming was also performed less on the first study day (P_{adj}<0.001). All data are presented as factor level LSM ± SE with the scatter of individual residual error points (N=154). Significant post hoc comparisons are indicated by differing letters within a panel. Y axes are shown on a log10 back transformed scale in panel A, and a square root back transformed scale in panels B-F.

Cage change

Escalated aggression, mediated aggression, and allo-grooming after cage change were not significantly altered by any factor in this study (Table 5.5). However, submissive behaviors were impacted by strain (Table 5.5), where SJL mice performed more than B6. Please refer to Table 5.4 for effect sizes and LSN calculations for the treatment predictor tested with mixed models. Since allo-grooming after cage change was analyzed with a Poisson regression, the treatment effect size

is reported here as the rate ratio for each factor level compared to the control: 3,4-dimethyl-1,2-cyclopentadione- 1.58; 3,5-diethyl-2-hydroxycyclopent-2-en-1-one- 0.95; 6-hydroxy-6-methyl-3-heptanone- 1.95; geranylacetone- 0.95.

Table 5.5. Fixed effects and model R_{adj}^2 for each behavior measured after cage change (N=39).

	Strain	Treatment	Strain*Treatment	Model R_{adj}^2
Escalated aggression	$F_{1,29}$=3.91, P=0.061	$F_{4,29}$=0.83, P=0.512	$F_{4,29}$=1.47, P=0.238	0.08
Mediated aggression	$F_{1,29}$=2.32, P=0.139	$F_{4,29}$=0.34, P=0.850	$F_{4,29}$=2.16, P=0.098	0.09
Submission	**$F_{1,29}$=31.07, P<0.001**	$F_{4,29}$=1.08, P=0.386	$F_{4,29}$=0.85, P=0.506	0.42
Allo-grooming*	$\chi(1)$< 0.01, P=0.976	$\chi(4)$= 3.29, P=0.511	$\chi(4)$= 2.24, P=0.692	0.10

Significant effects are shown in bold; "*" analyzed using Poisson regression, generalized R^2 is reported for the final model that contained only the main strain and treatment effects.

5.4.2 Wounding

Wounding was significantly altered by the interaction between the strain and pelt region ($F_{2,74}$=13.56, P<0.001). The lowest wounding scores were seen in the anterior region of SJL cages (Tukey: P<0.05, Figure 5.2A). This was followed by scores in the anterior region of B6 cages and the middle region of SJL cages (Tukey: P<0.05, Figure 5.2A). The highest wounding scores were seen in the middle region in B6 cages and the posterior region of both strains (Tukey: P<0.05, Figure 5.2A). Wounding differences were also seen between pelt region and the proportion of time escalated aggression was observed while active ($F_{2,74}$=13.71, P<0.001). Posterior wounding was higher as more escalated aggression was observed (Figure 5.2B; t(74)= 5.15, α/3, P<0.001). In contrast, anterior wounding was lower as more escalated aggression was observed (t(74)= -3.39, α/3, P=0.001). The effect size and LSN for treatment are reported in Table 5.4.

5.4.3 Fecal corticosterone metabolites

The concentration of FCMs was altered by strain ($F_{1,30.2}$=58.24, P<0.001), treatment ($F_{4,25.81}$=3.69, P=0.017), posterior PALS score ($F_{1,49.87}$=8.14, P=0.006), and the treatment x average posterior PALS score interaction ($F_{4,46.48}$=4.69, P=0.003). B6 mice, regardless of treatment, had higher FCM than SJL mice (Figure 5.3A). For only mice treated with 3,4-dimethyl-1,2-cyclopentadione, FCM increased as posterior wounding increased (Figure 5.3B; t(54.98)= 3.68,

α/5, P<0.001). However, mice that were treated with 3,5-diethyl-2-hydroxycyclopent-2-en-1-one, FCM decreased as wounding increased (t(40.10)= -2.82, α/5, P=0.008). Overall, posterior wounding had a positive effect on FCMs (t(49.87)= 2.85, P=0.006).

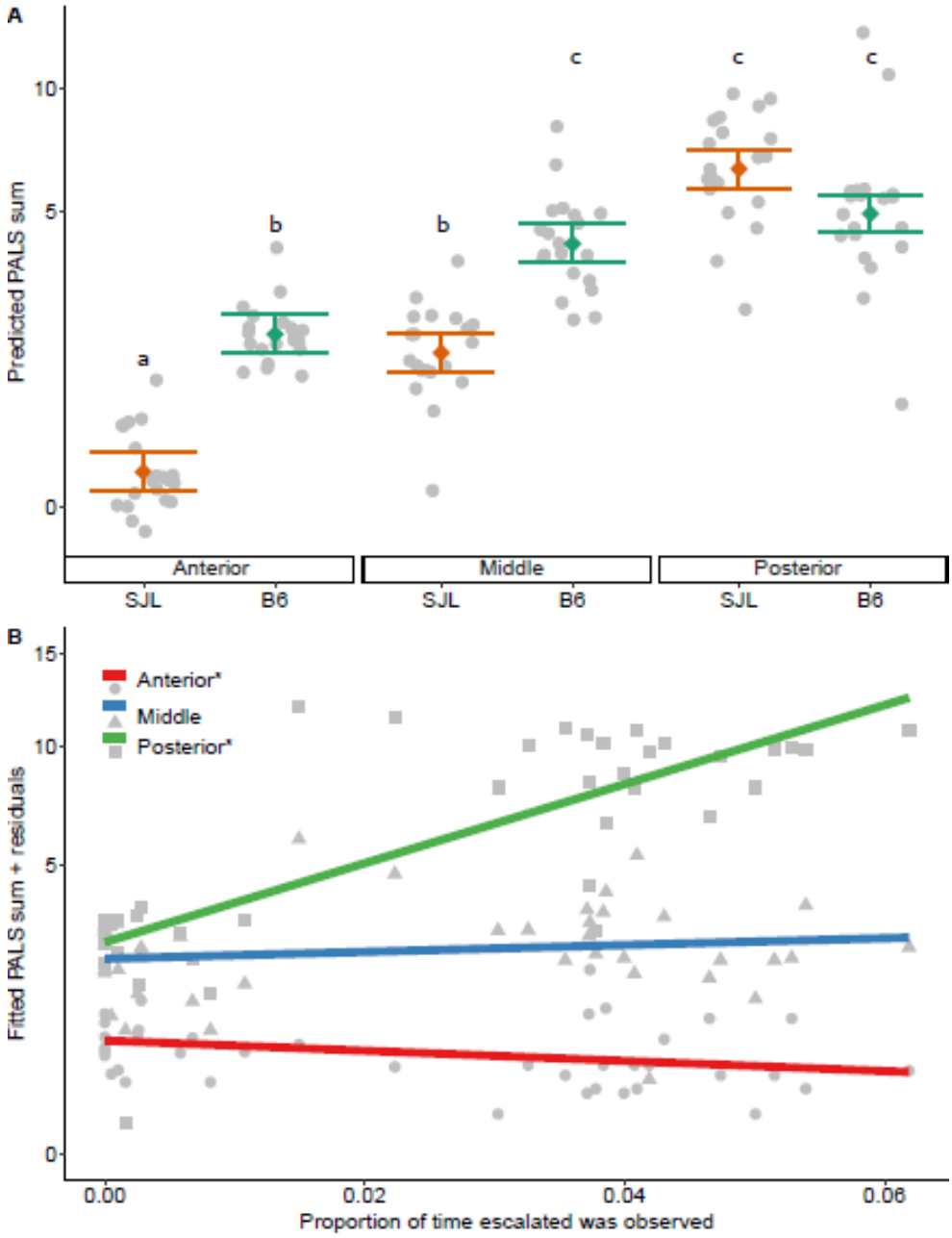

Figure 5.2. Wounding was impacted by (A) a strain x PALS region interaction and (B) a PALS region x proportion of escalated aggression interaction (R_{adj}^2 = 0.90, N=120). Data are presented as factor level LSM ± SE with the scatter of individual residual error points in panel A. Significant post hoc comparisons are indicated by differing letters within each panel. In panel B, data are presented as the best fit line per PALS region over a scatter of individual residual error points. Slopes that significantly differ from zero are marked by an "*" in the legend. Y axes are shown on a square root back transformed scale.

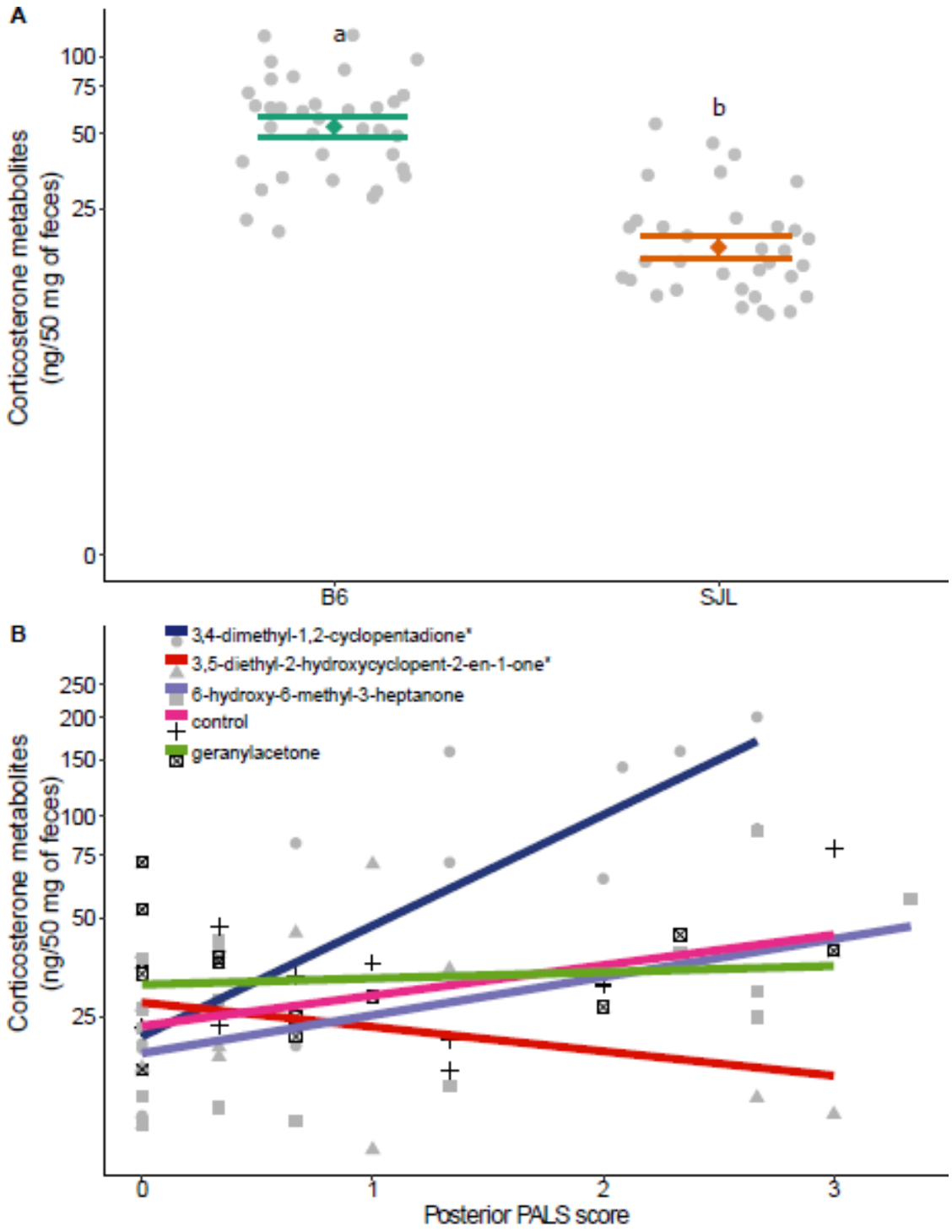

Figure 5.3. FCMs were impacted by (A) strain and (B) an interaction between posterior PALS score and treatment (R_{adj}^2 = 0.66, N=71). Data are presented as factor level LSM ± SE with the scatter of individual residual error points in panel A. Significant post hoc comparisons are indicated by differing letters within a panel. In panel B, data are presented as the best fit line per treatment over a scatter of individual residual error points. Slopes that significantly differ from zero are marked by an "*" in the legend. Y axes are shown on a log10 back transformed scale.

5.5 Discussion

This study aimed to test whether VOCs that previously correlated with male mouse social behavior directly influence home cage interactions and if they could be considered murine pheromones. Since geranylacetone negatively correlated with aggression (Barabas et al., 2021c), we expected it to reduce aggression here. We also expected 3,4-dimethyl-1,2-cyclopentanedione, 3,5-diethyl-2-hydroxycyclopent-2-en-1-one, and 6-hydroxy-6-methyl-3-heptanone to increase allo-grooming, since they previously correlated with this social behavior (Barabas et al., 2021c).

These data show that none of the VOC treatments tested here significantly altered social behavior in B6 or SJL mice. Based on η_p^2 calculations, these treatments had a small to intermediate statistical effect on most behaviors (Cohen, 1988). However, the LSN needed for a significant result is so large for each measure, that any biological effect is extremely low and likely not worth investigating. This could be due to the confounding effect of strain on the previous correlations as both behavior and VOC levels were largely strain dependent (Barabas et al., 2021c). Future endeavors could sample VOCs from cages with spontaneous occurrences of home cage aggression that are not so heavily strain biased. Further, the previous VOC datasets were reduced using Principal Component Analysis (Barabas et al., 2021c), and the components that explained the most variation were chosen to compare to behavior. It is possible that components with smaller explained variance had better predictive value (Jolliffe, 1982) and their respective high loading VOCs should be further examined.

That being said, there were also several factors in this study that could have led to the null results found. In order to detect VOC levels in the headspace of the cage, 5x the natural concentration was used. Using such a high concentration not only rules out the possibility of confirming pheromone activity, but it could also have been high enough to alter a behavioral response (Wyatt, 2017, 2009). Unfortunately, the true natural concentration of 3,4-dimethyl-1,2-cyclopentanedione and 3,5-diethyl-2-hydroxycyclopent-2-en-1-one have not been determined. Previously these compounds were identified in plantar sweat, which is produced in such low volumes that 1) pilocarpine is typically used to stimulate fluid production and 2) the samples were collected by directly rolling a Twister^{TM} stir bar on the foot which did not permit fluid volume to be recorded (Barabas et al., 2021c). While pilocarpine is often used in humans as a dry mouth remedy, there is individual variation in its effectiveness (Fox et al., 1991). Further analytical work is needed on plantar sweat itself to determine how pilocarpine may impact VOC content, how

much individual variation there is between mice injected with pilocarpine, and if VOCs can be collected without pilocarpine. This latter point would provide the most valid estimate of natural VOC concentrations in plantar sweat.

The application method could also have impacted the data seen here. The VOCs were administered in a 3% PEG, acetone solution as a first step to understand their efficacy at influencing behavior and to help rule out the effects of other molecules on behavior. However, two urinary murine pheromones known to increase inter-male aggression, 2-sec-butyl-thiazoline (SBT) and dehydro-exo-brevicomin (DHB), must be administered in castrate urine to provoke a behavioral response (Novotny et al., 1985). Both SBT and DHB are major urinary protein (MUP) ligands and need to interact with carrier proteins to be biologically active (Novotny et al., 1999b). The same may be true for the VOCs tested here. It is possible that 3,4-dimethyl-1,2-cyclopentanedione and 3,5-diethyl-2-hydroxycyclopent-2-en-1-one must be administered in murine sweat to increase allo-grooming. However, collecting enough sweat for a treatment would be challenging as mice produce less than 100nL of sweat without pilocarpine stimulation (Song et al., 2002) and creating a synthetic solution would not be possible without accurate compound concentrations (discussed above). While the concentration of geranylacetone used here was based on the levels found in used nesting material, it originates in both murine sweat and saliva (Barabas et al., 2021c), so it may need another component from one of these fluids to be biologically active. Along those lines, 6-hydroxy-6-methyl-3-heptanone may need to be administered in castrate urine to increase allo-grooming; it is a known MUP ligand (Novotny et al., 1999a) and may need to interact with carrier proteins to be effective. It is currently unknown if the other three VOCs are protein ligands, but the possibility that they need a transport protein cannot ruled out. Finally, SBT and DHB work synergistically to provoke a behavioral response (Novotny et al., 1985). It is possible that the VOCs tested here work in combination with one another, but this was not possible to test due to available time and resources.

While these specific compound treatments were not effective at improving male mouse social interactions, it cannot be denied that odor signals play a role in modulating home cage social behavior. General scent cue disruption can trigger aggression (Hurst et al., 1993; Jennings et al., 1998). The most common example of this effect is routine cage cleaning, after which aggression peaks are often seen. However, preserving used nesting material at cage cleaning can reduce aggression peaks, and it has been shown that used nesting material contains a variety of protein

associated odor signals used for identification purposes (Barabas et al., 2019; Van Loo et al., 2000). Since it is often recommended that male mice be kept in stable groups from an early age (Bartolomucci et al., 2002; Weber et al., 2017), perhaps odor profile familiarity is key for reducing aggression in the laboratory. Recognizing a cage mate's odor profile rather than individual appeasement odors may be sufficient to prevent fighting.

Social behavior was primarily impacted by strain, where SJL mice performed more aggression and submission while B6 mice performed more allo-grooming. These strain patterns are consistent with past work done by this group and another group's reported characterization of male SJL mice (Barabas et al., 2021c, 2021b; Festing, 1998). Interestingly, both mediated aggression and allo-grooming were performed less on the first study day than the others. This day effect was not previously reported, but past work found that cage level frequencies of allo-grooming are higher seven days after arrival compared to two days after (unpublished). The reduced levels of each behavior on the first night of the study may be because the mice were still acclimating to their new environment and spent less time engaging in these social behaviors. The similar pattern between these two behaviors is interesting as allo-grooming is often considered affiliative in mice (Brown, 1985). Anecdotally, allo-grooming in this study was often followed by chasing as the recipient tried to end the grooming bout and the actor followed in pursuit. This aligns with past work showing a correlation between an individual's place in a grooming network and their place in a chasing, but not fighting, network (So et al., 2015). This is not to suggest that allo-grooming is related to dominance, as the amount of allo-grooming performed and received did not predict social rank within the home cage (Barabas et al., 2021b).

Wound severity served as a secondary measure of escalated aggression and was impacted by an interaction between PALS region and the proportion of observed active time where escalated aggression was observed. At the cage level, wound severity in the posterior region increased with observed escalated aggression. This finding provides behavioral validation for past work showing that posterior PALS scores correctly predict fighting related wounding (Gaskill et al., 2016). Further, wounding was impacted by an interaction between strain and PALS region. The highest scores were seen in the posterior region of both strains as well as the middle section of B6 mice. This may suggest that while most aggression is directed at the hindquarters, B6 mice may have a larger target area that extends into the middle of the back.

Despite the lack of treatment effects on behavior, there was a significant interaction between treatment and posterior wounding on FCMs. Rodents undergoing repeated social defeat are known to have elevated plasma corticosterone levels in both short- and long-term measurements (Avitsur et al., 2001; Becker et al., 2008; Ely and Henry, 1978; Kinsey et al., 2007; Patki et al., 2013). The only treatment where this pattern extended to FCMs was 3,4-dimethyl-1,2-cyclopentanedione, despite similar wounding levels across treatments. It is unknown why this pattern was not seen in all mice, particularly the control mice. However, posterior wounding did have an overall positive effect on FCMs, implying that aggression related wounding has hormonal impacts that could alter a variety of research parameters. In contrast, mice treated with 3,5-diethyl-2-hydroxycyclopent-2-en-1-one had a negative relationship between wounding and FCMs. To the best of our knowledge, this pattern has not been documented before in mice. However, work in humans and non-human primates has shown that hypocortisolism can be a consequence of chronic stress, potentially protecting individuals from the consequences of prolonged HPA axis activity (Fries et al., 2005; Saltzman et al., 1998). It has been suggested that hypocortisolism in non-human primates can be an indicator of social stress (Mendoza et al., 2000), so a similar mechanism may explain these results in mice.

Finally, there was also a strain effect on FCMs: B6 mice had higher FCM concentration than SJL mice. Previous work has shown that strain can influence FCMs, with male B6 mice producing more FCMs than male BALB/c mice (Kalliokoski et al., 2012). In female mice, the strain effect has been variable across studies using B6, BALB/c and DBA mice (Walker et al., 2016, 2013). To the best of our knowledge, a comparison between male B6 and SJL mice has not been reported before.

5.5.1 Conclusion

This study served as a follow up to previous work demonstrating a correlation between four VOCs and reduced aggression or increased affiliative behavior in group housed male laboratory mice. While the treatments in this study did not impact social behavior in the home cage, it is possible that the administration methodology could have altered the VOCs' biological activity. It is worth pursuing future work using concentrations closer to natural levels and in solvents that better represent the natural fluids in which these VOCs were detected. Further, it is

possible that the tested VOCs were subjected to strain biases in the correlation study. Future sample analyses should focus on spontaneous occurrences of home cage aggression that are not so heavily strain biased.

5.6 Acknowledgments

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CHAPTER 6. CONCLUSIONS

Home cage aggression in male laboratory mice is one of the leading causes of poor animal welfare and reduces the quality of preclinical research data (Kappel et al., 2017; Poole, 1997; Weber et al., 2017). Since communication in mammalian species is largely dependent on olfaction (Apps et al., 2015), this sensory modality could be used as a tool to help improve social interactions in laboratory mice, by reducing aggression and/or increasing socio-positive (affiliative) behaviors. It is well known that urinary odor signals can promote aggression in male mice (Wyatt, 2014), but research on how odors can reduce social stress in captivity is lacking. Therefore, one purpose of the literature review was to quantify how same sex social behavior in captive mammals is impacted by odor treatments (Barabas et al., 2021a). This review showed that urine is the most prevalent source for odor treatments and aggression in a resident intruder paradigm (i.e., between unfamiliar animals) is the most commonly measured behavior. In general, urine treatments had a variable effect on aggression, likely due to the vast variety of tested components. In contrast, saliva was the next fluid source tested most often and it only accounted for 5% of treatments. Affiliative behaviors accounted for less than 2% of measured behaviors. This review emphasizes the present research gaps in our understanding of how odor signals affect social behavior in terrestrial mammals.

One step towards filling this gap is to explore the odors stored in used nesting material of laboratory mice. Aggression in male mice is often triggered at cage cleaning (Jennings et al., 1998), when mice are placed into a clean cage, devoid of odor signals. However, aggression can be reduced at this time if a portion of the nesting material is transferred to the new cage (Van Loo et al., 2000). Implementation of this practice has increased over the last two decades, yet no one can explain why it is effective. Many have speculated that the nest holds odor signals that reduce aggression, specifically odors produced in plantar sweat glands, but it has not been empirically proven. To clarify this assumption about what is in used nesting material, we conducted an exploratory study to document both protein and volatile organic compound (VOC) odors found in the nest. To identify the source of these odors, we also sampled plantar sweat, saliva, and urine. Home cage interactions were recorded to compare with odor profiles. This exploratory study found that both proteins and VOCs deposited in used nesting material come from plantar sweat, saliva, and urine sources (Barabas et al., 2021c, 2019). A majority of the proteins are from specific

families that give individual identity cues and bind VOCs that further indicate identity (Barabas et al., 2019). While the behavioral function of most of the VOCs in nesting material is unknown, one compound found in the nest (originating from sweat and saliva samples) had a negative correlation with home cage aggression (Barabas et al., 2021c). There were also three VOCs that correlated with more affiliative behavior: two were found in sweat samples and one in urine samples (Barabas et al., 2021c). The correlations between these four VOCs and home cage behavior suggest that the VOCs are candidates for future testing, but there is a caveat that both VOC profiles and behavior were largely strain dependent.

Before further VOC testing could occur, a challenge from the exploratory study needed to be addressed. Samples of plantar sweat, saliva, and urine were only analyzed from the dominant and subordinate mouse in each cage as social ranking can influence odor production (Harvey et al., 1989; Lee et al., 2017). Social ranking was determined by the tube test, from which scores indicate dominance, but test performance can be influenced by learning (Varholick et al., 2018; Wang et al., 2014). Social status based on tube test scores did not influence odor profiles of any fluid type, nor did it predict levels of specific compounds known to vary between dominant and subordinate mice. Therefore, we assessed the convergent validity of the tube test and potential physiological indicators of dominance (levels of darcin, a urinary pheromone; the preputial gland weight to body length ratio; wounding on subcutis tissue) with social ranking based on occurrences of home cage aggression (Barabas et al., 2021b). For each observed aggressive interaction, the aggressor and target mouse were recorded to calculate individual dominance scores. Then the following measures were obtained for individual mice: abundance of darcin in urine samples; scores from three rounds of tube tests; the ratio of preputial gland weight to body length; and postmortem wound severity. These six measures were condensed into two factors using factor analysis. The factor that represented urinary darcin and preputial gland: body length ratio was a significant predictor of dominance scores based on home cage aggression. This study showed that urinary darcin and the preputial gland: body length ratio show strong convergent validity with aggression based dominance in the home cage.

Finally, the four candidate VOCs from the exploratory study were tested for direct effects on social behavior in the home cage. Test solutions were formed by diluting each VOC in a 3% polyethylene glycol, acetone solution and spraying them on nesting material given to group housed male mice. Cages were randomly assigned to one of five treatments (four VOCs + control) and

home cage interactions were observed over four, 12-hour active periods and immediately after cage change. Postmortem wounding of the subcutis tissue was also assessed as well as fecal corticosterone metabolites, as indicators of social stress. Most study measures were not impacted by VOC treatment. However, several limitations could have contributed to these null results. First, the original exploratory study was heavily strain biased, and the candidate compounds could have correlated with behavior simply due to strain patterns. Second, the administration methods of this study could have impacted the observed behavior. The VOCs were administered in levels higher than their natural concentrations in order to verify their presence in the cage. This could have caused a sensory overload for the mice and negated any behavioral changes that may have occurred had biologically relevant concentrations been used. Further, the VOCs were administered in an acetone solution to rule out the effects of other molecules on behavior. This may have caused the VOCs to be ineffective as several known VOC pheromones must be administered in the fluid where they naturally occur. These fluids contain carrier proteins that aid with VOC biological activity and environmental stability. However, an alternative explanation is that simply preserving familiar odor signature mixtures in nesting material, instead of a specific appeasement signal, is enough to prevent home cage aggression. One of the most common recommendations for minimizing aggression is to maintain familiar social groups (Bartolomucci et al., 2002; Weber et al., 2017), so perhaps the presence of identity-specific proteins signals in the nest is key to cohesive groups. These signals could be maintained by housing male mice in stable groups from weaning and preserving used nesting material anytime the mice are moved to a new enclosure. This would include both cage cleaning and during transport.

Overall, these studies highlight the large research gap in how odor signals may improve social interactions and took the first step to filling that gap. Although the final study here had null results, work with pigs has shown that synthetic pheromones can effectively reduce aggression in groups of mixed weanlings (Guy et al., 2009; McGlone and Morrow, 1988). It is possible that the limitations mentioned above impacted the behavior observed here. Future studies could test the four VOCs identified here in combination with proteins to potentially improve signal transmission. However, more analytical work is needed first to confirm ligand potential of the VOCs. Further, more general information is needed on the properties of plantar sweat to determine the proper concentration of the two compounds uniquely detected in sweat samples. It would also be worth sampling VOC profiles from cages of mice displaying spontaneous aggression that are not

subjected to heavy strain bias. Perhaps additional sampling would highlight different compounds to be tested for behavioral effects.

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APPENDIX A. CHAPTER 1 SUPPLEMENTAL DATA

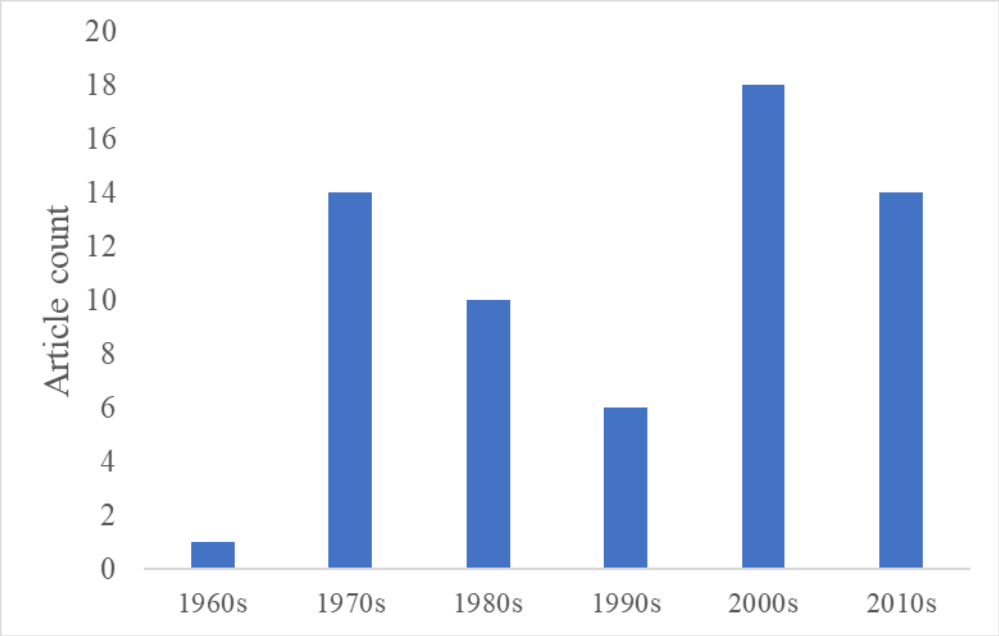


Figure A.1. Histogram of included article publication years, grouped by decade.

Table A.1. Risk of bias meta data.

SYRCLE question	Description
1. Randomized treatments/order	Did the authors describe a randomization method for assigning treatments/ treatment order, such as an automated sequence generator?
2. Similar baseline measures	Were baseline measures (behavior, olfactory ability) similar across groups/at each timepoint?
2a. If no, adjustments were made	If they were not equal, did the authors adjust for any differences?
3. Concealed treatment/order allocations	Were the treatment/ treatment order allocations concealed? Allocation should not be predictable in any way such as based on date of birth or cage/pen location.
4. Random housing	Were animals housed randomly throughout a facility, room, or cage rack?
4a. Unlikely that data was influenced by non-random housing	Is it unlikely that the outcome measure was influenced by non-random housing?

5. Blinded researchers	Were researchers and care staff blinded to the treatment assignment/ treatment order?
6. Random outcome assessment order	Were animals picked in a random order for outcome (behavior) assessment?
7. Blinded outcome assessment	Were researchers blinded to treatment when recording outcomes?
7a. Unlikely that data was influenced by lack of blinding	If they were not blinded, is the outcome unlikely to be influenced by this?
8. All samples were analyzed	Were all animals included in the analysis?
8a. Exclusion was unlikely to be related to the true outcome	Were reasons for exclusion unlikely to be related to the true outcome? A common example is a technical failure.
8b. Excluded data was balanced across groups	Were all excluded data balanced across treatment groups?
8c. Missing data was predicted appropriately	Were any missing data calculated or predicted with appropriate methods?
9. Protocol was available	Was the study protocol available and were all pre-specified outcomes listed?
9a. If protocol was not available, all outcomes were reported	If the protocol was not available, is it clear that all measured outcomes were reported?
10a. Treatments were free of contamination	Were the study treatments free of contamination? Contamination could occur if control and odor treatments were done simultaneously in the same room; if no cleaning procedures or wash out period were reported; or if endogenous odors could affect the treatment.
10b. Study was free of funder influence	Did the reported funder contribute to the study execution?
10c. Study was free of analysis errors	Was the study free of analysis errors? All model assumptions (homogeneity of variance/ normality of error) should be confirmed where appropriate. Individual data points should not be analyzed if the treatments were applied to a cage/pen.
10d. Excluded animals were replaced	If any animals were excluded, were new ones added as replacement?

Table A.2. List of articles included in the review.

First author	Title	Year	Journal
Andrist	Masking odour when regrouping rabbit does: effect on aggression, stress and lesions	2014	Livestock Science
Arakawa	Social features of scent-donor mice modulate scent marking of C57BL/6J recipient males	2009	Behav Brain Res
Arakawa	Scent marking behavior in male C57BL/6J mice: sexual and developmental determination	2007	Behav Brain Res
Bommel	Olfactory communication to protect livestock: dingo response to urine marks of livestock guardian dogs	2017	Australian Mammalogy
Cavaggioni	Absolute configuration of 2-sec-butyl-4,5-dihydrothiazole in male mouse urine	2003	Chem Senses
Chamero	Identification of protein pheromones that promote aggressive behaviour	2007	Nature
Connor	Olfactory control of aggressive and sexual behavior in the mouse (Mus musculus)	1972	Psychonomic Science
Corridi	Familiarity with conspecific odor and isolation-induced aggressive behavior in male mice (Mus domesticus)	1993	J Comp Psychol
Daly	Some Experimental Tests of the Functional Significance of Scent-Marking by Gerbils (Meriones unguiculatus)	1977	J Comp Physiol Psychol
Drea	Responses to olfactory stimuli in spotted hyenas (Crocuta crocuta): II. Discrimination of conspecific scent	2002	J Comp Psychol
Evans	Attempts to characterise and isolate aggression reducing olfactory signals from the urine of female mice Mus musculus L	1978	Physiol Behav
Fischer	Vaginal secretions increase the likelihood of intermale aggression in Syrian hamsters	1993	Physiol Behav
Gomes	The role of scent-marking in patchy and highly fragmented populations of the Cabrera vole (Microtus cabrerae)	2013	Zoolog Sci
Gray	The effects of cage cleaning on aggression within groups of male laboratory mice	1995	Animal Behaviour
Hattori	Self-Exposure to the Male Pheromone ESP1 Enhances Male Aggressiveness in Mice	2016	Curr Biol
Hopp	Odor cue determinants of urine marking in male rats (Rattus norvegicus)	1983	Behav Neural Biol
Hurst	The priming effects of urine substrate marks on interactions between male house mice, Mus musculus domesticus Schwarz & Schwarz	1993	Animal Behaviour
Hughes	Receiving behaviour is sensitive to risks from eavesdropping predators	2009	Oecologia

Idris	Behavioural responses of desert gerbil, Meriones hurrianae after removal of scent marking gland	2011	Indian J Exp Biol
Ingersoll	Latent aggression-promoting properties of mouse bladder urine activated by heat	1986	Behav Neurosci
Ingersoll	beta-Glucuronidase activation of latent aggression-promoting cues in mouse bladder urine	1982	Physiol Behav
Isogai	Multisensory Logic of Infant-Directed Aggression by Males	2018	Cell
Johnston	The causation of two scent-marking behaviour patterns in female hamsters (Mesocricetus auratus)	1977	Animal Behaviour
Johnston	Scent marking by male golden hamsters (Mesocricetus auratus). I. Effects of odors and social encounters. II. The role of the flank gland scent in the causation of marking. III. Behavior in a seminatural environment	1975	Zeitschrift fur Tierpsychologie
Jones	Effects of preputial and coagulating gland secretions upon aggressive behaviour in male mice: a confirmation	1973	J Endocrinol
Jones	Effects of clean and soiled sawdust substrates and of different urine types upon aggressive behavior in male mice	1975	Aggressive Behavior
Kaur	Murine pheromone proteins constitute a context-dependent combinatorial code governing multiple social behaviors	2014	Cell
Kleiman	The effects of exposure to conspecific urine on urine-marking in male and female degus (Octodon degus)	1975	Behav Biol
Lacey	The importance of exposure to other male scents in determining competitive behaviour among inbred male mice	2007	Applied Animal Behaviour Science
Lisberg	Effects of sex, social status and gonadectomy on countermarking by domestic dogs, Canis familiaris	2011	Animal Behaviour
Mackintosh	The effect of olfactory stimuli on the agonistic behaviour of laboratory mice	1966	Z Tierpsychol
Martínez-Macipe	Evaluation of an innovative approach for sensory enrichment in zoos: semiochemical stimulation for captive lions (Panthera leo)	2015	Animal Welfare
Maruniak	Urinary marking in male house mice: responses to novel environmental and social stimuli	1974	Physiol Behav
McGlone	Olfactory cues and pig agonistic behavior: evidence for a submissive pheromone	1985	Physiol Behav
McGlone	Reduction of Pig Agonistic Behavior by Androstenone	1988	Journal of Animal Science
McGlone	Synthetic maternal pheromone stimulates feeding behavior and weight gain in weaned pigs	2002	Journal of Animal Science
McGlone	Evidence for aggression-modulating pheromones in prepuberal pigs	1987	Behav Neural Biol

Monclus	Context-dependent responses to neighbours and strangers in wild European rabbits (Oryctolagus cuniculus)	2014	Behav Processes
Morgan	Melanocortin-5 receptor deficiency reduces a pheromonal signal for aggression in male mice	2001	Chem Senses
Mucignat-Caretta	Male urinary chemosignals differentially affect aggressive behavior in male mice	2004	J Chem Ecol
Mugford	Intermale fighting affected by home-cage odors of male and female mice	1973	J Comp Physiol Psychol
Mugford	Pheromones and their effect on aggression in mice	1970	Nature
Nakamura	The critical role of familiar urine odor in diminishing territorial aggression toward a castrated intruder in mice	2006	Physiol Behav
Nevison	The consequences of inbreeding for recognizing competitors	2000	Proc Biol Sci
Novotny	Synthetic pheromones that promote inter-male aggression in mice	1984	Proc Natl Acad Sci
Ogata	Clinical trial of a feline pheromone analogue for feline urine marking	2001	J Vet Med Sci
Payne	Pheromonal effects of Harderian gland homogenates on aggressive behaviour in the hamster	1977	J Endocrinol
Petrulis	Lesions centered on the medial amygdala impair scent-marking and sex-odor recognition but spare discrimination of individual odors in female golden hamsters	1999	Behav Neurosci
Petrulis	The role of the hippocampal system in social odor discrimination and scent-marking in female golden hamsters (Mesocricetus auratus)	2000	Behav Neurosci
Pettijohn	Reaction of male Mongolian gerbils to odors in a social situation	1982	J Comp Physiol Psychol
Plush	A synthetic olfactory agonist reduces aggression when sows are mixed into small groups	2016	Applied Animal Behaviour Science
Reasner	Scent marking by male dwarf hamsters (Phodopus sungorus campbelli) in response to conspecific odors	1987	Behav Neural Biol
Schell	Olfactory attractants and parity affect prenatal androgens and territoriality of coyote breeding pairs	2016	Physiol Behav
Stehn	Female odors and aggression among male Microtus	1976	Behav Biol
Svare	Aggressive behavior of juvenile mice: influence of androgen and olfactory stimuli	1975	Dev Psychobiol
Taha	Extracts from salivary glands stimulate aggression and inositol-1, 4, 5-triphosphate (IP3) production in the vomeronasal organ of mice	2009	Physiol Behav
Tang-Martinez	Individual odours and mating success in the golden hamster, Mesocricetus auratus	1993	Animal Behaviour

Taylor	Urinary odors and size protect juvenile laboratory mice from adult male attack	1982	Dev Psychobiol
Thompson	Chemosensory cues from the lacrimal and preputial glands stimulate production of IP3 in the vomeronasal organ and aggression in male mice	2007	Physiol Behav
Tinnes	Will Trespassers Be Prosecuted or Assessed According to Their Merits? A Consilient Interpretation of Territoriality in a Group-Living Carnivore, the European Badger (Meles meles)	2015	PLoS One
Van den Berghe	Dog appeasing pheromone prevents the androgen surge and may reduce contact dominance and active submission after stressful interventions in African wild dogs (Lycaon pictus)	2019	PLoS One
Van Loo	Modulation of aggression in male mice: influence of cage cleaning regime and scent marks	2000	Animal Welfare
Yonezawa	Appeasing pheromone inhibits cortisol augmentation and agonistic behaviors during social stress in adult miniature pigs	2009	Zoolog Sci

Table A.3. Animal subject demographics used in odor literature

Category	Percent
Age	
variable	1.04
juvenile	5.21
not reported	8.33
sexually mature	85.42
Sex	
female	8.33
male and female	20.83
male	70.83
Housing	
not reported	2.08
social + solitary	4.17
social	43.75
solitary	50
Enrichment	
NA (wild/pets)	3.13
yes	15.63
not clearly reported	81.25

Table A.4. Odor donor demographics used in odor literature

Category	Percent
Age	
variable	1.36
juvenile	8.18
not clearly reported	12.73
NA (synthetic)	14.55
sexually mature	63.18
Sex	
male-no preputial	0.45
male and female	4.09
not reported	4.09
male- castrated	4.09
NA (synthetic)	14.55
female	16.82
male	55.91
Housing	
social + solitary	0.9
NA (synthetic)	14.55
solitary	17.73
social	32.73
not reported	34.09
Familiarity to subject	
familiar	12.27
NA (synthetic)	14.55
not reported	25.91
unfamiliar	47.27
Fight status	
no observed fighting	3.18
fighting	4.09
variable*	4.55
NA (synthetic or solitary housing)	32.73
not reported	55.45

*mice exposed to own odors, fighting varied across cages

Text A.1. Database search strategies.

Pubmed.

1: (chemical*[Title/Abstract] OR "scent*"[Title/Abstract] OR "odor*"[Title/Abstract] OR "odour*"[All Fields] OR Odorants[Mesh] OR pheromones[Mesh] OR pheromon*[Title/Abstract])

2: (smell[MeSH Terms] OR olfactory[Title/Abstract] OR olfaction[Title/Abstract] OR "chemoreceptor cells"[MeSH Terms] OR chemoreceptor*[Title/Abstract] OR chemosignal* [Title/Abstract] OR "taste buds"[MeSH] OR “taste receptor*”[Title/Abstract] OR “taste buds”[Title/Abstract] OR "Vomeronasal Organ"[Mesh] OR vomeronasal[Title/Abstract] OR "Endocrine Disruptors"[Mesh] OR “endocrine disruptor*”[Title/Abstract] OR urine[MeSH Terms] OR urine[Title/Abstract] OR urinary[Title/Abstract] OR feces[MeSH Terms] OR feces[Title/Abstract] or faeces[Title/Abstract] OR fecal[Title/Abstract] OR faecal[Title/Abstract] OR "Lacrimal Apparatus"[Mesh] OR "Apocrine Glands"[Mesh] OR "Exocrine Glands"[Mesh] OR "Eccrine Glands"[Mesh] OR "musk" [Supplementary Concept] OR "Scent Glands"[Mesh] OR "Salivary Glands"[Mesh] OR lacrimal[Title/Abstract] OR salivary[Title/Abstract] OR apocrine[Title/Abstract] OR holocrine[Title/Abstract] OR merocrine[Title/Abstract] OR eccrine[Title/Abstract] OR preputial[Title/Abstract] OR ventral[Title/Abstract] OR sweat[Title/Abstract])

3: ("Behavior, Animal"[MeSH Terms] OR behavior*[Title/Abstract] OR behaviour*[Title/Abstract] OR Ethology[Mesh Terms] OR ethology[Title/Abstract] OR "Animal Welfare"[Mesh] OR welfare[Title/Abstract])

4: (aggression[MeSH Terms] OR "Agonistic behavior"[MeSH] OR "Stress, Physiological"[MeSH Terms] OR anxiety[MeSH Terms] OR "Social Behavior"[Mesh] OR aggression[Title/Abstract] OR aggressive[Title/Abstract] OR agonistic[Title/Abstract] OR stress[Title/Abstract] OR anxiety[Title/Abstract] OR "anti anxiety"[Title/Abstract] OR social[All Fields] OR antisocial[All Fields] OR "anti social"[Title/Abstract] OR interaction*[Title/Abstract] OR play[Title/Abstract] OR nonreproductive[Title/Abstract] OR "non-reproductive"[Title/Abstract] OR

nonsexual[Title/Abstract] OR "non-sexual"[Title/Abstract] OR investigative[Title/Abstract] OR defensive[Title/Abstract] OR affiliative[Title/Abstract])

1 AND 2 AND 3 AND 4
Apply PubMed filter “Other Animals”

CAB Abstracts.

1: TS=(chemical* OR scent* OR odor* OR odour* OR odorant* OR pheromone*)

2: TS=(smell* OR olfactory OR olfaction OR chemorecept* OR chemosignal* OR "taste bud*" OR "taste receptor*" OR vomeronasal OR "endocrine disrupt*" OR urine OR urinary OR feces OR faeces OR fecal OR faecal OR lacrimal OR apocrine OR exocrine OR eccrine OR musk OR "scent gland*" OR salivary OR holocrine OR merocrine OR preputial OR "ventral gland*" OR "sweat gland*")

3: TS=(behavior* OR behaviour* OR ethology OR welfare)

4: TS=(aggression OR aggressive OR agonistic OR stress OR anxiety OR "anti anxiety" OR antianxiety OR social OR "anti social" OR antisocial
OR interaction* OR welfare OR play OR "non reproductive" OR nonreproductive OR nonsexual OR “non sexual” OR affiliative OR investigative OR defensive)

5: 1 AND 2 AND 3 AND 4
5 AND BD=(mammals)

Agricola

1: (chemical* OR scent* OR odor* OR odour* OR odorant* OR pheromon*).ti,ab.

2: (smell* OR olfactory OR olfaction OR chemorecept* OR chemosignal* OR (taste ADJ bud*) OR (taste ADJ receptor*) OR vomeronasal OR (endocrine ADJ disrupt*) OR urine OR urinary OR feces OR faeces OR fecal OR faecal OR lacrimal OR apocrine OR exocrine OR eccrine OR musk OR (scent ADJ gland*) OR (salivary ADJ gland*) OR holocrine OR merocrine OR preputial OR (ventral ADJ gland*) OR (sweat ADJ gland*)).ti,ab.

3: (behavior* OR behaviour* OR ethology OR welfare).ti,ab.

4: (aggression OR aggressive OR agonistic OR stress OR anxiety OR anti-anxiety OR (anti ADJ anxiety) OR antianxiety OR social OR (anti ADJ social) OR anti-social OR antisocial OR interaction* OR play OR (non ADJ reproductive) OR nonreproductive OR nonsexual OR (non ADJ sexual) OR affiliative OR investigative OR defensive).ti,ab.

1 AND 2 AND 3 AND 4

APPENDIX B. CHAPTER 2 SUPPLEMENTAL DATA

Table B.1. Comprehensive list of all proteins detected across samples. Detected proteins had at least 2 MS/MS counts in two replicates of a single sample type. List is limited to the first two protein IDs where applicable and organized in descending order by how many sample types each protein was detected in.

Proteins in all 4 sample types		
Protein IDs	Protein names	Gene names
P00687;Q99KE6	Alpha-amylase 1	Amy1
B1ARR4	Carbonic anhydrase 6	Car6
A2AJD1	BPI fold-containing family B, member 9B	Bpifb9b
Q8R1E9;Q7TNY5	ABPBG27; Salivary androgen-binding protein beta subunit	Scgb2b27;Abpb
Q3UU48;P02816	Prolactin-inducible protein homolog	Pip
A2ANT5;P11590	Major urinary protein 4	Mup4
Q9D3H2	Odorant-binding protein 1a	Obp1a
Q546G4;P07724	Serum albumin	Alb
A2BHD2	Predicted gene 14743	Gm14743
O88968	Transcobalamin-2	Tcn2
O35176	Androgen binding protein A2	Scgb1b2
Q58ES8;A2CEL1	Major urinary protein 1	Mup1;Mup1
D2XZ31;E9PWZ2	Androgen binding protein A7; A20	Abpa29_a7;Scgb1b20
Q921I1;E9Q035	Serotransferrin	Tf;Gm20425
A2BIN1;Q4FZE8	Major urinary protein 10; Major urinary protein 1	Mup10;Mup1
Q5FW60	Major urinary protein 20	Mup20
E9QNP3;F8WJ23	Hornerin	Hrnr
A8DUK4;A8DUK7	Beta-globin	Hbbt1;Hbb-bs
Q3UAF7;Q3UAF6	Actin, cytoplasmic 1	Actb;Actg1
D2KHZ9;A0A0A0MQF6	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Q58E64;Q3UA81	Elongation factor 1-alpha 1;Elongation factor 1-alpha 2	Eef1a1;Eef1a2
Q3KQQ2;P04939	Major urinary protein 3	Mup3
Proteins in 3 sample types		
Protein IDs	Protein names	Gene names
Saliva, Sweat and Nest Proteins		
Q91WB5;G3UXN8	Androgen binding protein A27	Scgb1b27
A2AEN9	Predicted gene 5938	Gm5938
D2XZ37;G5E8B4	Secretoglobin family 2B member 2	Scgb2b2
Q5FW97;P17182	Alpha-enolase;Enolase	EG433182;Eno1
Q08189	Protein-glutamine gamma-glutamyltransferase E	Tgm3
Q3UEK9;Q3UEK5	Alpha-2-HS-glycoprotein	Ahsg
Q91X72	Hemopexin	Hpx

Saliva Sweat and Urine Proteins		
Q564E2;Q3TI99	L-lactate dehydrogenase; L-lactate dehydrogenase A chain	Ldha
P06745;B2RXT5	Glucose-6-phosphate isomerase	Gpi;Gpi1
Saliva Nest and Urine Proteins		
Q91XA9	Acidic mammalian chitinase	Chia
Q6PZE0	Mucin-19	Muc19
Q8C6C9;D3YTP1	Protein LEG1 homolog	Leg1;2310057J18Rik
Q3UU35;Q3TTY9	Ovostatin homolog	Ovos;BC048546
A0A1R3UFA0;P15949	Kallikrein 1-related peptidase b9	Klk1b9
A0A1R3UGI5;P07628	Kallikrein 1-related peptidase b8	Klk1b8
Q61114	BPI fold-containing family B member 1	Bpifb1
B1AVU4	Predicted gene 14744	Gm14744
A0A1R3UDC2;P36369	Kallikrein 1-related peptidase b26	Klk1b26
Q91WL7;Q14BW7	Deoxyribonuclease	Dnase1
Q5SW46;Q91WA0	Lactoperoxidase	Lpo
L7N1X9	Demilune cell and parotid protein 1	Dcpp1
A0A0B6VSQ6;P15947	Kallikrein-1	Klk1
L7N259	Demilune cell and parotid protein 3	Dcpp3
Q9CPP7	Gastric triacylglycerol lipase	Lipf
E9PYC2	Demilune cell and parotid protein 2	Dcpp2
A0A1R3UGK0;P00757	Kallikrein 1-related peptidase-like b4	Klk1b4
Q3V469;Q9JHY3	WAP four-disulfide core domain protein 12	Wfdc12
P11591	Major urinary protein 5	Mup5
Q8VC95;Q9EQG0	Mucin cell adhesion protein	Prol1
P08071;Q4FJR3	Lactotransferrin	Ltf
Q3UTR7;P11859	Angiotensinogen	Agt
D3YYY1	Androgen binding protein BG7	Scgb2b7
S4R244;Q544L5	Prostatic spermine-binding protein	Sbp
A2BHR2	Lipocalin 11	Lcn11
Q8VD07;P01132	Pro-epidermal growth factor; Epidermal growth factor	Egf
Q8BND5	Sulfhydryl oxidase 1	Qsox1
Q3TWM9;E9PZ00	Prosaposin	Psap
O70570	Polymeric immunoglobulin receptor; Secretory component	Pigr
Q3UCD9;Q3U7P0	Cathepsin D	Ctsd
A0A0R4J043;P28825	Meprin A subunit alpha; Metalloendopeptidase	Mep1a
P05533	Lymphocyte antigen 6A-2/6E-1	Ly6a
Sweat Nest and Urine Proteins		

C1KG51;A0A0A6YVU7	Truncated profilaggrin/filaggrin flaky tail mutant form	Flg
Q3MI12;Q9D6T8	2310057N15Rik protein (Fragment)	2310057N15Rik
Q91VB8;Q9CY10	Hemoglobin subunit alpha	haemaglobin alpha 2;Hbat1
Q5FWB7;P05064	Fructose-bisphosphate aldolase	Aldoa
G5E8Z3	MCG120169	2310050C09Rik
P52480;A0A1L1SU37	Pyruvate kinase PKM	Pkm
P11589	Major urinary protein 2	Mup2
Q58EV3;E9QA79	Major urinary protein 1; Major urinary protein 7	Mup1;Mup7
P22599	Alpha-1-antitrypsin 1-2	Serpina1b
A0A0R4J0I1;P07759	Serine protease inhibitor A3K	Serpina3k
A2CEK7	Major urinary protein 12	Mup14
P08228	Superoxide dismutase [Cu-Zn]	Sod1
Proteins in 2 sample types		
Protein IDs	Protein names	Gene names
Saliva and Sweat Proteins		
B2RTM0;P62806	Histone H4	Hist2h4;Hist1h4a
P01027	Complement C3	C3
Q00898	Alpha-1-antitrypsin 1-5	Serpina1e
P23953	Carboxylesterase 1C	Ces1c
Saliva and Nest Proteins		
B7ZCG3;P07743	BPI fold-containing family A member 2	Bpifa2
A0A1R3UCH4;P04071	Kallikrein 1-related peptidase b16	Klk1b16
Q540N3;P15948	Kallikrein 1-related peptidase b22	Klk1b22
Q8JZX1;Q7M745	Androgen binding protein BG26	Scgb2b26
A0A1R3UHM9;P00756	Kallikrein 1-related peptidase b3	Klk1b3
Q8K1H9	Odorant-binding protein 2a	Obp2a
B9EKG3;Q9Z0L8	Gamma-glutamyl hydrolase	Ggh
F6URP1	Predicted gene 6619	Gm6619
A0A1R3UCH5;Q9JM71	Kallikrein 1-related peptidase b27	Klk1b27
A0A1R3UCH6;P00755	Kallikrein 1-related peptidase b1	Klk1b1
Q545H0;Q03401	Cysteine-rich secretory protein 1	Crisp1
A0A1R3UDS6;P15946	Kallikrein 1-related peptidase b11	Klk1b11
A2BHR0	Odorant-binding protein 2b	Obp2b
A0A0G2JEK0;Q6LDU8	Beta-nerve growth factor	Ngf
Q80XI7	Vomeromodulin	Vom
E9QPG8;A0A140LI59	Deleted in malignant brain tumors 1 protein	Dmbt1
D2XZ39;Q7M747	Secretoglobin family 2B member 24	Scgb2b24
Q66VB7	Lacrein	Gm1553
A0A075B6A3;A0A0A6YXW6	Ig alpha chain C region	Igha;Igh;Igh-VJ558
Q3UP42;P31725	Protein S100-A9	S100a9

A8R0U8;A8R0U7	Exocrine gland secreted peptide 15	Esp15
Saliva and Urine Proteins		
E9Q5I3;Q8K1G6	Mucin 5, subtype B, tracheobronchial	Muc5b
Q9JM84	Cystatin 10	Cst10
Q3UKN6;P81117	Nucleobindin-2;Nesfatin-1	Nucb2
Q549A5;Q06890	Clusterin beta chain; Clusterin alpha chain	Clu
Q9D6Y8;Q9CPP2	Uncharacterized protein	Sbpl
Sweat and Nest Proteins		
Q9D6L6	RIKEN cDNA 2310079G19 gene	2310079G19Rik
E9QPZ3;Q2VIS4	Filaggrin-2	Flg2
P18165	Loricrin	Lor
D3Z724	Predicted gene 5965	Gm5965
Q5SXZ7;A0A0A0MQG3	Integrator complex subunit 2	Ints2
Q3TB63;Q3UDS0	Heat shock cognate 71 kDa protein; Heat shock-related 70 kDa protein 2	Hspa8;Hspa2
Q7TPC1;Q3V0M9	Corneodesmosin	Cdsn
Q61171;D3Z4A4	Peroxiredoxin-2	Prdx2
Q61838	Alpha-2-macroglobulin	A2m
Q5M9K1;P07309	Transthyretin	Ttr
Sweat and Urine Proteins		
E9Q557;E9PZW0	Desmoplakin	Dsp
P09411;S4R2M7	Phosphoglycerate kinase 1	Pgk1
Q52L87;Q3TIZ0	Tubulin alpha-1C chain; Tubulin alpha-1A chain	Tuba1c;Tuba1a
P00920;A0A0A6YX78	Carbonic anhydrase 2	Ca2;Car2
Q5SVY2;Q3UAJ1	Peptidyl-prolyl cis-trans isomerase	Ppia
Q00897	Alpha-1-antitrypsin 1-4	Serpina1d
G3UVV4;Q6GQU1	Hexokinase	Hk1
Urine and Nest Proteins		
Q91X17	Uromodulin	Umod
B7ZNS9;Q3UP47	Complement factor D	Cfd
L7MUC7	Major urinary protein 7 (Fragment)	Mup7
Q9JM79;Q9DCS8	Napsin-A	Napsa
P35459	Lymphocyte antigen 6D	Ly6d
Q07456	Protein AMBP	Ambp
Q547B5;Q3TND2	Osteopontin	Spp1
Q6S9I0;Q6S9I2	Kininogen 2	Kng2
Q149Y8;Q08423	Trefoil factor 1	Tff1
P00688;Q8C5B4	Pancreatic alpha-amylase	Amy2;Amy2a1
B8JI96	Major urinary protein 14 (Fragment)	Mup14
Q91XL1	Leucine-rich HEV glycoprotein	Lrg1
O09114	Prostaglandin-H2 D-isomerase	Ptgds
Q102J0;Q8R242	Di-N-acetylchitobiase	Ctbs
Q3UDD6;Q544Y8	Granulins; Acrogranin	Grn

E9PVG8	RIKEN cDNA 9530053A07 gene	9530053A07Rik
Q3UBS3;Q61646	Haptoglobin alpha chain; Haptoglobin beta chain	Hp
P09036	Serine protease inhibitor Kazal-type 3	Spink3
Unique Proteins		
Protein IDs	Protein names	Gene names
Saliva		
Q61902;Q61900	Submaxillary gland androgen-regulated protein 3A	Smr3a
A0A1R3UCI2;P15945	Kallikrein 1-related peptidase b5	Klk1b5
Q3U3J1	2-oxoisovalerate dehydrogenase subunit	Bckdha
Q3TTT1;P18761	Carbonic anhydrase 6	Car6;Ca6
A0A2I3BRY2;P02815	16.5 kDa submandibular gland glycoprotein	Spt1
A0A1R3UCH3;Q61754	Kallikrein 1-related peptidase b24	Klk1b24
P97361	BPI fold-containing family A member 1	Bpifa1
Q3UKV9;Q06318	Uteroglobin	Scgb1a1
Q3UNG6;Q61759	Kallikrein 1-related peptidase b21	Klk1b21
Q91X93	Proline-rich protein BstNI subfamily 1	Prb1
B7ZWD8;A0MA77	Uncharacterized protein	Dcpp2
Q14AV3;Q8C1E1	BPI fold-containing family B member 2	Bpifb2
A0A077S2U6;P08905	Lysozyme C-2	Lyz2
Q4FK86;O88593	Peptidoglycan-recognition protein	Pglyrp1
Q24JQ8;Q62472	Vomeronasal secretory protein 2	Lcn4
Q545I1;O09049	Regenerating islet-derived protein 3-gamma	Reg3g
O88309;P36368	Epidermal growth factor-binding protein type B	Egfbp2
Q61297	Alpha-amylase	NA
E9PWS6	RIKEN cDNA A630073D07	A630073D07Rik
Q14AJ3;Q62471	Vomeronasal secretory protein 1	Lcn3
Q3UWH6;Q9D0C0	Cathepsin L1	Ctsl
Q9D7Y7;Q9CPN9	NA	2210010C04Rik
Q3TVS6;Q3TC17	Cathepsin B	Ctsb
A0A077S9N1;P17897	Lysozyme	Lyz1
Q80ZU7;Q3V181	BPI fold-containing family B member 3	Bpifb3
Q0VDQ3;A0A0R4J0B9	Pancreatic secretory granule membrane major glycoprotein GP2	Gp2
Q53X15;P27005	Protein S100;Protein S100-A8	S100a8
Q9CQV3	Serpin B11	Serpinb11
A0A1C7CYU3;Q8BRD3	Nucleobindin-1	Nucb1
Q8VEH9;Q3UQ05	cDNA sequence BC018465; BPI fold-containing family B, member 5	Bpifb5
Q07797;E9Q5X5	Galectin-3-binding protein	Lgals3bp
O35744	Chitinase-like protein 3	Chil3
O08692	Neutrophilic granule protein	Ngp
G5E8B5;Q7M742	Secretoglobin family 1C member 1	Scgb1c1

E9Q704;F8VQA4	Peptidyl-glycine alpha-amidating monooxygenase; Peptidylglycine alpha-hydroxylating monooxygenase	Pam
Q544T7;A0A1W2P788	alpha-1,2-Mannosidase	Man1a;Man1a1
Q5SXG7	Vitelline membrane outer layer protein 1 homolog	Vmo1
Q8BKY2	Uncharacterized protein	Col3a1
E9PWB6;E9QAQ8	Mucin 5, subtypes A and C, tracheobronchial/gastric	Muc5ac
P21956;Q3TDU5	Lactadherin	Mfge8
F8WHM5;Q53WR6	Golgi apparatus protein 1	Glg1
Q3UDR2;Q3URP6	Protein disulfide-isomerase	P4hb
Q8BG86;G3X9V8	NA	Serpinb3a;Scca2; Serpinb3c
Q3UBP6;Q3UBQ4	NA	Actb
Q3TYW1;O55226	Chondroadherin	Chad
Sweat		
A5JUZ1;A0A0A6YW67	Ubiquitin-60S ribosomal protein L40; Ubiquitin-40S ribosomal protein S27a	Ubc;Gm8797
Q9D746	RIKEN cDNA 2310034C09	2310034C09Rik
Q02257	Junction plakoglobin	Jup
Q9D6S9	NA	2310061N02Rik
P17751;H7BXC3	Triosephosphate isomerase	Tpi1
Q9QUK9;Q3V2E0	MCG15083; Uncharacterized protein	Try5;Try4
Q8CE60;B2RXW1	Histidine ammonia-lyase	Hal
B2RQH0;Q7TSF1	Desmoglein-1-beta;Desmoglein-1-alpha	Dsg1b;Dsg1a
Q6WEH7;Q9JM83	Calmodulin-4	Calm4
Q9D7K4;Q9CRB1	Galectin;Galectin-7	Lgals7
A0JLR7;Q61484	Ahnak protein (Fragment); Desmoyokin (Fragment)	Ahnak
Q9D6U7;A2RTA0	Creatine kinase M-type	Ckm
Q9CZI7;Q542G9	Annexin A2	Anxa2
Q8VEE3;A0JLV3	Histone H2B	Hist2h2bb
P97350	Plakophilin-1	Pkp1
A0A0R4J293;Q9JLF6	Protein-glutamine gamma-glutamyltransferase K	Tgm1
Q3U7Z6;Q9DBJ1	Phosphoglycerate mutase 1	Pgam1
Q62266	Cornifin-A	Sprr1a
Q62267	Cornifin-B	Sprr1b
Q8BLX1	Protein S100	Hrnr
Q3TU85;A1E2B8	Heat shock 70 kDa protein 1A; Heat shock 70 kDa protein 1B	Hspa1b;Hspa1a
A0A0A6YW46;A0A0A6YX57	Filaggrin	Flg
Q8C605;Q9WUA3	ATP-dependent 6-phosphofructokinase	Pfkp
P40142;A0A286YE28	Transketolase	Tkt
Q6P6I3;Q91YH6	ATPase, H+ transporting, lysosomal V1 subunit B1	Atp6v1b1

Q6PAC1;Q3U9Q8	Gelsolin	Gsn
P21614	Vitamin D-binding protein	Gc
P50516	V-type proton ATPase catalytic subunit A	Atp6v1a
Q4FK88;Q4FJV4	Annexin A1	Anxa1
Q71LX8;P11499	Heat shock protein HSP 90-beta	Hsp90ab1
Q3TE06;Q3TNK2	WD repeat-containing protein 1	Wdr1
P14152	Malate dehydrogenase, cytoplasmic	Mdh1
Q548W7;P31786	Acyl-CoA-binding protein	Dbi
Q4FJX4;P97315	Cysteine and glycine-rich protein 1	Csrp1
P28665	Murinoglobulin-1	Mug1
G3X9T8;G3X8Q5	Ceruloplasmin	Cp
Q5HZY7;Q9CR51	V-type proton ATPase subunit G 1	Atp6v1g1
A0A338P7B8;Q6YJU1	Fetuin-B	Fetub
Q3ULT2;Q3UDJ7	Alpha-actinin-4	Actn4
Q6ZWX2	Thymosin, beta 4, X chromosome	Tmsb4x
A0A0A6YXG4	Filaggrin	Flg
Q99PT1	Rho GDP-dissociation inhibitor 1	Arhgdia
D3YTY9;A0A0R4J038	Kininogen-1	Kng1
A0A075B5P4;A0A0A6YWR2	Ig gamma-1 chain C region	Ighg1;HC
P21550;Q4FK59	Beta-enolase; Enolase	Eno3
P12382;Q8CD98	ATP-dependent 6-phosphofructokinase, liver type	Pfkl
Q5EBQ2;Q3TGC5	Phosphatidylethanolamine-binding protein 1	Pebp1
Q8CBU4;Q4KML7	Ezrin	Ezr
Q9CWS5	Uncharacterized protein	Uncharacterized protein
Q61509;Q99LT6	Elongation factor 2	Eef2
Q3U6E4;Q0VGU2	Prothymosin alpha; Thymosin alpha	Ptma;Gm12504
Q01853;Q8BNF8	Transitional endoplasmic reticulum ATPase	Vcp
Q60829	Protein phosphatase 1 regulatory subunit 1B	Ppp1r1b
Q545F0;P34884	Macrophage migration inhibitory factor	Mif
Q3TG37;P70441	Na(+)/H(+) exchange regulatory cofactor NHE-RF	Slc9a3r1
Q8BPH1;Q5SS40	14-3-3 protein epsilon	Ywhae
Q66JR8;Q9D0J8	Parathymosin	Ptms
P35700;B1AXW5	Peroxiredoxin-1	Prdx1
Q9QXD6;Q9QXC5	Fructose-1,6-bisphosphatase 1	Fbp1
Q3TZ44;Q3TSZ4	Aldose 1-epimerase	Galm
D3Z7F0;P16125	L-lactate dehydrogenase; L-lactate dehydrogenase B chain	Ldhb
Q544Y7;F8WGL3	Cofilin-1;Cofilin-2	Cfl1;Cfl2
Q91YT9;Q91V28	6-phosphogluconate dehydrogenase, decarboxylating	Pgd

Nest		
E9Q328;Q9D3N7	RIKEN cDNA 5430401F13 gene	5430401F13Rik
J3QK77;Q9JI02	Secretoglobin family 2B member 20	Scgb2b20
J3QME6;F6WYC8	Lipase	Gm5097
Q80XE3	BC051076 protein (Fragment)	BC051076
Q9D7P9	Serpin B12	Serpinb12
A8R0U0	Exocrine gland secreted peptide 6	Esp6
J3QJY4	Androgen binding protein A3	Scgb1b3
D3Z617;D3Z4E7	Seminal vesicle antigen-like 2	Sval2
S4R2L0;J3QM75	Androgen binding protein BG12; Androgen binding protein BG19	Scgb2b12;Scgb2b19
Q3UWK8	MCG20280	Serpinb6d
Q32ME9;Q2VPA9	Desmocollin-1	Dsc1
E9Q9C6;E9Q0B5	Fc fragment of IgG-binding protein	Fcgbp
Q9D3N5	RIKEN cDNA 5430402E10 gene	5430402E10Rik
Q0VGU8	BPI fold-containing family A, member 6	Bpifa6
Q7TT08;Q3UXH6	Lipase	Lipo1
Q9ES55;Q3TYQ9	Aldehyde oxidase 4	Aox4
Q3UW77;B9EKG4	MCG59630; Predicted gene, OTTMUSG00000008911	Gm12888
B1AVM1	Predicted gene 12887	Gm12887
Q0VDV3;W0UVC5	Ribonuclease 2B	Rnase2b
Q4KL81;Q3TSB7	Actin, cytoplasmic 2	Actg1
Q9D0H8	Uncharacterized protein	Uncharacterized protein
Q9QZ83	Gamma actin-like protein	Actg1
O88312	Anterior gradient protein 2 homolog	Agr2
P01592	Immunoglobulin J chain	Igj
O09133	Submaxillary gland androgen-regulated protein 2, isoform alpha	Smr2
S4R1X8;S4R2V3	Secretoglobin, family 2B, member 17; member 15	Scgb2b17;Scgb2b15
Q9ET22	Dipeptidyl peptidase 2	Dpp7
A0A0R4J077;Q8R1M8	Mucosal pentraxin	Mptx1
A0A089N3F1;D2XZ38	Androgen binding protein BG3	Abpbg3;Scgb2b3
O09131	Glutathione S-transferase omega-1	Gsto1
Urine		
Q80YV5;Q9QX97	Trefoil factor 2	Tff2
P11087	Collagen alpha-1(I) chain	Col1a1
A0A0N4SV66;A0AUV1	Histone H2A	Hist1h2ah
A9R9V7	Major Urinary Protein 21	Mup21
Q4KML8;A0A0R4IZW5	Cadherin-1	Cdh1
B7ZNZ9;Q02596	Glycosylation-dependent cell adhesion molecule 1	Glycam1

F6VHS4;Q5M9M1	Hepcidin-2	Hamp2
Q505K6;Q8BWN9	AI182371	AI182371
A0A087WRP7;A0A087WNZ5	Lymphocyte antigen 6C1;Lymphocyte antigen 6C2	Ly6c1;Ly6c2
Q8BHC0	Lymphatic vessel endothelial hyaluronic acid receptor 1	Lyve1
Q8JZM3;Q78ZN4	Resistin-like alpha	Retnla;Xcp2
A0A0N4SWB4;Q3UQF0	Kidney androgen-regulated protein	Kap
Q8R1I3;E9Q6G4	ATP-binding cassette sub-family A member 7	Abca7
Q5XFY8;A0A0M3KL49	Ig kappa chain C region	Igkc
Q5SSJ1	Activated macrophage/microglia WAP domain protein	Wfdc17
A2CEK6;L7N222	Major urinary protein 11; Major urinary protein 13	Mup13
Q62395	Trefoil factor 3	Tff3
Q91X23;Q60590	Alpha-1-acid glycoprotein;Alpha-1-acid glycoprotein 1	Orm1
A2ARV4;A2ARV5	Low-density lipoprotein receptor-related protein 2	Lrp2
Q920X5;Q91VE7	Cathelin-related antimicrobial peptide	Cramp
P68372;Q9CVR0	Tubulin beta-4B chain; Tubulin beta-4A chain	Tubb4b;Tubb4a
P13634	Carbonic anhydrase 1	Ca1
Q53ZF0;P97426	Eosinophil cationic protein 1	Ear1;R8;R9;Ear10
Q0VBA8;P06869	Urokinase-type plasminogen activator	Plau
A0A140T8N2;P01642	Ig kappa chain V-V region L7	Gm10881

Table B.2. List of loading values and contributions for proteins on the first two principal components (PC). Log_2 LFQ intensities for the 140 proteins common to at least two sample types were used in the principal component analysis. Bolded values signify loadings with higher than expected contribution to each PC as determined by the square of the loading divided by the sum of the square of all loadings on each PC

Protein names	PC1 loading	PC1 contribution (%)	PC2 loading	PC2 contribution (%)
Alpha-amylase 1	**0.798322**	**1.15684669**	0.142616	0.059547001
Carbonic anhydrase 6	**0.807033**	**1.182231837**	-0.02831	0.002347222
BPI fold-containing family B, member 9B	**0.79865**	**1.157798817**	-0.00331	3.21E-05
ABPBG27; Salivary androgen-binding protein beta subunit	**0.768089**	**1.07088558**	-0.1989	0.115825627
Prolactin-inducible protein homolog	**0.866815**	**1.363870657**	0.27678	0.224282802
Major urinary protein 4	**0.678011**	**0.834436943**	**0.547831**	**0.878657058**
Odorant-binding protein 1a	0.15211	0.041998573	**-0.63503**	**1.180646659**
Serum albumin	-0.35751	0.232010156	0.242068	0.171553507
Predicted gene 14743	0.324471	0.191105056	**-0.51526**	**0.777271467**
Transcobalamin-2	0.62512	0.709328451	-0.41643	0.507698768
Androgen binding protein A2	-0.05893	0.006303273	**-0.54272**	**0.862351646**

Major urinary protein 1	-0.0091	1.50E-04	**0.859771**	**2.164170871**
Androgen binding protein A7; A20	**0.644876**	**0.754871328**	0.228754	0.153201892
Serotransferrin	-0.36535	0.24228555	**-0.57261**	**0.959925112**
Major urinary protein 10; Major urinary protein 1	-0.29127	0.153992106	0.446103	0.58263389
Major urinary protein 20	0.174673	0.055382503	**0.937846**	**2.575069199**
Hornerin	-0.3263	0.193266853	-0.4495	0.591544847
Beta-globin	-0.5646	0.578631526	-0.12396	0.044987337
Actin, cytoplasmic 1	-0.55587	0.56087682	0.188971	0.104547557
Glyceraldehyde-3-phosphate dehydrogenase	-0.48893	0.433925726	-0.06484	0.012308331
Elongation factor 1-alpha	-0.49026	0.436294231	-0.07763	0.017645381
Major urinary protein 3	0.33133	0.199270355	**0.712766**	**1.487372728**
Androgen binding protein A27	0.34612	0.217456495	**-0.75965**	**1.689484077**
Predicted gene 5938	-0.08825	0.014135456	**-0.68919**	**1.390600221**
Secretoglobin family 2B member 2	**0.65872**	**0.787629644**	-0.48153	0.678859812
Alpha-enolase;Enolase	-0.60585	0.666267069	**-0.53726**	**0.845071425**
Protein-glutamine gamma-glutamyltransferase E	-0.29773	0.160908094	**-0.60456**	**1.070039369**
Alpha-2-HS-glycoprotein	-0.03128	0.00177635	**-0.661**	**1.279156172**
Hemopexin	0.015665	4.45E-04	**-0.6079**	**1.081899659**
L-lactate dehydrogenase	**-0.69667**	**0.880986196**	-0.37775	0.41776742
Glucose-6-phosphate isomerase	-0.59852	0.650237244	-0.32389	0.307125481
Acidic mammalian chitinase	**0.832652**	**1.258481875**	0.143859	0.060589852
Mucin-19	**0.901206**	**1.474238245**	-0.1174	0.040349124
Protein LEG1 homolog	**0.922667**	**1.545288835**	0.369059	0.39876598
Ovostatin homolog	**0.968056**	**1.701064721**	0.216393	0.137092404
Kallikrein 1-related peptidase b9	**0.850287**	**1.312353612**	-0.26031	0.19838195
Kallikrein 1-related peptidase b8	**0.842484**	**1.288377231**	-0.24739	0.17918074
BPI fold-containing family B member 1	**0.769847**	**1.075792894**	-0.12773	0.047768334
Predicted gene 14744	**0.849526**	**1.310005399**	-0.05074	0.00753626
Kallikrein 1-related peptidase b26	**0.822848**	**1.229021007**	-0.20659	0.124949633
Deoxyribonuclease	**0.90787**	**1.496122544**	0.40255	0.474421737
Lactoperoxidase	**0.927525**	**1.561603888**	0.36651	0.393275481
Demilune cell and parotid protein 1	**0.876866**	**1.395683456**	-0.04863	0.006923083
Kallikrein-1	**0.857968**	**1.336170512**	**0.506277**	**0.750415338**
Demilune cell and parotid protein 3	**0.908707**	**1.49888248**	0.409404	0.490714537
Gastric triacylglycerol lipase	**0.775113**	**1.090560163**	-0.11078	0.035930841
Demilune cell and parotid protein 2	**0.87878**	**1.401782637**	0.175806	0.090488276

Kallikrein 1-related peptidase-like b4	**0.833005**	**1.259550085**	-0.22457	0.147647091
WAP four-disulfide core domain protein 12	**0.869038**	**1.370875523**	-0.26994	0.213328528
Major urinary protein 5	**0.911506**	**1.508132167**	0.388251	0.441317856
Mucin cell adhesion protein	**0.853592**	**1.322575741**	-0.23187	0.157406887
Lactotransferrin	**0.892698**	**1.446535908**	0.134573	0.053020202
Angiotensinogen	**0.891231**	**1.441786403**	-0.09209	0.024826185
Androgen binding protein BG7	**0.85892**	**1.33913761**	**0.502101**	**0.738086804**
Prostatic spermine-binding protein	**0.865221**	**1.358858326**	0.186544	0.101880275
Lipocalin 11	**0.81701**	**1.211642375**	-0.19221	0.108163199
Pro-epidermal growth factor; Epidermal growth factor	**0.807961**	**1.184951062**	**0.581342**	**0.989437794**
Sulfhydryl oxidase 1	**0.83595**	**1.268470601**	-0.23429	0.160710506
Prosaposin	0.62519	0.709487314	**0.637878**	**1.191246016**
Polymeric immunoglobulin receptor; Secretory component	**0.843088**	**1.29022527**	-0.21252	0.132223189
Cathepsin D	**0.848946**	**1.308218179**	-0.19799	0.114761645
Meprin A subunit alpha; Metalloendopeptidase	0.25558	0.118569245	**0.838775**	**2.059762369**
Lymphocyte antigen 6A-2/6E-1	0.201072	0.073387737	**0.784296**	**1.800884982**
Truncated profilaggrin/filaggrin flaky tail mutant form	**-0.63868**	**0.740429271**	-0.17796	0.092715738
2310057N15Rik protein (Fragment)	**-0.67574**	**0.828854885**	0.026208	0.002010906
Hemoglobin subunit alpha	**-0.64906**	**0.764694443**	-0.19514	0.111481134
Fructose-bisphosphate aldolase	**-0.74145**	**0.997885559**	-0.2107	0.129969129
MCG120169	**-0.6269**	**0.713372973**	-0.16536	0.080055323
Pyruvate kinase PKM	**-0.825**	**1.235444881**	0.148551	0.064606836
Major urinary protein 2	-0.03919	0.002788514	**0.616163**	**1.111518946**
Major urinary protein 1; Major urinary protein 7	**-0.64358**	**0.751850694**	**0.629222**	**1.159135101**
Alpha-1-antitrypsin 1-2	-0.51566	0.482663155	**0.621607**	**1.131247661**
Serine protease inhibitor A3K	-0.518	0.487065878	**0.583216**	**0.995830346**
Major urinary protein 12	-0.07423	0.010000922	**0.839573**	**2.063683923**
Superoxide dismutase [Cu-Zn]	-0.47599	0.411265657	0.398747	0.465500096
Histone H4	0.173373	0.054561102	-0.44052	0.568130791
Complement C3	-0.33374	0.202180757	**-0.60396**	**1.067931254**
Alpha-1-antitrypsin 1-5	-0.13319	0.032198333	-0.33425	0.327086412
Carboxylesterase 1C	-0.13501	0.033085447	**-0.52901**	**0.819328525**
BPI fold-containing family A member 2	**0.86847**	**1.369082488**	-0.46544	0.634227176
Kallikrein 1-related peptidase b16	**0.85593**	**1.329829823**	-0.41881	0.513534887
Kallikrein 1-related peptidase b22	**0.855381**	**1.32812677**	-0.41833	0.51235487
Androgen binding protein BG26	**0.863623**	**1.353842297**	-0.43996	0.566711385

Kallikrein 1-related peptidase b3	**0.856219**	**1.330729125**	-0.42029	0.517163282
Odorant-binding protein 2a	**0.856791**	**1.332509366**	-0.42036	0.517328699
Gamma-glutamyl hydrolase	**0.793306**	**1.142357326**	-0.46584	0.635340674
Predicted gene 6619	**0.842855**	**1.289512571**	-0.39305	0.452286286
Kallikrein 1-related peptidase b27	**0.852914**	**1.320474863**	-0.4116	0.496005121
Kallikrein 1-related peptidase b1	0.531028	0.511863647	-0.24953	0.182292712
Cysteine-rich secretory protein 1	**0.7908**	**1.135150604**	**-0.49248**	**0.710074489**
Kallikrein 1-related peptidase b11	**0.856511**	**1.331637799**	-0.42087	0.518597672
Odorant-binding protein 2b	**0.852506**	**1.319212303**	-0.4114	0.495520507
Beta-nerve growth factor	0.3787	0.260321756	-0.14826	0.064352957
Vomeromodulin	**0.854462**	**1.325273778**	-0.4166	0.508113327
Deleted in malignant brain tumors 1 protein	**0.856042**	**1.330178804**	-0.42074	0.5182785
Secretoglobin family 2B member 24	0.575655	0.601513394	-0.15288	0.068424053
Lacrein	0.417484	0.316373716	-0.05883	0.010131111
Ig alpha chain C region	**0.692651**	**0.870860647**	-0.27956	0.228813115
Protein S100-A9	0.495082	0.444912088	-0.28099	0.231150303
Exocrine gland secreted peptide 15	0.411483	0.307342782	-0.04665	0.006372509
Mucin 5, subtype B, tracheobronchial	**0.673465**	**0.823285599**	0.017405	8.87E-04
Cystatin 10	**0.671375**	**0.818182183**	-0.29831	0.260539104
Nucleobindin-2;Nesfatin-1	**0.707252**	**0.907963871**	-0.30959	0.280615579
Clusterin	0.235572	0.10073187	**0.643805**	**1.213484521**
Uncharacterized protein	0.527721	0.505508759	-0.01616	7.65E-04
RIKEN cDNA 2310079G19 gene	-0.42739	0.331558261	-0.2418	0.1711752
Filaggrin-2	-0.60729	0.669445166	-0.3453	0.349067855
Loricrin	**-0.62553**	**0.710250252**	-0.35459	0.368114613
Predicted gene 5965	-0.0209	7.93E-04	-0.03731	0.004074735
Integrator complex subunit 2	**-0.73942**	**0.992434987**	-0.41808	0.511737432
Heat shock cognate 71 kDa protein;Heat shock-related 70 kDa protein 2	-0.39639	0.285212371	-0.24603	0.177210661
Corneodesmosin	-0.6108	0.677196769	-0.34655	0.351613214
Peroxiredoxin-2	-0.58429	0.619697259	-0.33328	0.325198177
Alpha-2-macroglobulin	-0.45466	0.375233548	-0.25858	0.195750513
Transthyretin	-0.41426	0.311505297	-0.23364	0.15981711
Desmoplakin	**-0.86613**	**1.361729454**	-0.22714	0.151043503
Phosphoglycerate kinase 1	**-0.87123**	**1.377791227**	-0.0508	0.007556025
Tubulin alpha-1C chain;Tubulin alpha-1A chain	**-0.84218**	**1.287435024**	0.423466	0.52500384
Carbonic anhydrase 2	-0.51531	0.482006062	-0.02119	0.001313978
Peptidyl-prolyl cis-trans isomerase	**-0.88572**	**1.424017049**	-0.08131	0.019354847
Alpha-1-antitrypsin 1-4	-0.27331	0.135590788	**0.776624**	**1.765821677**
Hexokinase	**-0.69366**	**0.873393534**	-0.16887	0.083493953

Uromodulin	0.118092	0.025313874	**0.943482**	**2.606109041**
Complement factor D	0.115793	0.024338047	**0.947204**	**2.626712567**
Major urinary protein 7 (Fragment)	0.122109	0.027065532	**0.933683**	**2.552256556**
Napsin-A	0.1229	0.027417263	**0.936562**	**2.568025124**
Lymphocyte antigen 6D	0.118445	0.025465668	**0.942604**	**2.601263541**
Protein AMBP	0.125464	0.028573311	**0.932924**	**2.548109747**
Osteopontin	0.117186	0.024926938	**0.945196**	**2.615587068**
Kininogen 2	0.123403	0.027642022	**0.936499**	**2.567675991**
Trefoil factor 1	0.12521	0.028457574	**0.933246**	**2.549867896**
Pancreatic alpha-amylase	0.130099	0.030723112	**0.926892**	**2.515266181**
Major urinary protein 14 (Fragment)	0.153852	0.042966317	**0.729679**	**1.558798648**
Leucine-rich HEV glycoprotein	0.126879	0.029221515	**0.930596**	**2.53541066**
Prostaglandin-H2 D-isomerase	0.123802	0.027820984	**0.935712**	**2.563364042**
Di-N-acetylchitobiase	-0.04289	0.003339061	**0.625016**	**1.143690016**
Granulins;Acrogranin	-0.04292	0.003343619	**0.62503**	**1.143741833**
RIKEN cDNA 9530053A07 gene	0.181213	0.059606895	**0.54118**	**0.857452315**
Haptoglobin	0.144224	0.037756911	**0.898921**	**2.365750669**
Serine protease inhibitor Kazal-type 3	0.133131	0.032172072	**0.919417**	**2.474862261**

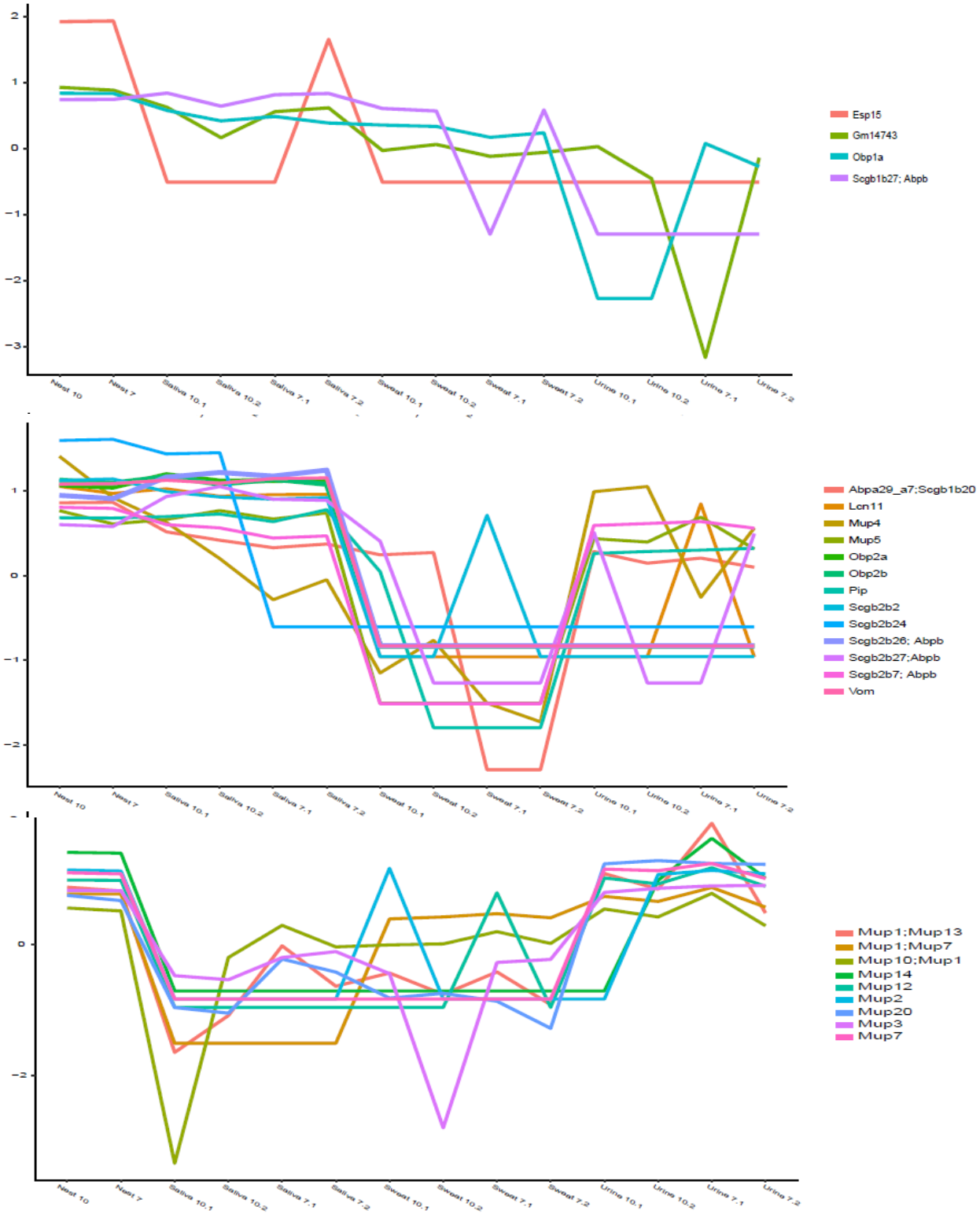


Figure B.1. Log_2 LFQ intensity expression patterns for 26 odorant proteins found across three hierarchical clusters as described in Figure 2.3

APPENDIX C. CHAPTER 3 SUPPLEMENTAL DATA

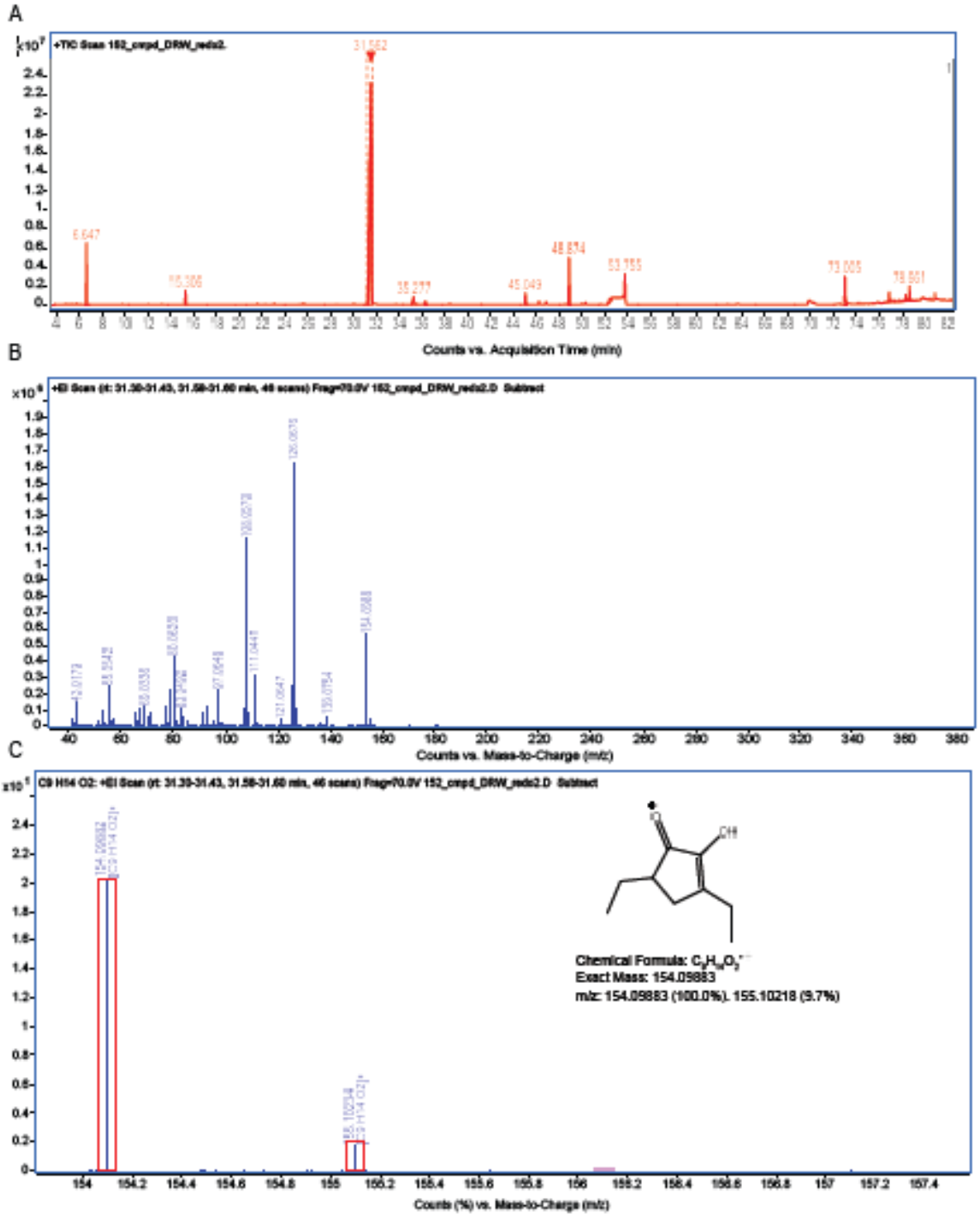


Figure C.1. (A) Total ion chromatogram (TIC); peak elutes at 31.562 minutes. (B) Full mass spectrum of 31.562 minute peak. (C) Molecular ion region. The boxes represent the theoretical distribution.

Supplemental text: Ketone preparation

General Procedure

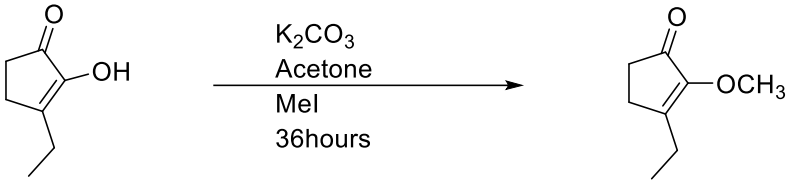
All reactions were performed in flame or oven-dried glassware under argon atmosphere unless otherwise noted. All commercial reagents were used as received unless otherwise noted. All materials were vacuum dried (1-5mmHg) to remove trace elements of solvent. "*in vacuo*" refers to bulk solvent removal which was performed by Buchi rotary evaporator linked to a water aspirator. Bulk solvent removal of solvents with boiling points above 80°C was performed on a Buchi rotary evaporator which was connected to Precision Scientific vacuum which allowed for pressures of 1 mmHg. Bulk grade solvents hexanes and ethyl acetate were distilled before use for chromatography. Diethyl ether (Et_2O), tetrahydrofuran (THF), methylene chloride (CH_2Cl_2), dimethylfomamide (DMF), and toluene (tol) were dried on a commercial solvent system before use in reactions. Hexamethylphosphoramide (HMPA) and N, N'-dimethylpropyleneurea (DMPU) were both distilled from CaH_2 and stored over 3 Å molecular sieves. Triethylamine (Et_3N), pyridine (pyr) and diisopropylethylamine (DIPEA) were distilled from CaH_2 under dry argon immediately before use.

Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were measured on a Varian VXR (400MHz), Varian INOVA-400 (400MHz), Varian INOVA 500 (500MHz) instruments. H^1 NMR and ^{13}C NMR are reported in parts per million (ppm) downfield from tetramethylsilane and calibrated using residual undeuterated chloroform as an internal standard which is set to δ 7.26. ^{1}H NMR spectra data were reported in the form δ (multiplicity, coupling constants (Hz), integration). Multiplicities are reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, dd = doublet of doublet, dt = doublet of triplet, ABq = AB quartet. Mass spectra data (GCMS, LCMS, HRMS) were recorded on an Agilent technologies 6890N 15973 (EI), Agilent Technologies 1200 series/6130(ESI), and Waters/Synapt Horns mass spectrometers using chemical ionization (CI) with methane and / or electrospray ionization (ESI).

Analytical thin-layer chromatography (TLC) was performed using glass backed 0.25 mm thickness silica gel 60 (F_{254}) plates which were visualized under UV light and/or by staining with ethanolic p-anisaldehyde, potassium permanganate, vanillin, dintrophenylhydrazine, and bromocresol green followed by heating on a hot plate. Iodine crystals were used to develop TLC plates in a glass

chamber. Flash chromatography was performed using Merck silica gel 60 (Kiesegel 60) from Whatman Scientific or Sorbent Technologies and pressure was obtained using an in-house airline.

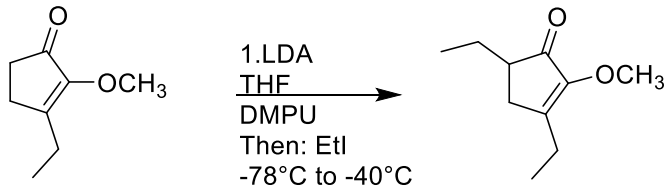
Synthesis of 3,5-diethyl-2-hydroxycyclopent-2-en-1-one (4)



(1) **(2)**

3-ethyl-2-methoxycyclopent-2-en-1-one (2)

Acetone (20mL) from a freshly opened bottle was added into a 50 mL round bottom flask charged with commercially available ketone **(1)** (500mg, 4 mmol, 1 equiv.). Following solvation, K_2CO_3 (1.05g, 7.9 mmol, 2 equiv) was added and followed by dropwise addition of methyl iodide (0.5 mL, 7.9 mmol, 2equiv). The mixture was allowed to stir for 48 hours until all starting material was consumed. The reaction mixture was then concentrated *in vacuo* and diluted with diethyl ether and water (1:1). The aqueous phase was separated and extracted with diethyl ether (2x 20 mL). The organic phases were combined and then washed with brine distilled water followed by drying over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The remaining crude oil was purified by column chromatography (10% EtOAc: 90%Hex) to yield 515mg (92%) of Ketone **(2)** as a colorless oil; characterized by R_f 0.31 (20%EtOAc:80%Hex); ^{1}H NMR (400MHz, $CDCl_3$) δ 3.86 (s, 3H), 2.38 (m,6H), 1.12 (t,3H) ^{13}C NMR (500MHz, $CDCl_3$) δ203.77, 159.88, 152.16, 58.66, 33.12, 24.62, 22.14, 11.74. IR (thin film): 2928, 2868, 1703 cm^{-1} HRMS m/z $[M]^+$ calcd for $C_8H_{12}O_2$ 140.08, found 140.0828. Derived from J.Org.Chem 2019,84,7166-7174

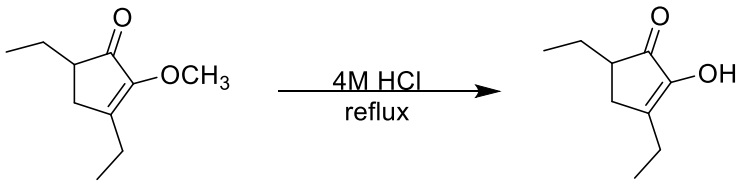


(2) **(3)**

3,5-diethyl-2-methoxycyclopent-2-en-1-one (3)

Ketone **(2)** (50mg, 0.36mmol) was added to 1.2 mL of dry THF and stirred at -78°C. A [0.5] molar solution of LDA (0.4mmol, 1 equiv) dissolved in THF was added dropwise followed by addition

of 0.6 mL of DMPU. This solution was stirred at -78°C for 3 hours then ethyl iodide (37.8 µL, 0.47 mmol, 1.3 equiv) was added and the reaction was warmed to- 40°C with an acetonitrile/dry ice bath. After stirring for 6 hours at -40°C the reaction was slowly quenched at -40°C by dropwise addition of distilled water. The mixture was extracted with diethyl ether (2x10mL). The organic layers were combined and washed with brine and distilled water followed by drying over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude oil was purified by column chromatography (7% EtOAc: 93%Hex) to yield 45mg (77%, 94% brsm) of the diethyl ketone **(3)** as a translucent yellow oil and 10 mg of starting material **(2)**; characterized by R_f 0.47 (20%EtOAc:80%Hex); ^{1}H NMR (400MHz, $CDCl_3$) δ 3.86 (s, 3H), 2.38 (m,6H), 1.12 (t,3H); ^{13}C NMR (500MHz, $CDCl_3$) δ 205.95, 158.55, 151.59, 58.66, 44.93, 31.24, 24.62, 22.03, 11.81, 11.15; IR (thin film) 2926, 2875, 1703 cm^{-1}; HRMS m/z $[M]^+$ calcd for $C_{10}H_{16}O_2$ 168.1145, found 168.1146.



(3) (4)

3,5-diethyl-2-hydroxycyclopent-2-en-1-one (4)

A 25 mL double necked round bottom flask was fitted with a condenser and charged with the diethyl ketone **(3)** (50mg, 0.29 mmol, 1equiv) and 5mL of 4M aqueous HCl was added while stirring. The solution was heated to reflux for 90 minutes until all starting material was consumed. The mixture was diluted with 5 mL of distilled water and then extracted with diethyl ether. The organic layer was washed with brine and then distilled water. The organic phase was then separated and then dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude material was purified by column chromatography (10% EtOAc: 90%Hex) to yield 40mg of ketone **(4)** (89%) as a colorless oil which later crystallized when stored at -20°C; characterized by R_f 0.36 (20%EtOAc:80%Hex); ^{1}H NMR (400MHz, $CDCl_3$) δ 3.86 (s, 3H), 2.38 (m,6H), 1.12 (t,3H); ^{13}C NMR (500MHz, $CDCl_3$) δ 205.71, 148.42, 147.68, 44.21, 31.57, 24.56,21.79, 11.41; IR(thin film): 3442, 2967, 1690 cm^{-1}; HRMS m/z $[M]^+$ calcd for $C_9H_{14}O_{21}$ 154.09883, found 154.09882.

Table C.1. Total number of data points used in each analysis.

Nesting material	Sweat	Urine	Behavior
Strain and VOC Profile Analysis [a]			
23	46	42	---
Urinary Pheromone Models [b]			
---	---	42	---
VOC Profile + Social Interaction Analysis [c]			
23	24	24	24
Strain + VOC PC Models [b]			
---	---	---	24
Strain and Behavior Models [b]			
---	---	---	72

[a] analyzed with non-metric multidimensional scaling and the Adonis test; [b] analyzed with mixed models; [c] analyzed with principal component analysis and mixed models; '---' indicates a variable was not used in the respective analysis

Table C.2. Effects of strain, social rank, and batch number on raw VOC proportions based on the Adonis test.

	Strain	Social Rank	Batch number
Nesting Material	$F_{2,17}$= 4.72, **p=0.003**	---	$F_{3,17}$= 1.83, p=0.099
Plantar Sweat	$F_{2,39}$= 8.29, **p=0.001**	$F_{2,39}$= 1.53, p=0.202	$F_{3,39}$= 1.92, p=0.060
Urine	$F_{2,35}$= 8.10, **p=0.001**	$F_{2,35}$= 1.82, p=0.116	$F_{3,35}$= 1.87, **p=0.034**

Significant p values are in bold.

Table C.3. Fixed effects on known urinary pheromones based on mixed models.

	Strain	Social Rank	Strain* Social Rank
β-farnesene	$F_{2,18.43}$= 0.30, p=0.746	$F_{1,21.32}$= 0.47, p=0.500	NS
SBT	$F_{2,16.13}$= 11.68, **p<0.001**	$F_{1,22.69}$= 0.91, p=0.350	NS

Interactions marked with 'NS' were non-significant and dropped from the final model. Significant p values are listed in bold.

APPENDIX D. CHAPTER 4 SUPPLEMENTAL DATA

Table D.1. Count of cages used for final analysis, broken down by strain and group size.

	Total Cages	SJL- 3	SJL- 5	Albino B6- 3[1]	Albino B6- 5
Social network analysis	20	5	6	4	5
Dominance measure- convergent validity	18	4	6	3	5
Dominance measure- discriminant validity	19	5	6	3	5

[1]One cage was euthanized between day 2 and 7 of video data. Data from day 2 was included in social network analyses.

Table D.2. Count of experiment units (either cage or mouse) used in each social network analysis and dominance measure model. The number of video days observed is indicated where applicable.

	Cages	Mice	Sampling Unit	Units with two days of behavior data	Units with one day of behavior data
Social network analysis					
Aim 1- power distribution	20	82	cage	18	2
Aim 2- influences on Glicko score	20	82	mouse	74	8
Aim 3- relationship between submission performed and aggression received	20	82	mouse	74	8
Aim 4- likelihood of submission following social investigation	18	74	mouse	74	0
Dominance measures					
Glicko score	19	38	mouse	36	2

Preputial gland ratio	19	38	mouse	---	---
Time in center of OFM	19	38	mouse	---	---
Fecal boli in OFM	19	38	mouse	---	---
Darcin	18	36	mouse	---	---
Tube test scores	19	38	mouse	---	---
Average posterior PALS score	19	38	mouse	---	---

Table D.3. Loading values from principal component analysis of tube test scores over three rounds. Only the first component had an eigenvalue over 1.

	Tube Test PC
Tube Test Round 1	0.90062
Tube Test Round 2	0.96658
Tube Test Round 3	0.90369
Eigenvalue	2.56
Total variance explained (%)	85.4

Table D.4. Least square mean ± SE for each strain*group size combination from general linear models of standardized measures tested for convergent validity.

	SJL – 3	**SJL – 5**	**Albino B6 - 3**	**Albino B6 - 5**
Change in Glicko-Agg score	-0.0101 ± 0.0616	-0.0068 ± 0.0601	0.1098 ± 0.0760	0.0747 ± 0.0685
Change in Glicko-Sub score	-0.0566 ± 0.0847	-0.0520 ± 0.0827	0.2011 ± 0.1046	0.0404 ± 0.0942
Preputial gland ratio	-0.2622 ± 0.3138	-0.3545 ± 0.3065	0.4057 ± 0.3876	0.6597 ± 0.3489
Urinary darcin	-0.5546 ± 0.2374	-0.6397 ± 0.2250	0.7949 ± 0.2836	1.1178 ± 0.2565
Tube test score-round 1	0.1077± 0.2221	-0.5201 ± 0.2169	-0.6075 ± 0.2743	-0.1921 ± 0.2470
Tube test score-round 2	0.2318 ± 0.2421	-0.3185 ± 0.2364	-0.4560 ± 0.2990	-0.1167 ± 0.2692
Tube test score-round 3	0.0885 ± 0.2778	-0.1854 ± 0.2713	-0.5723 ± 0.3431	-0.1225 ± 0.3089
Average posterior PALS score	0.6155 ± 0.2037	0.6368 ± 0.1989	-0.5242 ± 0.2515	-0.4631 ± 0.2265

Table D.5. Correlation coefficients of dominance measure residuals used in the factor analyses and subsequent general linear models.

	Change in Glicko-Agg score	**Change in Glicko-Sub score**	**Preputial gland ratio**	**Urinary darcin**	**Tube test-round 1**	**Tube test-round 2**	**Tube test-round 3**	**Average posterior PALS score**
Change in Glicko-Agg score	1.0000	0.9762	0.6528	0.4514	-0.3280	-0.2747	-0.3071	-0.5291
Change in Glicko-Sub score	0.9762	1.0000	0.6209	0.4004	-0.3143	-0.2893	-0.2996	-0.5869
Preputial Gland Ratio	0.6528	0.6209	1.0000	0.5583	-0.2983	-0.2524	-0.2808	-0.4193
Urinary darcin	0.4514	0.4004	0.5583	1.0000	-0.3169	-0.3460	-0.4817	-0.0280
Tube test - round 1	-0.3280	-0.3143	-0.2983	-0.3169	1.0000	0.7972	0.6274	0.4409
Tube test - round 2	-0.2747	-0.2893	-0.2524	-0.3460	0.7972	1.0000	0.8387	0.4873
Tube test - round 3	-0.3071	-0.2996	-0.2808	-0.4817	0.6274	0.8387	1.0000	0.4507
Average posterior PALS score	-0.5291	-0.5869	-0.4193	-0.0280	0.4409	0.4873	0.4507	1.0000

Figure D.4. Custom caging for home cage observations. Holes were drilled into polysulfone lids for air exchange on static racks. A metal feeder was secured to the lid using a nut and bolt. An external water bottle was accessible through a hole in the side of the lid and connected using medical grade silicone tubing and a metal water sipper. These cages allowed for overhead monitoring using CCTV cameras, one of which can be seen at the top of the figure.

APPENDIX E. CHAPTER 5 SUPPLEMENTAL DATA

Table E.1. Tested solution concentrations of the four compounds.

Compound	Natural concentration	5x natural concentration
geranylacetone	120ng/100µL	600ng/100µL
6-hydroxy-6-methyl-3-heptanone	4µg/100µL	20µg/100µL
3,4-dimethyl-1,2-cyclopentadione	40ng/100µL	200ng/100µL
3,5-diethyl-2-hydroxycyclopent-2-en-1-one	200ng/100µL	1µg/100µL

Table E.2. Solution test order for each room.

Batch	Facility	Treatment
1	A	3,4-dimethyl-1,2-cyclopentadione
2	A	control
3	A	3,5-diethyl-2-hydroxycyclopent-2-en-1-one
4	A	6-hydroxy-6-methyl-3-heptanone
5	A	geranylacetone
1	B	geranylacetone
2	B	6-hydroxy-6-methyl-3-heptanone
3	B	control
4	B	3,5-diethyl-2-hydroxycyclopent-2-en-1-one
5	B	3,4-dimethyl-1,2-cyclopentadione

Table E.3. Active period behavior proportions analyzed for treatment effects.

Cage	Day	Strain	Treatment	Batch	Facility	Mediated Proportion	Submissive Proportion	Escalated Proportion	Allo-groom Proportion
L1	3	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.013468	0.016835	0	0.075758
L1	4	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.004934	0.004934	0.009868	0.042763
L1	7	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.028846	0.034615	0.007692	0.076923
L2	3	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.007126	0.173397	0.033254	0.004751
L2	4	SJL	3,4-dimethyl-1,2-	1	A	0.01519	0.113924	0.017722	0.005063

			cyclopentadione						
L2	7	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.031496	0.217848	0.047244	0.007874
L3	3	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.017291	0.195965	0.040346	0.005764
L3	4	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.039437	0.185915	0.025352	0
L3	7	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.030534	0.195929	0.048346	0.012723
L4	3	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.021148	0.299094	0.093656	0
L4	4	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.026515	0.106061	0.068182	0.007576
L4	7	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.018405	0.058282	0.02454	0.003067
V1	1	SJL	geranylacetone	1	B	0.028777	0.208633	0.07554	0
V1	3	SJL	geranylacetone	1	B	0.032836	0.280597	0.062687	0.00597
V1	4	SJL	geranylacetone	1	B	0.050971	0.201456	0	0.004854
V1	7	SJL	geranylacetone	1	B	0.016173	0.188679	0.026954	0.005391
V2	1	B6	geranylacetone	1	B	0.015504	0.025194	0.001938	0.03876
V2	3	B6	geranylacetone	1	B	0.002012	0.002012	0	0.082495
V2	4	B6	geranylacetone	1	B	0.008803	0.021127	0.001761	0.033451
V2	7	B6	geranylacetone	1	B	0.007105	0.017762	0.007105	0.069272
V3	1	B6	geranylacetone	1	B	0.012681	0.012681	0	0.012681
V3	3	B6	geranylacetone	1	B	0.020619	0.025773	0	0.041237
V3	4	B6	geranylacetone	1	B	0.02521	0.02521	0.003361	0.031933
V3	7	B6	geranylacetone	1	B	0.02773	0.02773	0.006932	0.076256

V4	1	SJL	geranylacetone	1	B	0.009412	0.134118	0.037647	0.004706
V4	3	SJL	geranylacetone	1	B	0.033186	0.210177	0.061947	0.004425
V4	4	SJL	geranylacetone	1	B	0.024331	0.20438	0.048662	0.009732
V4	7	SJL	geranylacetone	1	B	0.019704	0.046798	0	0.012315
L5	1	SJL	control	2	A	0.004695	0.161972	0.032864	0
L5	3	SJL	control	2	A	0.006135	0.171779	0.046012	0
L6	1	B6	control	2	A	0	0.011952	0.003984	0.025896
L6	3	B6	control	2	A	0.015625	0.083333	0.017361	0.038194
L6	4	B6	control	2	A	0.026217	0.067416	0.011236	0.024345
L6	7	B6	control	2	A	0.017143	0.051429	0.009524	0.038095
L7	1	B6	control	2	A	0.003984	0.033865	0.003984	0.013944
L7	3	B6	control	2	A	0.022727	0.068182	0.011364	0.034091
L7	4	B6	control	2	A	0.009579	0.02682	0.003831	0.034483
L7	7	B6	control	2	A	0.036329	0.059273	0.007648	0.072658
L8	1	SJL	control	2	A	0.011204	0.151261	0.039216	0
L8	3	SJL	control	2	A	0.050802	0.13369	0	0.002674
L8	4	SJL	control	2	A	0.027248	0.245232	0.051771	0.013624
L8	7	SJL	control	2	A	0.008427	0.157303	0.030899	0.005618
V5	1	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.00367	0.053211	0.012844	0.007339
V5	3	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.006329	0.120253	0.037975	0.006329
V5	4	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.015504	0.096899	0.034884	0.003876
V5	7	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.006349	0.031746	0.012698	0.019048
V6	1	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.002475	0.131188	0.029703	0.002475
V6	3	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.041534	0.124601	0	0
V6	4	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.012012	0.168168	0.048048	0
V6	7	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.050926	0.300926	0.0625	0.002315
V7	1	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.020833	0.266204	0.060185	0.006944

V7	3	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.018672	0.257261	0.045643	0.018672
V7	4	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.037736	0.296226	0.04717	0.007547
V7	7	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.027719	0.219616	0.034115	0.010661
V8	1	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.009653	0.042471	0.011583	0.009653
V8	3	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.020522	0.039179	0.003731	0.033582
V8	4	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.018367	0.061224	0.022449	0.040816
V8	7	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.024242	0.10303	0.027273	0.024242
L10	1	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.011211	0.139013	0.024664	0
L10	3	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.016432	0.223005	0.044601	0.016432
L10	4	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.020958	0.095808	0.026946	0.020958
L10	7	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.024922	0.205607	0.056075	0.009346
L11	1	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.017903	0.255754	0.048593	0
L11	3	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.014742	0.235872	0.054054	0.004914
L11	4	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.028351	0.224227	0.046392	0.010309
L11	7	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.030227	0.214106	0.040302	0.012594
L12	1	B6	3,5-diethyl-2-hydroxycyclo	3	A	0	0.008316	0	0

			pent-2-en-1-one						
L12	3	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.015086	0.017241	0.002155	0.081897
L12	4	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.013725	0.017647	0	0.068627
L12	7	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.011538	0.017308	0.007692	0.048077
L9	1	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.003781	0.009452	0	0.003781
L9	3	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.006565	0.008753	0	0
L9	4	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.002203	0.002203	0	0
L9	7	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.003906	0.003906	0.003906	0.011719
V10	1	B6	control	3	B	0.011407	0.013308	0	0.043726
V10	3	B6	control	3	B	0.008032	0.006024	0	0.062249
V10	4	B6	control	3	B	0.011385	0.01518	0	0.047438
V10	7	B6	control	3	B	0.006036	0.014085	0	0.078471
V11	1	B6	control	3	B	0.005545	0.005545	0	0.027726
V11	3	B6	control	3	B	0.020794	0.018904	0.00189	0.064272
V11	4	B6	control	3	B	0.003766	0.013183	0.001883	0.054614
V11	7	B6	control	3	B	0.009191	0.003676	0	0.040441
V12	1	SJL	control	3	B	0.01355	0.189702	0.062331	0.00271
V12	3	SJL	control	3	B	0.022556	0.180451	0.035088	0.002506
V12	4	SJL	control	3	B	0.011429	0.188571	0.051429	0
V12	7	SJL	control	3	B	0.0271	0.176152	0.02439	0.01084
V9	1	SJL	control	3	B	0.017632	0.307305	0.070529	0.002519
V9	3	SJL	control	3	B	0.028011	0.240896	0.056022	0
V9	4	SJL	control	3	B	0.005305	0.159151	0.047745	0
V9	7	SJL	control	3	B	0.035422	0.188011	0.024523	0
L13	1	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.009579	0.009579	0	0.065134

L13	3	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.006198	0.012397	0.014463	0.11157
L13	4	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.004132	0.006198	0	0.136364
L13	7	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.014644	0.020921	0.018828	0.100418
L14	1	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.013928	0.203343	0.050139	0.005571
L14	3	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.014327	0.26361	0.068768	0
L14	4	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.028796	0.212042	0.049738	0
L14	7	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.045161	0.209677	0.041935	0
L15	1	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.020921	0.320084	0.069038	0.004184
L15	3	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.037915	0.151659	0.00237	0.009479
L15	4	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.008734	0.268559	0.054585	0.010917
L15	7	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.030457	0.175127	0.027919	0.010152
L16	1	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.012	0.008	0	0.032
L16	3	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.008715	0.008715	0	0.026144
L16	4	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.016807	0.023109	0	0.05042
L16	7	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.022173	0.019956	0.006652	0.031042
V13	1	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.032468	0.251082	0.051948	0
V13	3	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.03599	0.192802	0.030848	0.002571
V13	4	SJL	3,5-diethyl-2-hydroxycyclo	4	B	0.016556	0.168874	0.039735	0.009934

			pent-2-en-1-one						
V13	7	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.011662	0.125364	0.014577	0
V14	1	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.001938	0.001938	0	0.034884
V14	3	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.005607	0.005607	0	0.020561
V14	4	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.010381	0.019031	0	0.043253
V14	7	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.006656	0.011647	0	0.039933
V15	1	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.002222	0.002222	0	0.055556
V15	3	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.015066	0.013183	0	0.041431
V15	4	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.008897	0.014235	0	0.042705
V15	7	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.020443	0.020443	0	0.054514
V16	1	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.013483	0.285393	0.060674	0
V16	3	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.02963	0.283951	0.054321	0.002469
V16	4	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.020455	0.243182	0.047727	0.004545
V16	7	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.015831	0.195251	0.042216	0.002639

L17	1	SJL	geranylacetone	5	A	0.005666	0.376771	0.082153	0.008499
L17	3	SJL	geranylacetone	5	A	0.027431	0.259352	0.057357	0
L17	4	SJL	geranylacetone	5	A	0.020045	0.253898	0.053452	0.004454
L17	7	SJL	geranylacetone	5	A	0.033557	0.196868	0.029083	0.006711
L18	1	B6	geranylacetone	5	A	0.003914	0	0	0.088063
L18	3	B6	geranylacetone	5	A	0.00998	0.00998	0	0.061876
L18	4	B6	geranylacetone	5	A	0	0	0	0.039352
L18	7	B6	geranylacetone	5	A	0.003953	0.003953	0	0.06917
L19	1	B6	geranylacetone	5	A	0.00404	0.00404	0	0.022222
L19	3	B6	geranylacetone	5	A	0.005693	0.005693	0	0.047438
L19	4	B6	geranylacetone	5	A	0.012526	0.008351	0.002088	0.025052
L19	7	B6	geranylacetone	5	A	0.011881	0.011881	0	0.073267
L20	1	SJL	geranylacetone	5	A	0.016432	0.13615	0.023474	0
L20	3	SJL	geranylacetone	5	A	0.015873	0.177249	0.050265	0
L20	4	SJL	geranylacetone	5	A	0.028351	0.221649	0.048969	0.005155
L20	7	SJL	geranylacetone	5	A	0.026954	0.245283	0.043127	0.005391
V17	1	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0	0	0	0.08498
V17	3	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0	0	0	0.048027
V17	4	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0.00369	0.00369	0	0.064576
V17	7	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0.007477	0	0	0.084112
V18	1	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.042959	0.167064	0	0
V18	3	SJL	3,4-dimethyl-1,2-	5	B	0.015424	0.208226	0.051414	0

			cyclopentadione						
V18	4	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.0375	0.24	0.04	0.0025
V18	7	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.029613	0.314351	0.075171	0.002278
V19	1	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.018293	0.213415	0.046748	0.002033
V19	3	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.02834	0.194332	0.034413	0.018219
V19	4	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.035928	0.233533	0.045908	0.011976
V19	7	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.028103	0.199063	0.035129	0.04918
V20	1	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0.011583	0.009653	0	0.03668
V20	3	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0.008016	0.008016	0	0.058116
V20	4	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0.010288	0.00823	0	0.053498
V20	7	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0	0	0	0.0282

Table E.4. Cage change behavior analyzed for treatment effects.

Cage	Strain	Treatment	Batch	Facility	Mediated Proportion	Submissive Proportion	Escalated Proportion	Allo-groom Count
L1	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.083333	0.066667	0.05	1
L2	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.05	0.383333	0.016667	0
L3	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.018868	0.264151	0.056604	2
L4	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0	0.236842	0.078947	0
V1	SJL	geranylacetone	1	B	0.041667	0.375	0.083333	0
V2	B6	geranylacetone	1	B	0.051724	0.086207	0.103448	0
V3	B6	geranylacetone	1	B	0	0.016949	0.050847	1
V4	SJL	geranylacetone	1	B	0	0.136364	0.045455	0
L6	B6	control	2	A	0.035714	0.232143	0.017857	0
L7	B6	control	2	A	0.033898	0.067797	0	0
L8	SJL	control	2	A	0.016949	0.474576	0.118644	0
V5	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0	0.276596	0.085106	0
V6	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.04878	0.219512	0.04878	0
V7	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.019608	0.078431	0.019608	1
V8	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0	0.076923	0.019231	0
L10	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0	0	0	0
L11	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.016667	0.116667	0	1
L12	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.033333	0.066667	0.05	0
L9	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0	0	0	2

V10	B6	control	3	B	0.033333	0.083333	0	0
V11	B6	control	3	B	0	0	0	1
V12	SJL	control	3	B	0	0.315789	0.070175	0
V9	SJL	control	3	B	0.036364	0.290909	0.036364	0
L13	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0	0	0	2
L14	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.066667	0.3	0.033333	0
L15	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.089286	0.178571	0	3
L16	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0	0	0	0
V13	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0	0.298246	0.070175	0
V14	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.033333	0.083333	0.066667	0
V15	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0	0	0	0
V16	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.037037	0.37037	0.092593	0
L17	SJL	geranylacetone	5	A	0.05	0.416667	0.083333	1
L18	B6	geranylacetone	5	A	0	0	0	0
L19	B6	geranylacetone	5	A	0.018182	0.036364	0	1
L20	SJL	geranylacetone	5	A	0	0.293103	0.068966	0
V17	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0	0	0	1
V18	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.051724	0.189655	0.017241	0
V19	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0	0.137931	0.017241	1
V20	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0	0	0	0

Table E.5. Fecal corticosterone metabolites analyzed for treatment effects.

Cage	Mouse	Strain	Treatment	Social Rank	Batch	Facility	Posterior PALS	FCM ng/50mg feces
L1	L	B6	3,4-dimethyl-1,2-cyclopentadione	Sub	1	A	0.333333	52.9
L1	2R	B6	3,4-dimethyl-1,2-cyclopentadione	Dom	1	A	0.666667	40.2
L2	L	SJL	3,4-dimethyl-1,2-cyclopentadione	Sub	1	A	2.333333	78.6
L2	LR	SJL	3,4-dimethyl-1,2-cyclopentadione	Dom	1	A	2.666667	46.7
L3	2R	SJL	3,4-dimethyl-1,2-cyclopentadione	Sub	1	A	2.083333	70.3
L3	R	SJL	3,4-dimethyl-1,2-cyclopentadione	Dom	1	A	1.333333	80.6
L4	2L	B6	3,4-dimethyl-1,2-cyclopentadione	Sub	1	A	2.666667	390.6
L4	R	B6	3,4-dimethyl-1,2-cyclopentadione	Dom	1	A	0.666667	166
V1	R	SJL	geranylacetone	Sub	1	B	2	15.5
V1	LR	SJL	geranylacetone	Dom	1	B	0	21.8
V2	LR	B6	geranylacetone	Dom	1	B	0	61.1
V2	2R	B6	geranylacetone	Sub	1	B	0	19.3
V3	L	B6	geranylacetone	Dom	1	B	0	56.3
V3	2L	B6	geranylacetone	Sub	1	B	0	59
V4	2L	SJL	geranylacetone	Sub	1	B	0.666667	12.6
V4	R	SJL	geranylacetone	Dom	1	B	0	43.9
L6	L	B6	control	Dom	2	A	1	64.8
L6	2R	B6	control	Sub	2	A	0.666667	57.4
L8	L	SJL	control	Sub	2	A	3	44.2
V5	R	B6	6-hydroxy-6-methyl-3-heptanone	Sub	2	B	3	83.6
V5	2L	B6	6-hydroxy-6-methyl-3-heptanone	Dom	2	B	0.333333	26.5
V6	2L	SJL	6-hydroxy-6-methyl-3-heptanone	Dom	2	B	0	7.1
V6	R	SJL	6-hydroxy-6-methyl-3-heptanone	Sub	2	B	3.333333	27.4
V7	2L	SJL	6-hydroxy-6-methyl-3-heptanone	Sub	2	B	2.333333	18.8
V7	LR	SJL	6-hydroxy-6-methyl-3-heptanone	Dom	2	B	0.333333	21
V8	2L	B6	6-hydroxy-6-methyl-3-heptanone	Dom	2	B	0.333333	56.6
V8	LR	B6	6-hydroxy-6-methyl-3-heptanone	Sub	2	B	2.666667	178.7
L10	LR	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Dom	3	A	0	19.6

L10	2R	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Sub	3	A	1	51.8
L11	LR	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Sub	3	A	2.666667	10.1
L11	R	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Dom	3	A	0	17.1
L12	LR	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Sub	3	A	0.666667	59.2
L12	2L	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Dom	3	A	1.333333	47.3
L9	2L	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Sub	3	A	1	13
L9	LR	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Dom	3	A	0.333333	25.6
V10	L	B6	control	Sub	3	B	0.333333	80.9
V10	2L	B6	control	Dom	3	B	1.333333	30.5
V11	2R	B6	control	Dom	3	B	1.333333	37.6
V11	2L	B6	control	Sub	3	B	0.333333	40.6
V12	2R	SJL	control	Dom	3	B	1.333333	11.8
V12	R	SJL	control	Sub	3	B	2	16.9
V9	R	SJL	control	Sub	3	B	2	17.1
V9	L	SJL	control	Dom	3	B	0	12.9
L13	R	B6	6-hydroxy-6-methyl-3-heptanone	Dom	4	A	0.666667	24.2
L13	2L	B6	6-hydroxy-6-methyl-3-heptanone	Sub	4	A	0	51.6
L14	L	SJL	6-hydroxy-6-methyl-3-heptanone	Dom	4	A	1.333333	7.6
L14	LR	SJL	6-hydroxy-6-methyl-3-heptanone	Sub	4	A	2.666667	14.4
L15	LR	SJL	6-hydroxy-6-methyl-3-heptanone	Dom	4	A	0	18.7
L15	L	SJL	6-hydroxy-6-methyl-3-heptanone	Sub	4	A	2.666667	12
L16	R	B6	6-hydroxy-6-methyl-3-heptanone	Dom	4	A	0	46.2
L16	2L	B6	6-hydroxy-6-methyl-3-heptanone	Sub	4	A	0	23
V13	R	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Dom	4	B	0	12.8
V13	L	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Sub	4	B	3	9

V14	2R	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Dom	4	B	0.333333	34.3
V14	LR	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Sub	4	B	0.333333	53.3
V15	2R	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	Dom	4	B	0.333333	27.4
L17	LR	SJL	geranylacetone	Sub	5	A	0	10
L17	2L	SJL	geranylacetone	Dom	5	A	3	23.6
L18	L	B6	geranylacetone	Sub	5	A	0.666667	40.5
L18	2R	B6	geranylacetone	Dom	5	A	0	88.3
L19	2R	B6	geranylacetone	Sub	5	A	0.333333	59.3
L19	2L	B6	geranylacetone	Dom	5	A	0.333333	63.1
L20	2R	SJL	geranylacetone	Dom	5	A	1	17.1
L20	R	SJL	geranylacetone	Sub	5	A	2.333333	25.7
V17	LR	B6	3,4-dimethyl-1,2-cyclopentadione	Dom	5	B	0	39.4
V17	2R	B6	3,4-dimethyl-1,2-cyclopentadione	Sub	5	B	0	39.7
V18	2R	SJL	3,4-dimethyl-1,2-cyclopentadione	Dom	5	B	0	6.3
V18	2L	SJL	3,4-dimethyl-1,2-cyclopentadione	Sub	5	B	2	32.2
V19	LR	SJL	3,4-dimethyl-1,2-cyclopentadione	Sub	5	B	1.333333	36.1
V20	R	B6	3,4-dimethyl-1,2-cyclopentadione	Dom	5	B	0	52
V20	2R	B6	3,4-dimethyl-1,2-cyclopentadione	Sub	5	B	0.666667	44.4

Table E.6. Wound scores analyzed for treatment effects.

Cage	Strain	Treatment	Batch	Facility	Overall Escalated Proportion	Pelt Region	PALS Cage Sum
L1	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.005807	Posterior	2.333333
L1	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.005807	Middle	4.333333
L1	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.005807	Anterior	1.666667
L2	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.032581	Posterior	10
L2	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.032581	Middle	1.333333
L2	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.032581	Anterior	0
L3	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.038356	Posterior	10.08333
L3	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.038356	Middle	1.666667
L3	SJL	3,4-dimethyl-1,2-cyclopentadione	1	A	0.038356	Anterior	0
L4	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.061889	Posterior	9.583333
L4	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.061889	Middle	4
L4	B6	3,4-dimethyl-1,2-cyclopentadione	1	A	0.061889	Anterior	1.333333
V1	SJL	geranylacetone	1	B	0.037249	Posterior	9.333333
V1	SJL	geranylacetone	1	B	0.037249	Middle	1.666667
V1	SJL	geranylacetone	1	B	0.037249	Anterior	0.333333
V2	B6	geranylacetone	1	B	0.002799	Posterior	2
V2	B6	geranylacetone	1	B	0.002799	Middle	2.833333
V2	B6	geranylacetone	1	B	0.002799	Anterior	1.916667
V3	B6	geranylacetone	1	B	0.002602	Posterior	0.666667
V3	B6	geranylacetone	1	B	0.002602	Middle	2.583333
V3	B6	geranylacetone	1	B	0.002602	Anterior	1.333333
V4	SJL	geranylacetone	1	B	0.03778	Posterior	3.666667
V4	SJL	geranylacetone	1	B	0.03778	Middle	1.333333
V4	SJL	geranylacetone	1	B	0.03778	Anterior	0
L5	SJL	control	2	A	0.038564	Posterior	8.666667
L5	SJL	control	2	A	0.038564	Middle	3.333333
L5	SJL	control	2	A	0.038564	Anterior	0.666667
L6	B6	control	2	A	0.010763	Posterior	3.333333
L6	B6	control	2	A	0.010763	Middle	3.666667
L6	B6	control	2	A	0.010763	Anterior	2.25
L7	B6	control	2	A	0.006747	Posterior	2.333333
L7	B6	control	2	A	0.006747	Middle	3.166667
L7	B6	control	2	A	0.006747	Anterior	2.583333
L8	SJL	control	2	A	0.030261	Posterior	10.33333
L8	SJL	control	2	A	0.030261	Middle	2.333333
L8	SJL	control	2	A	0.030261	Anterior	0
V5	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.022315	Posterior	9.75
V5	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.022315	Middle	6.25

V5	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.022315	Anterior	1.25
V6	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.037112	Posterior	12
V6	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.037112	Middle	2.333333
V6	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.037112	Anterior	0
V7	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.046524	Posterior	8
V7	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.046524	Middle	1
V7	SJL	6-hydroxy-6-methyl-3-heptanone	2	B	0.046524	Anterior	0.333333
V8	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.014941	Posterior	10.33333
V8	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.014941	Middle	7.666667
V8	B6	6-hydroxy-6-methyl-3-heptanone	2	B	0.014941	Anterior	1.666667
L10	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.037328	Posterior	4.666667
L10	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.037328	Middle	1.666667
L10	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.037328	Anterior	0.666667
L11	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.047378	Posterior	10
L11	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.047378	Middle	1
L11	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.047378	Anterior	0
L12	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.002532	Posterior	3.666667
L12	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.002532	Middle	3.583333
L12	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.002532	Anterior	2.833333
L9	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.001025	Posterior	2.666667
L9	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.001025	Middle	4.333333
L9	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	3	A	0.001025	Anterior	2
V10	B6	control	3	B	0	Posterior	3.333333
V10	B6	control	3	B	0	Middle	4
V10	B6	control	3	B	0	Anterior	2.333333
V11	B6	control	3	B	0.000932	Posterior	3.333333
V11	B6	control	3	B	0.000932	Middle	5.666667
V11	B6	control	3	B	0.000932	Anterior	5
V12	SJL	control	3	B	0.04304	Posterior	12.66667
V12	SJL	control	3	B	0.04304	Middle	2.666667
V12	SJL	control	3	B	0.04304	Anterior	0.333333
V9	SJL	control	3	B	0.050067	Posterior	10.33333
V9	SJL	control	3	B	0.050067	Middle	1
V9	SJL	control	3	B	0.050067	Anterior	0
L13	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.00813	Posterior	1
L13	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.00813	Middle	1.666667

L13	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.00813	Anterior	1
L14	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.052857	Posterior	11.33333
L14	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.052857	Middle	1.333333
L14	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.052857	Anterior	0.333333
L15	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.039954	Posterior	10
L15	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.039954	Middle	1.333333
L15	SJL	6-hydroxy-6-methyl-3-heptanone	4	A	0.039954	Anterior	0
L16	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.001591	Posterior	0
L16	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.001591	Middle	1.666667
L16	B6	6-hydroxy-6-methyl-3-heptanone	4	A	0.001591	Anterior	1
V13	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.035428	Posterior	11.33333
V13	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.035428	Middle	1
V13	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.035428	Anterior	0
V14	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0	Posterior	3.333333
V14	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0	Middle	5.666667
V14	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0	Anterior	3.416667
V15	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0	Posterior	2.916667
V15	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0	Middle	5.166667
V15	B6	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0	Anterior	2.333333
V16	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.051528	Posterior	10.33333
V16	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.051528	Middle	1
V16	SJL	3,5-diethyl-2-hydroxycyclopent-2-en-1-one	4	B	0.051528	Anterior	0
L17	SJL	geranylacetone	5	A	0.053939	Posterior	11
L17	SJL	geranylacetone	5	A	0.053939	Middle	2.333333
L17	SJL	geranylacetone	5	A	0.053939	Anterior	0
L18	B6	geranylacetone	5	A	0	Posterior	1.666667
L18	B6	geranylacetone	5	A	0	Middle	3.333333
L18	B6	geranylacetone	5	A	0	Anterior	1
L19	B6	geranylacetone	5	A	0.000499	Posterior	1.666667
L19	B6	geranylacetone	5	A	0.000499	Middle	1.333333
L19	B6	geranylacetone	5	A	0.000499	Anterior	0.666667
L20	SJL	geranylacetone	5	A	0.040947	Posterior	12
L20	SJL	geranylacetone	5	A	0.040947	Middle	3.666667
L20	SJL	geranylacetone	5	A	0.040947	Anterior	0
V17	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0	Posterior	2.666667
V17	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0	Middle	4.666667

V17	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0	Anterior	2
V18	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.041894	Posterior	9.666667
V18	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.041894	Middle	0
V18	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.041894	Anterior	0
V19	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.040752	Posterior	8
V19	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.040752	Middle	0.666667
V19	SJL	3,4-dimethyl-1,2-cyclopentadione	5	B	0.040752	Anterior	0
V20	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0	Posterior	1.666667
V20	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0	Middle	3.333333
V20	B6	3,4-dimethyl-1,2-cyclopentadione	5	B	0	Anterior	2.333333

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