Unravelling information flow and olfactory eavesdropper networks in murine rodent communities

by

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Abstract

Functional roles of mammalian pheromones have routinely been investigated in an intraspecific context, such as territorial marking and sexual signaling. Sex pheromones, being innately conspicuous against a 'noisy' background to enhance detection by the intended receiver, are especially susceptible to interception, or eavesdropping, by heterospecific community members. Only recently was it discovered that predators are attracted to heterospecific predator scent, demonstrating intra-guild eavesdropping, but the underlying semiochemicals (message bearing chemicals) remained unknown. Here, I investigated the olfactory interceptive eavesdropper network and information flow in a murine rodent community.

First, I identified new pheromone components of female and male house mice, *Mus musculus*, as well as male deer mice, *Peromyscus maniculatus.* Headspace volatiles emanating from urine and feces excreta of males or females were collected and analysed by comparative gas chromatography-mass spectrometry (GC-MS). Candidate pheromone components were synthesized or purchased and tested for their attractiveness to rodents in both laboratory and field experiments. I discovered three new sex attractant pheromone components produced by female house mice (butyric acid, 2-methyl butyric acid and 4-heptanone) that attract conspecific males, two new pheromone components produced by male house mice (1-hexanol and 2,3,5-trithiahexane) that synergistically attract conspecific females, and a blend of nine ketones produced by male deer mice (3-methyl-2-pentanone, 5-methyl-2-hexanone 4-heptanone, 2-heptanone, 6-methyl-2-heptanone, 3-octanone, 2-octanone, 2-nonanone) that, together with testosterone, attracts conspecific females.

With these pheromone components in hand, I then investigated their exploitation by murine community members to elucidate the flow of olfactory information between species, guilds, and trophic levels. First, I tested for, and in field experiments experimentally demonstrate, intra-guild eavesdropping by wild brown rats, *Rattus norvegicus* (predator of mice), and wild house mice (prey of brown rats). Next, I tested for, and in animal shelter and field experiments demonstrate, inter-guild predatory eavesdropping by domestic and feral cats, *Felis catus* (predator of mice), on rodent-derived pheromones and sound signals or cues. Finally, I investigated olfactory information flow between two distantly related phyla, rodents and bumble bees, *Bombus* spp.. I show that queen bumble bees sense, and behaviorally respond to, (synthetic) rodent odor when they seek abandoned rodent burrows as nesting sites.

Keywords: Murine rodent communities; olfactory interceptive eavesdropper network; information flow; Behavioral two-choice experiments; GC-MS

To my animal family who shared their lives with we me and taught me so much. I will treasure you all forever.

To Oliver (Ollie, Irish Terrier),

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Chapter 1.

Introduction

Functional roles of mammalian pheromones have routinely been investigated in an intraspecific context, such as territorial marking, sexual signaling, and health status conveyance[1]. Mating signals, being innately conspicuous against a 'noisy' background and 'designed' to increase the likelihood of detection by the intended receiver[2], are especially susceptible to interception, or eavesdropping, by heterospecific community members. However, eavesdropping in mammals has focused on audio and visual communication systems, leaving interceptive eavesdropping on olfactory signals understudied[3]. That scent marks have received little attention is surprising because scent marks – deposited as glandular secretions, feces or urine – are used by most territorial mammals and in all aspects of social interactions[4]. This introductory chapter summarizes findings of interceptive eavesdropping on scent marks and discusses the likelihood that volatile sex attractant pheromone components are exploited in interceptive eavesdropping.

1.1. Interceptive eavesdropping on scent communication: A network perspective

Interceptive eavesdropping on scent communication has long been viewed as a one-way dyadic interaction, with prey sensing the scent of predators[5,6] because the ability of prey to detect and avoid predators is fundamental to prey survival[7]. Consequently, the majority of research has addressed the question whether prey sense predator scent[5]. However, interceptive eavesdropping is defined as "the use of information in signals by individuals other than the primary target"[2], and expanded views of auditory and visual communication systems now portray a multi-directional eavesdropper community network[3]. In this network perspective, the role of scent, in general, is hardly understood. Only recently was it discovered that predators are attracted to heterospecific predator scent[8], and that competing rodent species eavesdrop on each other's scent marks in a way that is affected by predation risk from a shared predator[9], both examples demonstrating intra-guild eavesdropping. Although these discoveries have added olfaction as a new sensory modality to the eavesdropper

community network (Figure 1), the underlying semiochemicals (message bearing chemicals) remain unknown.

1.2. Susceptibility of volatile pheromones to eavesdropping

The exploitation of pheromones as kairomones (inter-specific chemicals benefitting the receiver) is well documented in insects[10–13] but has hardly been studied in mammals[6]. Unlike acoustic and visual signals, pheromones often persist in the environment[8], and thus are particularly susceptible to inter-species exploitation as kairomones[14]. House mice recognize the presence of predators based on their major urinary proteins (MUPs)[15] but these high-molecular-weight proteins are less suitable than volatile sex attractant pheromone components for inter-species eavesdropping[5]. Volatile sex attractant pheromone components contrive long-range mate attraction[16] and thus are exceedingly susceptible to eavesdropping[3].

1.3. Selection of house mice as model organisms and murine rodents as a modal community

I selected the house mouse, *Mus musculus*, as a model species for my study for three reasons: (1) the house mouse has global distribution; (2) it relies on olfaction for communication and has well-documented use of pheromones, and (3) it inhabits urban ecosystems. Below, I elaborate on each of these reasons.

1.3.1. Near-global distribution of house mice

Of all mammalian families, the rodent family *Muridae* is the most diverse and abundant[17], including approximately 1,383 species that are distributed across the globe except for Antarctica and secluded oceanic islands. A few murid rodents such as Brown rats, *Rattus norvegicus*, roof rats, *Rattus rattus*, and house mice, *Mus musculus*, have dispersed with humans in the late Pleistocene (~11,700 years ago) and, as a result, now have near global distribution[18,19] and are well adapted to a wide variety of ecosystems[20].

1.3.2. Pheromonal communication of house mice

Scent-marking is a major form of pheromonal communication in house mice[21]. Males, in particular, are prolific scent markers[22]. Dominant (territorial) males urine-mark up to 100 times per hour[23], reflecting a major time and energy investment. Indeed, the energy investment (20-40 mg of MUP per 1 mL of urine[24]) is so significant that heavily marking (dominant) males experience a reduced growth rate compared to less frequently marking (subordinate) males[25].

House mice are archetypal of mammals that communicate by olfaction[9]. With approx. 950 intact and functional olfactory receptor genes in their genome, compared to only approx. 360 in humans[26], house mice have a very keen sense of smell. Their contact and airborne sex pheromone components are chemically diverse (see below), serve multiple functions (see below), and are sensed at sub-nanomolar levels by two types of receptors, the main olfactory epithelium and the vomeronasal organ (VNO)[27–32], the VNO sensing primarily compounds of little or no volatility[33].

The sex pheromone blend of male house mice consists of major histocompatibility complex (MHC) peptides[34], major urinary proteins (MUPs)[27], volatile sex attractant pheromone component[35–37] and sex steroid pheromone components[38]. These pheromone components offer a wealth of information about the signaller, including its age[39], health[40], breeding status[41,42], dominance[43], kinship and individual identity[44,45]. Urine deposits even have a timestamp informing the receiver of how recently they were placed[24]. Even though sex pheromone components of male mice have been intensely studied for decades, there is strong evidence for components that are still to be identified.

The sex pheromone blend of female house mice, in contrast, has hardly been studied but the sex steroids estradiol and progesterone have been assigned a pheromonal function, contributing to the attraction of both juvenile and adult males[38].

1.3.3. Information flow in the murine rodent community

Studying the pheromone signals that murine rodent communities members, including house mice, deer mice, *Peromyscus maniculatus,* and brown rats use to communicate information, and the semiochemical foraging cues that other community

members exploit, such as cats, *Felis catus*, hunting rodent prey, and bumble bees, *Bombus* spp., seeking abandoned rodent burrows as nesting sites, will allow us to gain a better understanding of the information flow within and between species, guilds, and trophic levels. This information, in turn, will inform the design of control tactics for pest species (all murine rodents studied here as well as feral cats), and the conservation of bumble bees which serve a vital pollination function. I elaborate on all of these aspects, in detail, in the introductions of respective research chapters.

1.4. Brief life histories of study organisms

1.4.1. House mice

House mice are listed as one of the world's worst invasive species[46]. They exploit and are uniquely adapted to habitats within and around human settlements, including homes, buildings, farms, food stores, and waste facilities[47]. Structural changes in house mouse molars indicate that the human/house mouse commensalism began when human hunter-gatherer communities became more sedentary, approximately 15,000 years ago[48]. As stowaways on ships, house mice expanded with human settlements and dispersed with humans in the late Pleistocene (~11,700 years ago)[19], eventually giving rise to the global distribution of house mice[49].

House mice are omnivores with continuously growing incisors[47] that can even flourish ferally on remote islands eating insects, seeds, eggs, and flowers[50]. House mice reach sexual maturation in only 28 days and have a gestation period of just 18 days[51]. These traits, combined with high genetic adaptability[47], enabled house mice to adapt to a wide variety of ecosystems[20]. House mice thrive in diverse environments ranging from equatorial to sub-Antarctic[49].

1.4.2. Brown rats

Brown rats are among the most invasive species[46]. They are native to the plains of Asia[52,53] but as stowaways on ships have invaded, and now inhabit, all continents except the Artic and Antartica[54]. In favorable conditions, Brown rats reproduce year-round. They become sexually mature in just 3 weeks and give birth to their young after only 3 weeks of gestation. As omnivores[55], Brown rats have an exceptionally broad diet.

With continuously growing and iron laden incisors[56], Brown rats can obtain food resources that would otherwise be inaccessible. Laboratory strains of Brown rats, and house mice, are premier mammalian models used in research across disciplines[57]. Nonetheless, little is known about the behavior of wild Brown rats.

1.4.3. Deer mice

Deer mice are native to, and nearly ubiquitous across, North America. They are likely the most common small mammal in North America, being present everywhere from high-elevation deserts to low-elevation forests[58]. The diet of deer mice is diverse and includes seeds, vegetables and bird eggs[58]. As prolific breeders, females have up to four litters of nine pups each per year[16]. The seasonal reproductive activity of deer mice is linked to photoperiod. Days with a decreasing photophase prompt females to delay the onset of sexual maturity and prompt males to lower the weight of their testes[20]. Deer mice are strictly nocturnal and even limit their foraging activities during full moons to reduce predation risk[18]. Being nocturnal, deer mice rely on olfaction to navigate their environment. Deer mice can be serious agricultural pests, particularly in orchards[59].

1.4.4. Domestic cats

Feral and domestic cats are descendants of the Near Eastern wildcat, *Felis silvestris lybica*[60]. They are thought to have self-domesticated between 9,500-3,600 B.P., essentially adapting to the human environment that is co-inhabited with commensal house mice and Brown rats as prey[61]. Perceived as rodent control agents, cats were common on ships which contributed to their global spread[61], now ranging from the sub-Antartic to the sub-Artic[62]. Feral and domestic cats, as obligate carnivores[63] and generalist predators, prey on birds, rodents, amphibians, reptiles, invertebrates, and wild rabbits[64–66]. Remarkably, domestic cats can survive without access to fresh water[62]. These traits, combined with a high reproductive rate, make domestic cats highly adaptable. With near-global distribution and generalist predatory behavior, feral cats are listed as one of the world's worst invasive species[46]. Feral cats are responsible, at least in part, for 8% of global bird, mammal, and reptile extinctions, and they pose a significant threat to an additional 10% of critically endangered bird, mammal, and reptile species[62].

1.4.5. Bumble bees

Bumble bees, *Bombus spp.,* are found in temperate climates and exhibit a yearly lifecycle[67]. Bumble bee queens, but not worker bees or males, overwinter often nestled in leaf litter and mulch, or in downed trees[68]. Species-specifically, sooner or later in spring, queens emerge[69] and seek above- or below-ground cavities as nesting sites[68]. At this stage, every queen is essentially solitary, founding her nest and provisioning it with pollen and nectar. At first, the queen uses her wax gland secretions to build wax pots in which she stores nectar from early-blooming flowers. Eventually, she forms a mound of pollen and wax, the 'brood clump'[69], and lays her first complement of eggs on it. She incubates the eggs until the larvae hatch and feeds the larvae on pollen and nectar collected from nearby flowers. Within 2-3 weeks, the larvae complete their development and then spin a cocoon within which they metamorphose to adult bees.

Bumble bees feed on the pollen and nectar of flowering plants throughout the spring and summer. Colonies, at their peak, can range from 30 to 400 individuals[67]. As the summer season progresses, queens lay unfertilized eggs, which give rise to male drones, and new queens are produced by feeding larvae a special diet (royal jelly) or possibly by exposing them to queen pheromone[68]. Once males and new queens have mated, queens go into hibernation and the cycle begins again[68].

Bumble bees are exceptional pollinators and measurably more effective than honey bees. Bumble bees visit and pollinate more flowers per minute, and pollinate flowers that honey bees cannot. The bumble bees' shaking of flowers with a distinctive buzzing is crucial for pollination of blueberries and cranberries[67]. Overall, the pollination service of bumble bees is crucial for numerous world crops and high crop yield[67].

1.5. Overview of research chapters

My thesis consists of seven chapters. Chapter 1 (this chapter) is a concise introduction of my field of study, and Research Chapters 2–7 report new findings. The thesis is presented in article format. Chapter 2 (Journal of Chemical Ecology) and Chapter 5 (Scientific Reports) have already been published. All other chapters have been submitted to various journals for peer review (Chapter 3: PLOS ONE; Chapter 4: Scientific Reports; Chapter 6: Biological Invasions; Chapter 7: Biological Conservation).

Each chapter is presented in the format that is required by the journal where the corresponding manuscript has been submitted for review. Furthermore, each research chapter includes an abstract, introduction, methods, results, discussion, and a reference list.

In Chapter 2, I identify volatile sex attractant pheromone components of female house mice. At three-day intervals, I collected headspace volatiles emanating from urine- and feces-soiled bedding of male and female mice as they progressed from juveniles to adults (from 21 to 56 days of age). Volatile analyses by GC-MS revealed three candidate pheromone components (CPCs) that were adult female-specific: butyric acid, 2-methyl butyric acid and 4-heptanone. In a two-choice laboratory experiment, we show that adult male mice spent significantly more time in the treatment chamber baited with both the synthetic steroids (progesterone, estradiol) and the synthetic CPCs than in the paired control chamber baited only with the synthetic steroids. In a field experiment, we show that trap boxes baited with both the CPCs and the steroids captured 6.7-times more adult males and 4.7-times more juvenile males than trap boxes baited with the steroids alone. We conclude that butyric acid, 2-methyl butyric acid and 4-heptanone are the first volatile sex attractant pheromone components identified in female house mice.

In Chapter 3, I reanalyzed the male odorant data from Chapter 2. In the process, I found three new candidate pheromone components (CPCs) that were significantly more abundant in headspace odorants of males than females: 1-hexanol, 2,3,5-trithiahexane, and 3-methyl-2-pentanone. Drawing on these data, I tested the hypothesis that these CPCs are part of the male house mouse sex pheromone. As males progressed from juveniles to adults, 1-hexanol, 2,3,5-trithiahexane, 3-methyl-2-pentanone and 2-*sec*-butyl-4,5-dihydrothiazole (thiazole; a previously known pheromone component) markedly increased in abundance. Drawing further on laboratory behavioral experiments showing that 1-hexanol and 2,3,5-trithiahexane are the key CPCs, I designed a critically important paired-trap field experiment. In this field experiment, I show that trap lures containing both the two CPCs, 1-hexanol and 2,3,5-trithiahexane, and a ternary blend ('TB') of known pheromone components [thiazole; 3,4-dehydro-*exo*-brevicomin; testosterone] attracted 11-times more adult females and 5.3 times more juvenile females than trap lures containing only the TB. These data support the conclusion that 1-hexanol and 2,3,5-trithiahexane are novel sex attractant pheromone components of house mouse males.

Having identified new volatile sex attractant pheromone components of male and female house mice (Chapters 2 & 3), my research objective in Chapter 4 was to identify sex attractant pheromone components of male deer mice which reportedly produce a sex pheromone that attracts female mice. Working with laboratory-strain and wild deer mice, I identified the male-produced volatile sex pheromone components that attract female mice and investigated whether the sex steroid testosterone enhances female attraction to volatile pheromone components. In comparative analyses of headspace volatiles from urine and feces excreta of male and female mice, one ketone (5-methyl-2-hexanone) was male-specific, and eight others (3-methyl-2-pentanone, 4-heptanone, 2-heptanone, 6-methyl-2-heptanone, 3-octanone, 2-octanone, 2-nonanone) were significantly more abundant in male samples than in female samples. In field experiments, I showed (*i*) that trap boxes baited with the ketone lure captured significantly more females than corresponding unbaited boxes, and (*ii*) that synthetic testosterone enhanced the attractiveness of the ketone blend to female deer mice, but not to male deer mice. Deer mice are the third rodent species (following house mice and Brown rats) shown to use a combination of volatile sex attractant pheromone components and a less volatile sex steroid pheromone component.

In Chapter 5, I study interceptive olfactory eavesdropping between predator and prey, commonly understood as a one-way dyadic interaction, where prey sense and respond to the scent of a predator. Here, I tested the “counterespionage” hypothesis that both predator and prey co-opt each other’s pheromone as a cue. I worked with wild brown rats (predator of mice) and wild house mice (prey of brown rats) as model species, testing their responses to pheromone-baited traps at infested field sites. The treatment trap in each of two trap pairs per replicate received sex attractant pheromone components (including testosterone) of male mice or male rats, whereas corresponding control traps received only testosterone, a pheromone component shared between mouse and rat males. I show that trap pairs disseminating male rat pheromone components captured significantly fewer mice than trap pairs disseminating male mouse pheromone components, and that no female mice were captured in rat pheromone-baited traps, indicating predator aversion. Indiscriminate captures of rats in trap pairs disseminating male rat or male mouse pheromone components, and fewer captures of rats in male mouse pheromone traps than in (testosterone-only) control traps indicate that rats do eavesdrop on the male mouse sex pheromone but do not exploit the

information for mouse prey location. I conclude that my counterespionage hypothesis is supported by trap catch data of both mice and rats but that only the mice data are in keeping with our predictions for motive of the counterespionage.

Chapter 6 is the first of two applied ecology chapters. Essentially, I tried to develop technology that would help control free-ranging domestic and feral cats that have caused population declines and extinctions of multiple prey species. Specifically, I tested the hypotheses that (H1) prey-derived pheromone and sound lures, in combination, attract and help capture cats, and (H2) pheromone lures or sound lures on their own mediate cat attraction. I prepared pheromone lures that contained synthetic sex attractant and sex steroid pheromone components of male and female house mice and male deer mice, and I assembled a sound lure comprising playback recordings of house mouse vocalizations and Brown rat food-chewing sounds. In field settings, 12 feral cats fully entered live traps baited with pheromone and sound lures, whereas three cats entered paired unbaited control traps. To tease apart the effect of pheromone and sound lures on cat attraction, shelter cats were then presented with two mouse toys that were either pheromone-baited and scentless, or that were sound-baited and silent. Nine out of 11 cats tested contacted or closely approached first the pheromone-baited toys, but cats did not exhibit a similar preference for sound-baited mouse toys. I show, for the first time, that cats are attracted to sex pheromone components of mice. Whereas prey sound, on its own, did not prompt behavioural responses by cats, it seemed to entice entry of feral cats into pheromone-baited live traps. This study provides proof of concept that rodent pheromone and sound lures can be developed to expedite captures of feral cats in trap-neuter-release (TNR) programs.

Chapter 7 is the second of two applied ecology chapters. Here, I investigated whether there is information flow between two distantly related phyla, rodents and bumble bees, and whether this information flow can be used towards bumble bee conservation. Drawing on reports that bumble bees in spring establish colonies in abandoned rodent burrows, I hypothesized (1) that queen bumble bees sense, and behaviorally respond to, rodent odor, and (2) that lures of synthetic rodent odor can guide spring queens to nesting sites. Currently, artificial nest boxes have low occupancy (10%) and thus are impractical for conservation use. To field-test attraction of queens to mouse excreta odorants, I tree-mounted paired nest boxes in florally rich locations. I randomly assigned clean bedding to one box in each pair and bedding soiled with urine

and feces of house mice to the other box. In this experiment, queens established colonies in 17 mouse-scented boxes and in six unscented boxes. This 43% occupancy rate of mouse-scented boxes represents a significant improvement over the 10% occupancy rate common for unscented boxes. I then collected headspace odorants from soiled bedding, identifying 10 odorants that elicited responses from queen antennae. In a further field experiment, I baited one box in each pair with a *synthetic* mouse odor lure and found that queens established colonies in 13 baited boxes and in six unbaited control boxes. Specifically, *Bombus mixtus* established seven colonies in baited boxes and only one colony in an unbaited box. With this proof-of-concept that synthetic lures can guide queens to nest boxes, I anticipate that bumble bee conservation programs will soon be able to offer both expanded floral resources and baited nest boxes readily detectable by queens.

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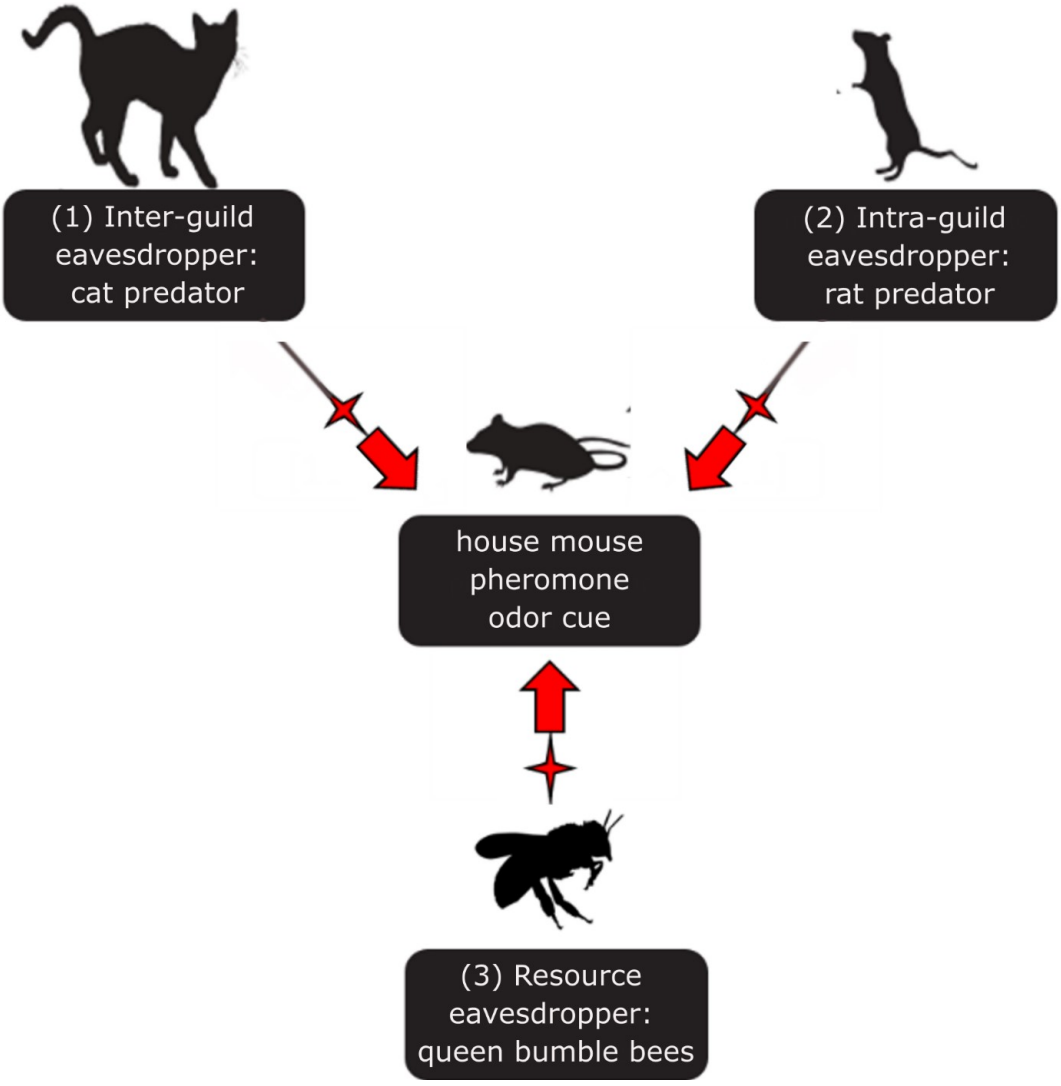

Figure 1.1. **Adapted illustration[8] depicting the murine rodent community olfactory eavesdropper network investigated in this thesis research. Red arrows and stars indicate research projects that I investigated, including (1) inter-guild eavesdropping by domestic cats on house mice (Chapter 6), (2) intra-guild eavesdropping by Brown rats on house mice (Chapter 5), and (3) eavesdropping by queen bumblebees on house mouse odorants as indicators of potential nesting sites (abandoned rodent burrows) (Chapter 7).**

Chapter 2.

Identification and field testing of volatile components in the sex attractant pheromone blend of female house mice

A very similar version of this chapter has been published: Elana Varner, Regine Gries, Stephen Takács, Stephanie Fan, Gerhard Gries (2018) Journal of Chemical Ecology 45:18-27.

2.1. Abstract

Recently, it was reported (*i*) that the sex pheromone blend of male house mice, *Mus musculus*, comprises not only volatile components (3,4-dehydro-*exo*-brevicomin; 2-*sec*-butyl-4,5-dihydrothiazole) but also a component of low volatility (the sex steroid testosterone), and (*ii*) that the sex steroids progesterone and estradiol are sex pheromone components of female house mice. Here we tested the hypothesis that the sex attractant pheromone blend of female mice, analogous to that of male mice, also comprises volatile pheromone components. Analyzing by GC-MS the head space volatiles of bedding soiled with urine and feces of laboratory-kept females and males revealed three candidate pheromone components (CPCs) that were adult female-specific: butyric acid, 2-methyl butyric acid and 4-heptanone. In a two-choice laboratory experiment, adult males spent significantly more time in the treatment chamber baited with both the synthetic steroids (progesterone, estradiol) and the synthetic CPCs than in the paired control chamber baited only with the synthetic steroids. In field experiments, trap boxes baited with both the CPCs and the steroids captured 6.7-times more adult males and 4.7-times more juvenile males than trap boxes baited with the steroids alone. Conversely, trap boxes baited with both the CPCs and the steroids captured 4.3-times more adult males and 2.7-fold fewer adult females than trap boxes baited with the CPCs alone. In combination, these data support the conclusion that butyric acid, 2-methyl butyric acid and 4-heptanone are part of the sex attractant pheromone of female house mice. With progesterone and estradiol being pheromone components of both female brown rats, *Rattus norvegicus*, and female house mice, these three volatile components could impart specificity to the sexual communication system of house mice, brown rats and possibly other rodent species.

2.2. Introduction

Communication of murine rodents is remarkably complex, involving multiple modalities of information conveyance, including olfaction (Hurst 1989; Arakawa et al. 2008), contact chemoreception (e.g., Luo et al. 2003; Fuss et al. 2005; Kimoto et al. 2005; Breer et al. 2006; Moncho-Bogani et al. 2002) and sound (e.g., Holy and Guo 2005). Each signalling pathway comprises a plethora of intricate and nuanced signals. For example, vocalizations of house mouse, *Mus musculus*, vary depending upon both the age and sex of the signaller (Clancy et al. 1984; Wysocki and Lepri 1991; Stowers et al. 2002; Osada et al. 2008) and the context of signalling (Grimsley et al. 2011). Ultrasonic courtship songs of male mice rival the complexity of courtship songs in birds (Holy and Guo 2005; Heckman et al. 2016).

Olfaction and contact chemoreception of mice and rats are equally sophisticated. Two types of receptors, the main olfactory epithelium and the vomeronasal organ, sense pheromone components of diverse chemical classes and functions (Leinders-Zufall et al. 2000; Kimoto et al. 2005; Haga et al. 2010; Chamero et al. 2007, 2011; Haga-Yamanaka et al., 2014; Fu et al., 2015; Takács et al. 2017) at sub-nanomolar levels (Spehr et al. 2006).

Pheromones in urine deposits of mice and rats offer a wealth of information about the signaller, including its age (Osada et al. 2008), health (Kavaliers et al. 2005), breeding status (Hurst 1990; Mossman and Drickamer 1996), dominance (Jones and Nowell 1973), kinship and individual identity (Barnard and Fitzsimons 1988; He et al. 2008). Urine deposits even have a timestamp informing the receiver of how recently they were placed (Hurst and Beynon 2004).

Mate location, attraction and assessment in house mice take place at night and thus are reliant primarily on chemical and (ultra) sonic signals. Chemical communication is mediated by sex pheromone components, comprising major histocompatibility complex (MHC) peptides (Leinders-Zufall et al. 2004; Haga et al. 2010), major urinary proteins (MUPs) (Chamero et al. 2007), volatile odorants (Jemiolo et al. 1985; Novotny et al. 1985; Schwende et al. 1986) and sex steroids (Takács et al. 2017). MHC peptides reveal the individual identity of a male and may trigger abortion in females, inducing new heat (Bruce 1960; Brennan 2009). MUPs exhibit their own pheromonal characteristics

(Chamero et al. 2007) and are highly concentrated in urine deposits of territorial males (Wyatt 2014). The MUP Darcin of males, e.g., not only improves spatial memory in MUP-sensing males but through associative learning also stimulates female memory and sexual attraction to an individual male's odour (Roberts et al. 2010, 2012, 2014). Furthermore, the tertiary structure of MUPs binds to, and facilitates slow release of, more volatile pheromone components such as 2-*sec*-butyl-4,5-dihydrothiazole (thiazole) (Bacchini et al. 1992; Robertson et al. 1993), thereby enhancing the longevity of pheromonal marks (Hurst et al. 1998; Armstrong et al. 2005). Thiazole together with the other volatile pheromone components 3,4-dehydro-*exo*-brevicomin (brevicomin) and α- and β-farnesene signals a male's dominance (Novotny et al. 1985), induces oestrus in adult females (Jemiolo et al. 1986), and accelerates puberty of juvenile females (Novotny et al. 1999; Flanagan et al. 2011). Conversely, thiazole and brevicomin synergistically contribute to aggressive behaviour in males (Novotny et al. 1985). The level of the sex steroid testosterone in urine deposits of males reflects their health (Zala et al. 2004), and enables females both to discern between males and to select healthy ones with high levels of testosterone (Zala et al. 2004). Testosterone also serves as a sex attractant pheromone component that synergistically with thiazole and brevicomin attracts females (Takács et al. 2017).

Much less is known about the sex attractant pheromone of female house mice. The reasons for this are not immediately obvious. Females may have been deemed, primarily, signal recipients that respond to the sex pheromone of territorial males rather than signallers themselves. Similarly, the chemicals fluctuating in accordance with the females' estrus cycle (Achiraman et al. 2011) seem to have been regarded mainly as indicators of sexual receptivity, or not, rather than as potential sex attractant pheromone components. Yet, in a recent field experiment, corncob bedding soiled with urine and feces of laboratory-kept female mice had a strong effect on attraction and capture of wild male mice (Musso et al. 2017), indicating the presence of sex attractant pheromone components deposited by female mice. In yet another field experiment, the female sex steroids progesterone and estradiol proved to be effective sex pheromone components for attraction and capture of both juvenile and adult male mice (Takács et al. 2017). Nonetheless, if the sex pheromone blend of female mice were to mirror that of male mice, and – analogously – were to comprise both non-volatile (sex steroid) and volatile

sex pheromone components, then these volatile pheromone components of female mice are yet to be unravelled.

Our objectives (O) were (1) to identify volatile sex pheromone components of female house mice, and (2) to test these compounds for their ability to enhance the attractiveness of the known pheromone components progesterone and estradiol.

2.3. Materials and Methods

2.3.1. Lab Animals

House mice (CD-1® strain), 21 days of age, were obtained from Charles River Laboratories International Inc. (Saint-Constant, QC J5A 2E7, Canada) and cared for by Animal Care Services of Simon Fraser University (SFU). On arrival, mice were assigned to nine groups of five females each and nine groups of five males each. Each group was accommodated in a cage (50 × 40 × 20 cm) lined with corncob bedding (Anderson's Bed o'cobs, The Andersons Inc. Maumee, OH 43537, USA) and enriched with Nalgene toys and running wheels (Jaimesons Pet Food Distributers, Richmond, BC V4G 1C9, Canada). All mice were provisioned with rodent food (LabDiet® Certified Rodent Diet, LabDiet, St. Louis, MO 64144, USA), and were given water *ad libitum*. All cages were kept at a photoperiod of 12L:12D in rooms maintained at 50% relative humidity and 21 °C.

2.3.2. O1: Identify volatile sex attractant pheromone components of female house mice

As mice progressed from juveniles to adults during days 21 to 56, urine- and feces-soiled bedding from nine groups of five females each, and nine groups of five males each, was collected and replaced with fresh bedding at three-day intervals. The combined soiled bedding from each of three groups of females (450 g), and each three groups of males (450 g), was placed into separate Pyrex glass chambers (30 × 15 cm) each connected to a Pyrex glass tube (15 cm × 5 mm OD) filled with the adsorbent Porapak Q (200 mg) serving as a volatile trap. Charcoal-filtered air was drawn through each chamber and the Porapak Q volatile trap at a flow of 1 L min^{-1}. After capturing urine and feces odorants on Porapak Q for 24 h, odorants were desorbed with consecutive

rinses of pentane (2 ml) and ether (2 ml). After adding dodecyl acetate as an internal standard, extracts were concentrated to 250 µl per sample.

Aliquots (2 µl) of each sample were analyzed on a Varian Saturn Ion Trap GC-MS fitted with a DB-5 MS GC column (30 m × 0.25 mm ID; Agilent Technologies Inc., Santa Clara, CA 95051, USA) using helium as the carrier gas (35 cm^{-1s}), and running the following temperature program: 40 °C for 5 min, 10 °C per min until 280 °C (5 min). The injector port was set at 250 °C and the ion trap at 200 °C. Odorants were identified by comparing their retention indices (relative to straight chain alkanes) and mass spectra with those of authentic standards purchased from suppliers (Table 1) or synthesized in our laboratory (3-methyl-2-buten-1-ol, *E*5-2-heptenone, 6-methyl-3-heptanone, 3,4-dehydro-*exo*-brevicomin, 2-*sec*-butyl-4,5-dihydrothiazole). Odorants that were either female-specific [butyric acid, 2-methylbutyric acid, 4-heptanone (see Results)] at the average time of female sexual maturity (Dutta and Sengupta 2016) or that increased in abundance as females progressed to sexual maturity were considered candidate pheromone components.

To determine whether the female-specific odorants originated from the urine or feces of female mice, three groups of four female mice each were removed on separate occasions from their home cage in SFU's animal care facility and placed on top of a stainless-steel grid in the upper part of a metabolic chamber (Techniplast® Metabolic Cage Systems, Braintree Scientific, Inc. Braintree, MA 02185, USA). While the females were kept in the chamber, they were provided with food and water *ad libitum*. Their elimination products fell through the stainless-steel grid, with feces and urine accumulating in separate catchment vials. Urine and feces were collected over 6 h from each group. Urine and feces samples from each of the three group were placed on separate filter papers and the emanating odorants were captured on Porapak Q for 24 h, after which they were desorbed and analyzed as described above.

The absolute configuration of the 2-methylbutryic acid produced by female house mice was determined by derivatizing it to the corresponding ethyl ester (Neises and Steglich 1978), and by analyzing this ester derivative and that of authentic standards [(racemic) 2-methylbutryic acid, (*S*)-2-methylbutryic acid] on a chiral GC column (see below). (*S*)-2-Methylbutryic acid was obtained by oxidizing (*S*)-(-)-2-methylbutanol.

To esterify mouse-produced 2-methylbutryic acid, 75-µl aliquots of each of three Porapak Q headspace volatile extracts were combined in one sample that then contained circa 500 $ng^{-1\ \mu l}$ of 2-methylbutryic acid. After concentrating the sample to 50 µl, 10 µl each of dimethylamino-pyridine (50 µg/µl) and absolute ethanol as well as 20 µl of dicyclohexylcarbodiimide (220 µg/µl) were added. This sample was kept overnight at room temperature before 100 µl of pentane were added. Aliquots (2 µl) of this sample were analyzed isothermally (70° C) by GC, using a 6890 Agilent GC (Agilent Technologies Inc.) fitted with a CP Chirasil Dex CB column [25 m × 0.25 mm ID; Varian Inc. (now Agilent), Lake Forest, CA 92630, USA], and setting the injector and FID detector to 240° C.

2.3.3. O2: Test volatile pheromone components of female house mice for their ability to enhance the attractiveness of progesterone and estradiol

The ability of the candidate pheromone components (CPCs) butyric acid, racemic 2-methylbutyric acid, and 4-heptanone to enhance the attractiveness of progesterone and estradiol as a lure was tested in laboratory and field experiments.

The laboratory experiment (Exp. 1) followed a protocol previously described (Musso et al. 2017). Briefly, for each replicate a single (reproductively active) male mouse (n = 22) was placed into a “release” chamber (40 cm × 20 cm × 30 cm) interconnected by a Pyrex glass T-tube (stem: 65 cm long, side arms: 45 cm long, all 10 cm in diameter) to a treatment and a control chamber (each 60 cm × 30 cm × 40 cm). Both chambers were baited with a piece of filter paper (Whatman #1, 120 mm, Maidstone, England, 01622) treated with a blend of the known pheromone components progesterone (250 ng) and estradiol (125 ng) dissolved in 50 µl of ether. The randomly assigned treatment chamber also received a CPC formulation [butyric acid (14 mg), 2-methylbutyric acid (1.4 mg), 4-heptanone (0.1 mg) in 1 ml of mineral oil] of which 200 µl were pipetted into a 400-µl polyethylene microcentrifuge tube (Evergreen Scientific, 18704 South Ferris Place, Rancho Dominguez, CA 90220) with a pierced (1.5 mm) lid. This tube was placed next to the steroid-treated filter paper. The amounts and ratios of the CPCs in this mineral oil formulation were carefully and repeatedly adjusted until they generated a headspace volatile blend equivalent to that emanating from soiled bedding of one female over 24 h. This headspace volatile blend remained the same during 0-24 h

and 24-48 h, justifying the replacement of CPC lures in field experiments (see below) only every second or third day. The control chamber in the laboratory experiment received a microcentrifuge tube filled with 200 µl of mineral oil. For each 10-min bioassay, the male mouse (n = 22) was allowed to enter the stem of the T-tube on its own accord in response to test stimuli, and the following data were recorded: (i) the treatment or control chamber he entered first with all four paws ("first choice data"), and (*ii*) his position at each of 40 15-sec intervals. Position data were then used to calculate the proportion of time the male spent in the treatment or the control chamber ("time spent data").

The corresponding field experiment (Exp. 2) (June - December 2017) was run in three locations in the Greater Vancouver Area and the Fraser Valley of British Columbia, Canada. Experimental replicates were set up along interior or exterior walls of a bird conservatory, livestock production facility, and a horse stable. Each replicate (n = 186) consisted of paired trap boxes (PROTECTA® Mouse, Bell Laboratories Inc. Madison, WI 53704, USA) (Fig. 1), with 0.5-m spacing between the boxes in each pair, and approximately 2 m between pairs. Each trap box contained a Victor® snap trap (M325 M7 Pro mouse Woodstream Co., Lititz, PA 175543, USA) that was baited with a food bait (Takács et al. 2018) which prompted feeding and thus capture of responding mice. Both the treatment and the control trap box in each pair were also baited with a piece of filter paper treated with progesterone (250 ng) and estradiol (125 ng) dissolved in 50 µl of ether (see above). The dose of each of the two steroids was – conservatively – 2-fold lower than previously tested (Takács et al. 2017). The treatment trap in each pair received a microcentrifuge tube filled with the CPC formulation in mineral oil (see above), whereas the corresponding control trap box in each pair received a microcentrifuge tube filled with mineral oil.

A second field experiment (Exp. 3) (n = 27) (May – August 2018; five locations in the Greater Vancouver Area and the Fraser Valley), tested the ability of progesterone and estradiol to enhance the attractiveness of the CPCs. The design was identical to the first field experiment except that both trap boxes in each pair were baited with the CPCs but only the treatment trap box received progesterone and estradiol.

In both field experiments, twice or thrice every week, traps were checked, and food baits and pheromone lures replaced. Whenever a mouse had been captured, its

sex [based on ano-genital distance (Schneider et al. 1978)] and its age [juvenile or adult based on genitalia development (visibly discernable testes (Montoto et al. 2012) of adult males (approx. 45 days of age); vaginal opening (Safranski et al. 1993) of adult females (approx. 27 days of age)] were recorded, and a new trap box and snap trap were deployed. This procedure ensured that the odor of captured mice did not affect future captures. The position of the treatment and the control trap within a trap pair was re-randomized after each capture.

2.3.4. Statistical analyses

For (laboratory) experiment 1, first-choice data were analyzed by a χ^2 test, and proportion time-spent data were analyzed by the Student's t-test. Field capture data of mice in treatment and control traps were analyzed by χ^2 tests with Yate's correction for continuity (Exps. 2, 3) and by a logistic regression model (Exp. 2) performed in SAS® statistical software version 9.4., with age-sex (juvenile, adult, male, female) and location as factors. The probabilities of trapping mice in these four groups were analyzed by multiple comparisons using a Tukey test ($\alpha = 0.05$). Limited trap captures in experiment 3 did not warrant data analyses with the logistic regression model.

2.4. Results

2.4.1. O1: Identify candidate sex attractant pheromone components of female house mice

GC-MS analyses of headspace volatiles emanating from urine- and feces-soiled bedding of female mice revealed a plethora of odorants comprising acids, ketones, alcohols, sesquiterpenes, as well as sulfur- and nitrogen-containing compounds that varied greatly in relative abundance (Table 1). Compared to headspace volatiles of bedding soiled with urine and feces from mature male mice (> 33 days of age; Table 1), three odorants were female-specific: butyric acid, 2-methylbutyric acid, and 4-heptanone. While these components differed in absolute amounts, their relative abundance did not increase as female mice progressed from juveniles to adults (Fig. 2). Comparative chiral GC analyses of ethyl ester derivatives of (*i*) 2-methylbutyric acid present in soiled bedding headspace volatiles, (*ii*) racemic synthetic 2-methylbutyric acid

and (*iii*) synthetic (*S*)-2-methylbutyric acid (Fig. 3) revealed that female mice produce the *S*-enantiomer of 2-methylbutyric acid.

2-Methylbutyric acid and 4-heptanone were present in headspace volatiles of urine, but not feces, collected from female mice in metabolic chambers. Conversely, butyric acid was present in headspace volatiles of feces but not urine.

2.4.2. O2: Test volatile pheromone components of female house mice for their ability to enhance the attractiveness of progesterone and estradiol

In (laboratory) experiment 1, nearly as many males (n = 12) entered first the treatment chamber baited with both the steroids (progesterone, estradiol) and the CPCs as entered first the control chamber (n = 10) baited only with the steroids ($\chi^2 = 0.045$; $P > 0.05$). However, males spent significantly more time (14.2%) in the treatment chamber than in the control chamber ($t = 2.92$, $P < 0.0082$).

In (field) experiment 2, treatment trap boxes baited with both the two steroids (progesterone, estradiol) and the CPCs captured significantly more adult males ($\chi^2 = 25.13$ $P < 0.0001$; Fig. 4) and more juvenile males ($\chi^2 = 26.68$ $P < 0.0001$; Fig. 4). Out of 46 adult males captured, 40 were captured in traps baited with the CPCs. As a trap lure, the CPCs had no significant effect on captures of adult females ($\chi^2 = 2.0$ $P = 0.16$; Fig. 4) and juvenile females ($\chi^2 = 3.25$ $P = 0.07$; Fig. 4). The probability of capturing a mouse of a particular age or sex differed based on the trap lure (logistic regression analysis; Tukey's test for multiple comparisons of trapping probabilities; $P < 0.05$). In treatment trap boxes baited with both the CPCs and the steroids, the probability of capturing adult males and juvenile males was 0.78 and 0.82, respectively, whereas the probability of capturing adult females and juvenile females was 0.66 and 0.37, respectively.

In (field) experiment 3, treatment trap boxes baited with both the CPCs and the steroids captured significantly more adult males ($\chi^2 = 5.06$ $P = 0.024$; Fig. 5), and significantly fewer adult females ($\chi^2 = 3.27$ $P = 0.07$; Fig. 5), than control trap boxes baited only with the CPCs. Trap catch data of juvenile males and juvenile females in this experiment were too low to reveal a behavior-modifying effect attributable to the CPCs.

2.5. Discussion

Our data support the hypothesis that female house mice produce volatile sex attractant pheromone components that enhance the pheromonal effect of the steroid pheromone components progesterone and estradiol. Below, we elaborate on our conclusion.

A previous study (Takács et al. 2017) reported that trap boxes baited with synthetic progesterone alone or in combination with synthetic estradiol captured significantly more adult and juvenile male mice than corresponding unbaited control traps, thus establishing a pheromonal function for these two female sex hormones, particularly progesterone. Both steroids had no effect on captures of juvenile and adult female mice, further supporting the conclusion that they are sex attractant pheromone components affecting the behavior of males. Both of these steroids are compounds of high molecular weight (progesterone: 314 Da; estrogen: 272 Da) with low volatility and thus limited active space over which they may attract males. As the sex attractant pheromone blend of house mouse males comprises both volatile pheromone components [3,4-dehydro-*exo*-brevicomin, 2-*sec*-butyl-4,5-dihydrothiazole (Jemiolo et al. 1985; Novotny et al. 1985; Schwende et al. 1986; Musso et al. 2017)] and a less volatile steroid pheromone component [testosterone (Takács et al. 2017)], it seemed plausible that the sex attractant pheromone blend of female mice also comprises components of low and high volatility. With progesterone and estradiol already identified as components of low volatility (Takács et al. 2017), our study here focused on finding volatile pheromone components that may attract males over a longer range.

Our search started with the acquisition and analyses of headspace volatiles emanating from bedding soiled with urine and feces of female mice that progressed during days 21-56 of age from juveniles to adults. Using the same procedures for male mice, and comparing odor profiles of females and males, we could determine three odorants that were female-specific in mature mice: butyric acid, 2-methylbutyric acid and 4-heptanone (Table 1). Of these, the absolute amount of butyric acid exceeded that of 2-methylbutyric acid and 4-heptanone about 10-fold (Fig. 2). A fourth female-specific compound, 3-methylbutyric acid, became evident only after the field experiment was well under way. This component may, or may not, have pheromonal activity.

The first field experiment was designed to test whether the candidate pheromone components (CPCs) butyric acid, 2-methylbutyric acid and 4-heptanone enhance the well-established pheromonal effect of progesterone and estradiol. Consequently, both the treatment and the control trap box in each pair were baited with progesterone and estradiol but only the treatment trap box received the CPCs. Significantly higher trap captures of adult males (6.6-fold) and of juvenile males (4.7-fold) in treatment boxes baited with both the CPCs and the two steroids than in control boxes baited only with the two steroids (Fig. 3) clearly established a pheromonal function of the CPCs. This pheromonal function was also evident in the laboratory experiment where adult males spent more time in the treatment chamber baited with both the CPCs and the steroids than in the control chamber baited only with steroids. Moreover, the combined data of the two field experiments reveal a synergistic effect between the CPCs and the steroids which – in combination – attracted 6.6 times more adult males than the steroids alone (Exp. 2, Fig. 4) and 4.33 times more adult males than the CPCs alone (Exp. 3, Fig. 5). Field experiments with wild rodents are the best measure to reliably assess the behavioral effect of candidate pheromone components, as we have argued before (Takács et al. 2016).

The sex attractant pheromone blend of female and male house mice bears compositional resemblance in that each blend contains one or two components of low volatility [testosterone in males; progesterone and estradiol in females (Takács et al. 2017)] and two or three components of high volatility [3,4-dehydro-*exo*-brevicomin and 2-*sec*-butyl-4,5-dihydrothiazole in males (Jemiolo et al. 1985; Novotny et al. 1985; Schwende et al. 1986; Musso et al. 2017); butyric acid, 2-methylbutyric acid, and 4-heptanone in females (this study)]. In both the male and the female pheromone blend, components of low and high volatility seem to act synergistically in the attraction of prospective mates. Analogous to results in this study (Fig. 4), the more complex sex attractant pheromone blend of males attracted (*i*) 15-times more adult females and 2.4-times more juvenile females than testosterone alone (EV, ST et al. unpubl) and (*ii*) 15-times more adult females and 3.6-times more juvenile females than the volatile pheromone components alone (Takács et al. 2017).

Butyric acid, 2-methylbutyric acid and 4-heptanone seem to convey “female scent”, signalling the presence of a female but not likely “informing” males about her sexual maturity or receptivity. This conclusion is based on data that the relative amount

of these three components did not increase as females progressed from juveniles to adults (Fig. 2). Similarly, most (five out of six) volatile sex pheromone components of female brown rats did not increase in abundance as females became sexually mature (Takács et al. 2016), suggesting that other mechanisms reveal sexual maturity or receptivity of females. It is conceivable that both male rats and male mice “read” the sexual receptivity of females by monitoring the dynamic ratio of progesterone and estradiol during estrus cycles **(**Butcher el. 1974**)**.

While the data of this study advance our understanding of sexual communication in house mice, there may be additional pheromone components that await identification. However, these components appear to function in a context other than sexual communication between mature females and males. Our inference is based on differential captures of juvenile and adult mice in traps that were baited with either bedding soiled by females (Musso et al. 2017) or synthetic female sex pheromone (this study). Synthetic sex pheromone attracted juvenile males (Exp. 2, Fig. 4), whereas female-soiled bedding had no apparent effect on responses of juvenile males (Musso et al., 2017), suggesting that urine or feces deposits of adult female mice contain pheromone components that suppress attraction of sexually immature males. These as yet unknown pheromone components may be particularly adaptive in non-commensal house mouse populations that inhabit large territories with a limited food supply or access to water (Gray and Hurst 1997**)**. Here, adult females accrue no apparent benefit from attracting juvenile males that may compete with them for food and water resources.

With the sex steroids testosterone, progesterone and estradiol serving as sex attractant pheromone components in both house mice and brown rats (Takács et al. 2017), one might wonder about the mechanisms that impart specificity to sexual communication and habitat partitioning. In the presence of rats, house mice rarely co-infest buildings (de Masi et al. 2009), possibly because brown rats are strong food competitors (Barnett and Spencer 1951) and also prey on house mice (Molina et al. 1987; Ferreira et al. 2013; Yang et al. 2004). House mice may sense the presence of rats based – in part – on the volatile sex pheromone components that emanate from urine deposits of male and female brown rats. The ketone blend in urine deposits of male rats (2-heptanone, 4-heptanone, 3-ethyl-2-heptanone, 2-octanone, 2-nonanone, 4-nonanone) markedly differs from 3,4-dehydro-*exo*-brevicomin and 2-*sec*-butyl-4,5-dihydrothiazole in urine deposits of male house mice. Similarly, the volatile sex

pheromone components of female brown rats (2-methylbutyric acid, 3-methylbutyric acid, heptanal, hexanoic acid, 2-phenyl acetaldehyde, nonanal, decanal) differ from those of female house mice (butyric acid, 2-methylbutyric acid, 4-heptanone). The noticeable presence of 2-methylbutyric acid in pheromone blends of both female house mice and female brown rats implies that species-specificity of pheromonal communication is more contingent upon the blend composition than upon specific blend constituents (Johnston et al. 2003). This conclusion is reinforced by findings that 4-heptanone is a pheromone blend constituent of male brown rats (Takács et al. 2016) and female house mice (this study).

The identification of sex attractant pheromone blends of both male and female house mice, and the demonstrated efficacy of synthetic pheromone as a trap lure to attract and capture mice (Musso et al., 2017; Takács et al., 2016, 2017), offer new opportunities for mice management. Striking evidence that mouse and rat (anticoagulant) poisons enter food chains and wreak havoc on avian and mammalian rodent predators (Van Den Brink et al. 2018) necessitates the development and implementation of alternative (earth-friendly) methods of rodent control (Van Den Brink et al. 2018). Pheromone technology, combined with superior food baits (Takács et al. 2018), and coupled with a type of mechanical trap capable of resetting itself after rodent capture (Bond et al. 2011), has the potential to make rodent trapping as effective as rodent poisoning.

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2.8. Figures

Table 2.1. List of odorants identified in the headspace of corncob bedding soiled by three separate groups of 15 female and 15 male house mice each, progressing from 33-day-old juveniles to 56-day-old adults (see methods for details). Bold-face compounds (butyric acid, 2-methylbutyric acid, and 4-heptanone) are female-specific.

	Mean abundance (%)		
Compound[1]	Female	Male	Supplier
2-pentanone	0.18	0.4	Sigma-Aldrich[2]
3-methyl-3-buten-1-ol	6.96	4.6	Sigma-Aldrich[2]
3-methyl-2-pentanone	0.01	0.33	Sigma-Aldrich[2]
1-pentanol	0.27	0.35	Fisher Chemical[3]
3-methyl-2-buten-1-ol	0.20	0.42	Synthesized in Gries-lab[7]
butyric acid	**29.74**	**0.0**	Sigma-Aldrich[2]
Unknown	0.37	2.93	n/a
3-methylbutyric acid	**3.3**	**0.0**	Sigma-Aldrich[2]
2-methylbutyric acid	**1.14**	**0.0**	Sigma-Aldrich[2]
1-hexanol	0.15	1.88	Sigma-Aldrich[2]
4-heptanone	**1.52**	**0.0**	Sigma-Aldrich[2]
2-heptanone	32.83	43.6	Sigma-Aldrich[2]
*E*5-2-heptenone	5.6	4.06	Synthesized in Gries-lab[7]
2-acetyl-pyrroline	0.12	1.03	Toronto Research Chemicals[4]
*E*3-2-heptenone	2.3	2.15	Sigma-Aldrich[1]
Unknown	0.39	2.12	n/a
6-methyl-3-heptanone	6.39	4.44	Synthesized in Gries-lab[7]
dimethyl trisulfide	0.46	1.2	Sigma-Aldrich[2]
1-octen-3-ol	1.68	2.46	Sigma-Aldrich[2]
3,4-dehydro-*exo*-brevicomin	2.84	7.4	Synthesized in Gries-lab[7]
Acetophenone	1.96	3.61	Sigma-Aldrich[2]
2-*sec*-butyl-4,5-dihydrothiazole	0.00	5.56	Synthesized in Gries-lab[7]
2,3,5-trithiahexane	0.12	8.91	DeLong Chemicals America[5]
2-undecanone	0.21	0.13	Oxidized from 2-undecanonl
trans-caryophyllene	0.21	0.3	Sigma-Aldrich[2]
geranylacetone	0.24	0.69	Sigma-Aldrich[2]
β-farnesene	0.39	1.37	Bedoukian Research[6]
α-humulene	0.73	0.74	Sigma-Aldrich[2]

[1](5Z)-Tetradecenol (Yoshikawa et al. 2013), 1-hexadecanol and 1-hexadecanol acetate (Zhang et al. 2007) as well as *E*-β-farnesene and *E*,*E*-α-farnesene (Harvey et al. 1989; Novotny et al. 1990; Jemiolo et al. 1991; Ma et al. 1999) produced by male mice were not detected in this study possibly due to the specific laboratory strain of mice (CD-1) studied here or the lack of dominance and aggression displays by males;[2]Sigma Aldrich (SA), St. Louis, MO 63103, USA; [3]Fisher Chemical, Fisher Scientific, Fair Lane, NJ 07410, USA; [4]Toronto Research Chemicals Inc., North York, ON M3J 2J8, CA; [5]DeLong Chemicals America, New Haven, CT 06473, USA; [6]Bedoukian Research Inc., Danbury CT 06810, USA; [7]Synthesized in Gries-lab by Santosh K. Alamsetti, Grigori Khaskin or Huimin Zhai.

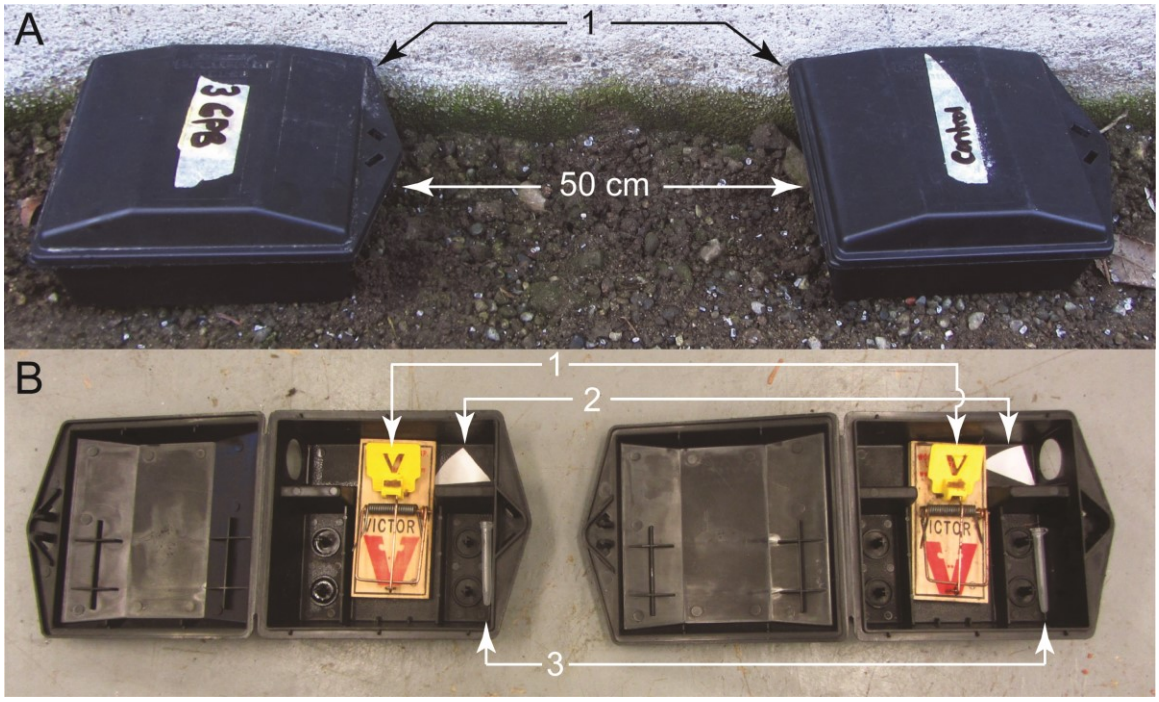

Figure 2.1. **Photographs illustrating (A) the experimental paired-trap design deployed in field experiments, and (B) details of the bait and lure tested in randomly assigned treatment and control traps, as follows: 1 = trap box, 2 = snap trap baited with a food bait (Takács et al. 2017), 3 = filter paper treated with the steroids progesterone and estradiol dissolved in ether or an ether control, 4 = 400-µl polyethylene micro-centrifuge tube (with pierced lid) containing 200 µl of mineral oil with, or without (control), the volatile candidate pheromone components butyric acid (2.8 mg), 2-methylbutyric acid (0.28 mg) and 4-heptanone (0.02 mg).**

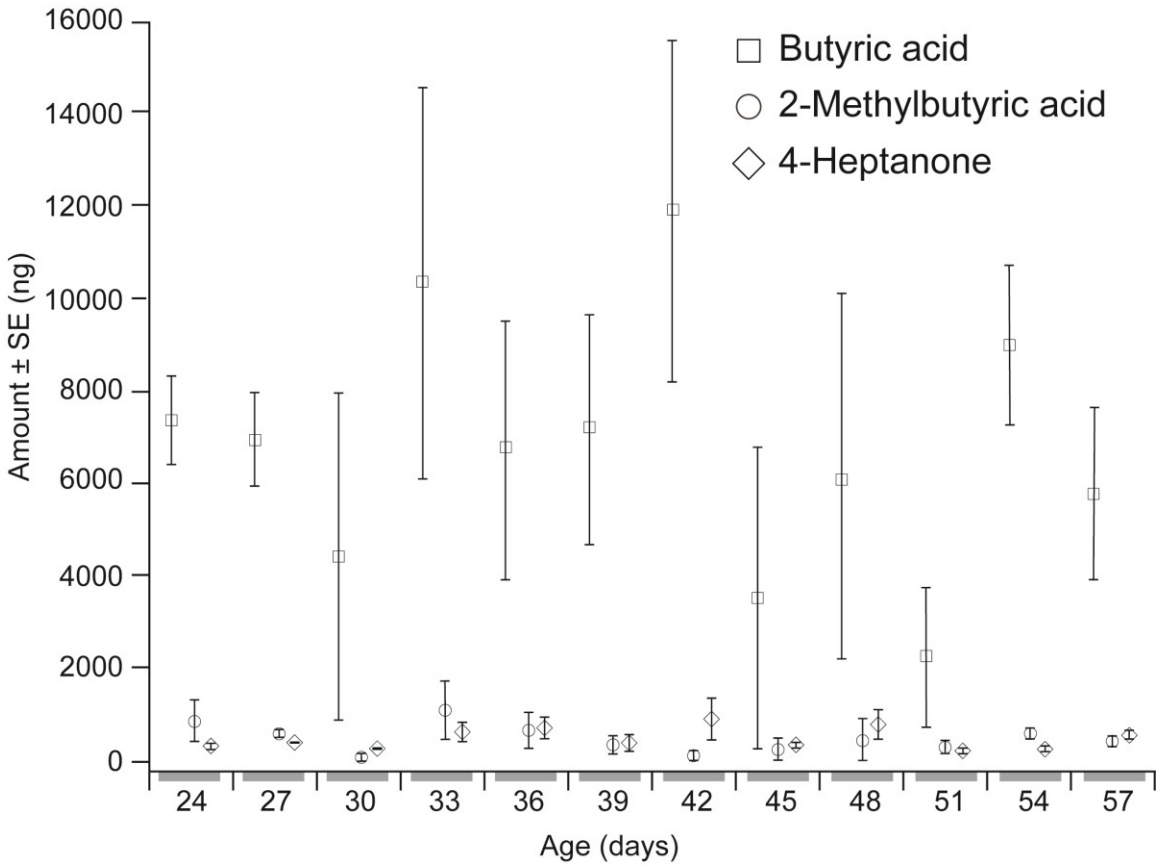

Figure 2.2. **Mean amounts (+ SE) of the pheromone components butyric acid, 2-methylbutyric acid, and 4-heptanone in the headspace of corncob bedding soiled by three separate groups of 15 female house mice each, progressing from 21-day-old juveniles to 56-day-old adults. Bedding was replaced at 3-day intervals (see methods for detail).**

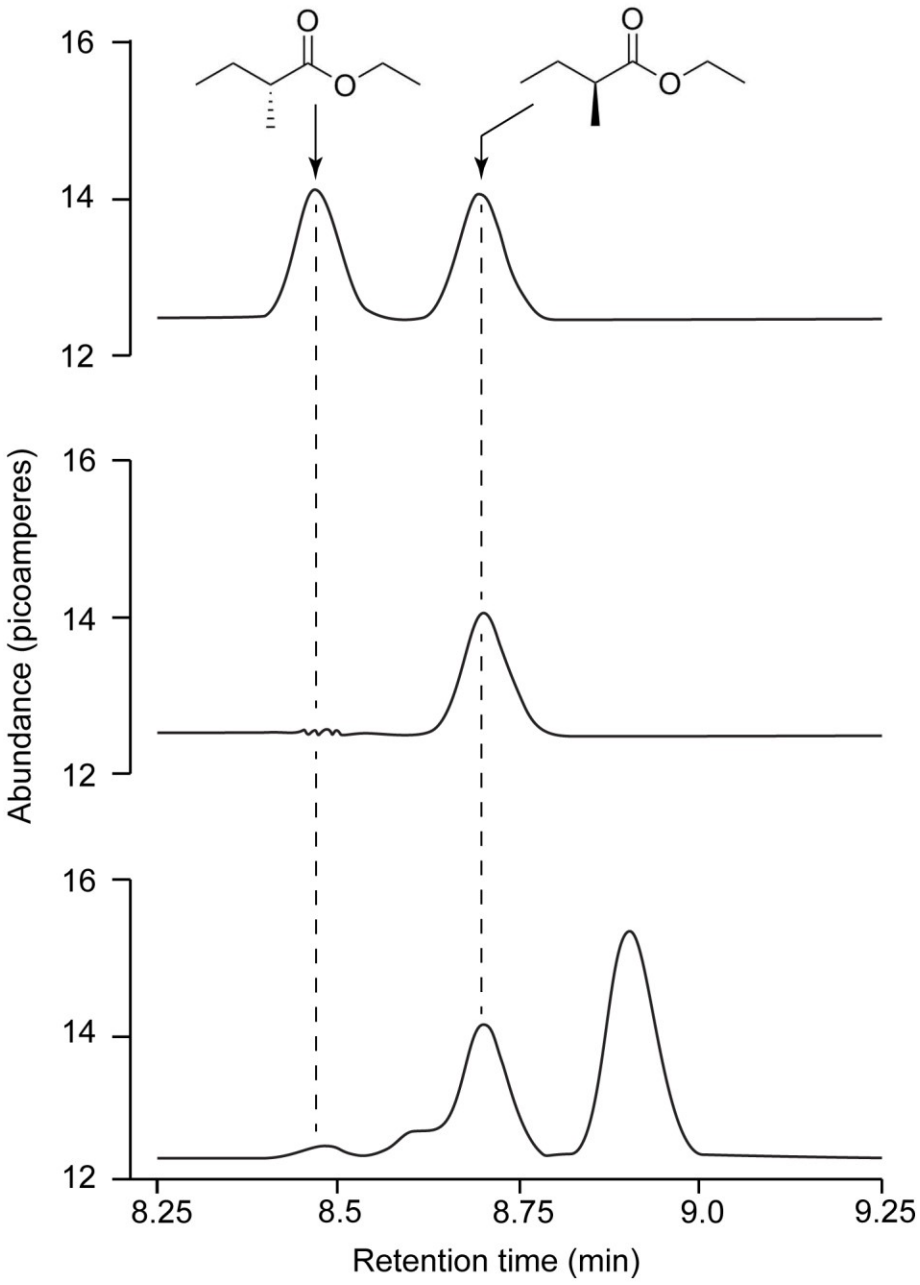

Figure 2.3. **Comparative gas chromatograms of the ethyl ester derivatives of 2-methylbutryic acid (top), (S)-2-methylbutryic acid (middle), and 2-methylbutryic acid produced by female house mice (bottom). Esterification enabled separation of the enantiomers on a chiral GC column and revealed that female house mice produce predominantly the (S)-enantiomer of 2-methylbutryic acid.**

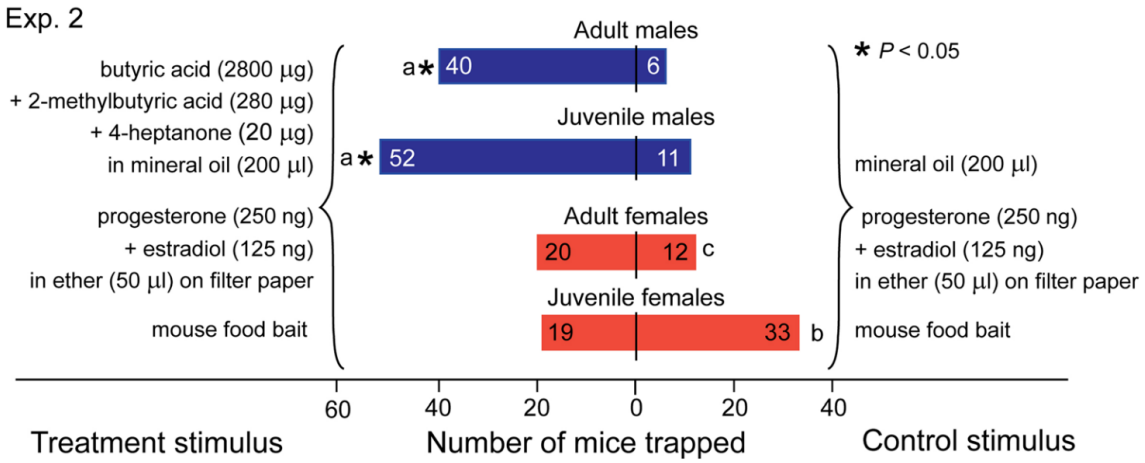

Figure 2.4. **Mean (+ SE) captures of adult and juvenile male and female house mice in paired traps (193 replicates) (see Fig. 1) baited with the steroid pheromone components progesterone and estradiol alone (control) or in combination with the candidate pheromone components (CPCs) butyric acid, 2-methylbutyric acid and 4-heptanone (treatment). An asterisk denotes a significant preference for the specific trap lure (χ^2-test using Yate's correction for continuity, $P < 0.05$). Bars with different letter superscripts indicate a different probability of capturing a mouse of a particular age or sex (logistic regression analysis; Tukey's test for multiple comparisons of trapping probabilities; $P < 0.05$). In traps baited with both the steroids and CPCs the probability of capturing adult males and juvenile males was 0.78 and 0.82, respectively, whereas the probability of capturing adult females and juvenile females was 0.66 and 0.37, respectively. There was no effect of location, indicating that data were consistent over all three field sites.**

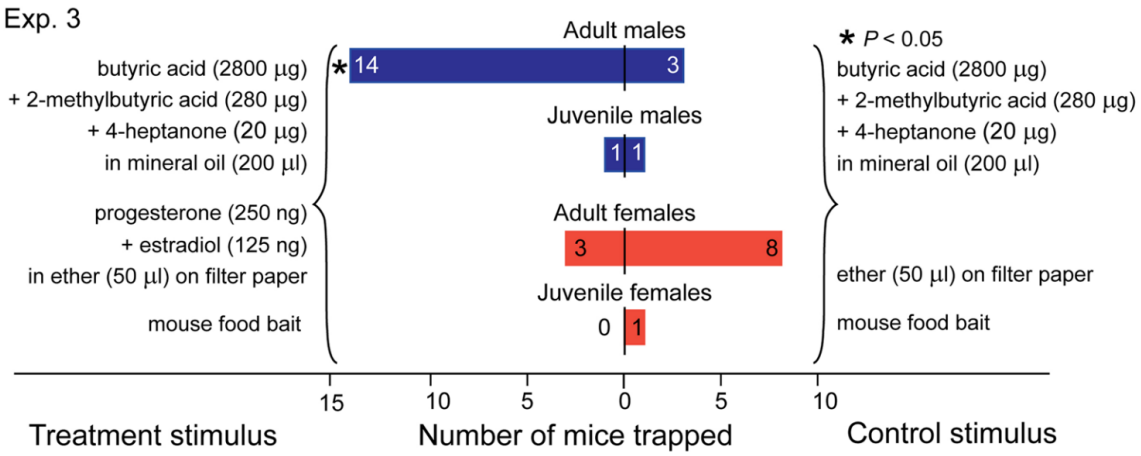

Figure 2.5. **Mean (+ SE) captures of adult and juvenile male and female house mice in paired traps (27 replicates) (see Fig. 1) baited with the candidate pheromone components (CPCs) butyric acid, 2-methylbutyric acid and 4-heptanone alone (control) or in combination with the steroid pheromone components progesterone and estradiol (treatment). An asterisk denotes a significant preference for the specific trap lure (χ^2-test using Yate's correction for continuity, $P < 0.05$).**

Chapter 3.

1-Hexanol and 2,3,5-trithiahexane – novel sex attractant pheromone components of house mouse males

A very similar version of this chapter has been resubmitted as a revised manuscript to PLoS ONE, with the following authors: Elana Varner, Daniella Gofredo, Kaya Vukovic, Kendal Singleton, Raissa Sourabh, Pabil Adhikari, Sahib Janjua, Adam Blake, Regine Gries, Gerhard Gries

3.1. Abstract

In a recent study, we reported the sex attractant pheromone components of female house mice, *Mus musculus.* Here, we closely analyzed the male odorant data from the same study and found three new candidate pheromone components ('CPCs') that were significantly more abundant in headspace odorants of males than females: 1-hexanol, 2,3,5-trithiahexane, and 3-methyl-2-pentanone. Drawing on these data, we tested the hypothesis that these CPCs are part of the male *M. musculus* sex pheromone. As males progressed from juveniles to adults, 1-hexanol, 2,3,5-trithiahexane, 3-methyl-2-pentanone and 2-*sec*-butyl-4,5-dihydrothiazole (thiazole; a previously known pheromone component) markedly increased in abundance. In a laboratory two-choice olfactometer experiment, significantly more female mice entered first the chamber baited with the CPCs in combination with a ternary blend ('TB') of known pheromone components [thiazole; 3,4-dehydro-*exo*-brevicomin (brevicomin); testosterone] than the chamber baited with the TB alone. In a corresponding field experiment testing the same stimuli in a paired-trap design, traps baited with the CPCs in combination with the TB captured 3.4-times more adult females and 5.5-times fewer adult males than traps baited with the TB alone. In two follow-up parallel field experiments, traps baited with the TB, but not traps baited with the CPCs and testosterone, captured significantly more female mice than control traps baited with testosterone alone, indicating that the synergistic pheromonal activity of the CPCs is contingent upon the presence of the TB. The final paired-trap field experiment drew on laboratory data showing that 1-hexanol and 2,3,5-trithiahexane are the key CPCs. In this field experiment, trap lures containing both the two CPCs 1-hexanol and 2,3,5-

trithiahexane and the TB captured 11-times more adult females and 5.3 times more juvenile females than trap lures containing only the TB. Our data support the conclusion that 1-hexanol and 2,3,5-trithiahexane are novel sex attractant pheromone components of house mouse males.

3.2. Introduction

Pheromone-based sexual communication of house mice, Mus musculus, is remarkably complex and reflects significant evolutionary advances in both signal "design" and signal perception. Contact and airborne sex pheromone components are chemically diverse (see below), serve multiple functions (see below), and are sensed at sub-nanomolar levels by two types of receptors, the main olfactory epithelium and the vomeronasal organ (VNO) [1–6], the VNO sensing primarily compounds of little or no volatility [7].

Sex pheromone components of M. musculus males are present in, and disseminate from, their urine markings and feces. Pheromone components consist of major histocompatibility complex (MHC) peptides [3,6], major urinary proteins (MUPs) [1], sex steroids [8], and volatile sex attractants [9–12]. In accordance with their molecular structure, all pheromone components contribute to the message of the signal, or its delivery, and cause behavioral or physical responses in female mice as signal recipients.

MHC peptides reveal a male's identity and may trigger abortion and ensuing new receptivity in females [13, 14]. Major urinary proteins on their own exhibit pheromonal activity [1,15–17], bind to sex attractant pheromone components, and facilitate their slow release [18,19], thus prolonging the effectiveness of pheromonal urine markings [20,21]. Dominant (territorial) males may urine-mark up to 100 times per hour [22], reflecting a major time and energy investment. Indeed, the energy investment (20–40 mg of MUP per 1 mL of urine [23]) is so significant that heavily marking (dominant) males experience a reduced growth rate compared to less frequently marking (subordinate) males [24].

The males' sex attractant pheromone components [2-sec-butyl-4,5-dihydrothiazole (thiazole) and 7-exo-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]-3-octene (= 3,4-dehydro-exo-brevicomin = brevicomin) [11] not only attract females in field settings

[10], they also signal – together with α- and β-farnesene – dominance [11], accelerate puberty (onset of first estrous cycle) in juvenile females [25,26], and induce estrus in adult females [9]. Although the sex steroid testosterone is a less volatile sex attractant pheromone component, together with thiazole and brevicomin it synergistically attracts females [8,27].

Sex pheromone components of M. musculus females have only recently been thoroughly studied. However, there is now convincing evidence that females communicate with sex attractant pheromone components over distance. In a field experiment, bedding material soiled with urine and feces of laboratory-kept females strongly attracted wild M. musculus males [10] and in a follow-up field experiment, synthetic female steroids (progesterone and estradiol) attracted both juvenile and adult males [8]. The attractiveness of these moderately volatile sex steroids was significantly enhanced by admixture of three rather volatile sex attractant pheromone components specific to adult females: butyric acid, 2-methyl butyric acid and 4-heptanone [28], indicating that the sex attractant pheromone blend of M. musculus females, analogous to that of males, comprises components of both low and high volatility.

Even though the sex attractant pheromone blend of M. musculus males has been intensely studied for decades [7,29,30], we expect that additional pheromone components are used in specific contexts and state-dependently [31]. Indeed, there is strong evidence for pheromone components that are still to be identified. For example, exposure of pre-pubertal female mice (Swiss strain) to urine of sexually mature males prompted accelerated puberty, shorter latency to vaginal opening, increased uterine weight, and behavioral attraction to male urine scent [32–35], all such physical or behavioral responses often being referred to as the “Vandenbergh” effect. Yet, the known sex attractant pheromone components of M. musculus males did not trigger the full complement of the “Vandenbergh” effect in juvenile females of a Swiss house mouse strain [36,37], and failed to elicit any Vandenbergh effect in pre-pubertal females of BALB/cJ house mice [26], supporting the conclusion that some sex pheromone components of M. musculus males have eluded identification [26,36,37].

Important clues to the missing pheromone components of male M. musculus came from a recent study reporting the sex attractant pheromone components of female M. musculus [28]. Analyzing urine and feces headspace odorants of female and male

mice by comparative gas chromatography-mass spectrometry, we found three female-specific odorants (butyric acid, 2-methyl butyric acid, 4-heptanone) that proved to be sex attractant pheromone components [28]. Here, we closely analyzed the male odorant data from the same study and found three new candidate pheromone components (CPCs) that were significantly more abundant in headspace odorants of males than females: 1-hexanol, 2,3,5-trithiahexane and 3-methyl-2-pentanone (Table 1). In addition to being sex-specific or -biased, these CPCs may also be linked to sexual maturity. For example, sex pheromone components of male brown rats, Rattus norvegicus, increase in abundance in sexually maturing males [38], and M. musculus males produce thiazole and brevicomin in accordance with their level of testosterone [11,12,39,40]. As sexually mature and female-exposed M. musculus males have the highest testosterone level [41–46], it follows that the CPCs, if they were sex pheromone components, would be more abundant in adult than in juvenile males.

As 3-methyl-2-pentanone emanates not only from urine deposits of male house mice (see above), but also of male deer mice, *Peromyscus maniculatus* (Varner et al., unpubl.), we predicted that this compound is least likely to contribute to the specificity of sexual communication in these two murine rodents, and for that reason is probably the least important of the three CPCs. This, however, does not exclude the possibility that a compound shared between closely related species may serve combinatorial code or modulator functions [47].

Working with laboratory-strain and wild M. musculus, in both laboratory and field experiments, we tested the hypothesis (H) that some of the CPCs are sex pheromone components of M. musculus males. We tested the specific hypotheses that: (H1) CPCs increase in abundance as males progress from juveniles to adults; (H2) CPCs synergistically increase the blend attractiveness of brevicomin, thiazole and testosterone; (H3) CPCs, as effectively as thiazole and brevicomin, enhance the attractiveness of testosterone; and (H4) 3-methyl-2-pentanone does not contribute to pheromonal attraction of female house mice.

3.3. Materials and methods

3.3.1. Laboratory mice

For laboratory experiment 1, reproductively unexperienced adult female house mice (C-57® strain) >6 weeks of age were obtained from the Christian-laboratory at Simon Fraser University (SFU), whereas house mice (CD-1® strain) for laboratory experiments 5–8 were obtained from Charles River Laboratories International Inc. (Saint-Constant, QC J5A 2E7, CA). Females were housed in groups of four or five in cages (50 × 40 × 20 cm) lined with corncob bedding (Anderson's Bed o 'cobs, The Andersons Inc., Maumee, OH 43537, USA), provisioned with rodent food (LabDiet® Certified Rodent Diet, LabDiet, St. Louis, MO64144, USA) and water *ad libitum,* and enriched with Nalgene toys and running wheels (Jaimesons Pet Food Distributers, Richmond, BC V4G 1C9, CA). Cages were maintained at 50% relative humidity and 21 °C and kept at a reverse photoperiod (12L:12D) to facilitate behavioral bioassays. For behavioral testing, individual mice were recognized based on a single ear punch in the right or the left ear, both ears, or no ear. Mice were allowed one week to recover after ear punching. All mice were cared for by Animal Care Services of SFU. Following completion of laboratory bioassays, mice were CO_2-euthanized. The research protocol was approved and supported by the Animal Care Committee of Simon Fraser University (protocol #1159B-15 and #1295B-19) which abides by the Canadian Council on Animal Care guidelines.

3.3.2. (H1) CPCs increase in abundance as males progress from juveniles to adults

To test H1, we drew on data that we had collected on female mice in the context of our 2019 study [28] but that we had not yet analyzed with respect to males for testing H1. As methods for collecting these data have already been detailed at length in this 2019-study, they will only be outlined here. Briefly, as mice (CD-1® strain) progressed from juveniles to adults during days 21 to 57 of age, urine- and feces-soiled bedding was collected and replaced with fresh bedding every three days during this 36-day period. Specifically, bedding was collected from nine cages with females and nine cages with males, each cage housing five mice. Three composite samples were prepared at each date's collection of bedding, with each sample comprising bedding from three cages.

The headspace odorants from each composite sample were captured on Porapak Q adsorbent, desorbed with pentane and ether, and analyzed on a Varian Saturn Ion Trap GC-MS fitted with a DB-5 MS GC column.

3.3.3. (H2) CPCs synergistically increase the blend attractiveness of brevicomin, thiazole and testosterone

Laboratory experiment (Exp. 1)

Experiment 1 (Table 2) used the design (Fig 1, a) and followed a protocol previously described [9]. Briefly, for each replicate (n = 20) a single reproductively mature female house mouse was placed into a release chamber (**1**) interconnected by a Pyrex glass T-tube (**2**) to a treatment and a control chamber (**3a, 3b**). Both the treatment and the control chamber were baited with (*i*) a piece of filter paper (**4**; Whatman #1, 120 mm, Maidstone, England, 01622) treated with testosterone (750 ng) dissolved in ether (50 µL) and (*ii*) a synthetic blend of brevicomin (1 mg) and thiazole (1 mg) formulated in mineral oil (10 mL) and contained in a 20-mL glass scintillation vial (**5**; VWR International, LLC Randor, PA 19087, USA) (Table 2). The randomly assigned treatment chamber in each replicate also received 700 µL of a CPC formulation [3-methyl-2-pentanone (0.3 mg), 1-hexanol (0.3 mg), and 2,3,5-trithiahexane (50 mg) in 100 mL of mineral oil] presented in a 0.5-dram glass vial (**6**). The amounts and ratios of the CPCs in this mineral oil formulation were carefully adjusted until they generated a headspace odorant blend equivalent to that emanating from previously soiled bedding (see above) of one male mouse over the course of 24 h. The control chamber received a 0.5-dram glass vial containing mineral oil only (**7**). Each of 20 mice was tested only once, and the olfactometer was thoroughly cleaned after each replicate [10]. The behavior of all mice was observed and video recorded (Akaso EK7000, Las Vegas, NV, 89117, USA).

To prevent the female mouse from contacting and knocking over the vial, it was placed inside a 600-mL beaker (**7**) next to the testosterone-treated filter paper. The control chamber in the laboratory experiment received a 0.5-dram glass vial (**8**) filled with 700 µL of mineral oil. For each 10-min bioassay, we allowed the female mouse to enter the stem of the T-tube on her own accord and we recorded whether she first entered (with all four paws) the treatment or the control chamber.

Field experiment (Exp. 2)

The corresponding field experiment (Exp. 2) (May to December 2018; Table 2) used the design (Fig 1, b) and followed the protocol previously detailed [10,28]. Briefly, experimental replicates were set up along interior and exterior walls of buildings in Greater Vancouver and the Lower Mainland of British Columbia. Each replicate consisted of paired trap boxes (**9a, 9b**; PROTECTA® Mouse, Bell Laboratories Inc., Madison, WI 53704, USA), with 0.5-m spacing between the boxes in each pair, and circa 2 m between pairs. Each trap box was fitted with a Victor® snap trap (**10**; M325 M7 Pro mouse Woodstream Co., Lititz, PA 175543, USA) that was set with a food bait (**11**; [48]) which prompted feeding and thereby capture of responding mice. Whenever a mouse had been captured, its sex (based on ano-genital distance [49]) and its age [juvenile or adult based on genitalia development (visibly discernable testes [50] of adult males (approx. 45 days of age); vaginal opening [51] of adult females (approx. 27 days of age)] were recorded, and a new trap box and snap trap were deployed [28]. If both trap boxes in a pair had captured a mouse within weekly intervals, it was not possible to ascertain which box captured first. Therefore, these data were not included in statistical analyses, and both boxes in such pairs were replaced with new ones. This protocol ensured that the odor of captured mice did not affect future captures. The position of the treatment and the control trap within a trap pair was re-randomized after each capture.

Treatment and control stimuli in this field experiment were the same as in the preceding laboratory experiment (Table 2) and were randomly assigned to one trap box in each of 49 trap box pairs (experimental replicates). The headspace pheromone blend of lures remained the same over the course of a week, allowing us to replace pheromone lures and food baits, and to record trap captures, once a week.

3.3.4. (H3) CPCs, as effectively as thiazole and brevicomin, enhance the attractiveness of testosterone

Field experiments (Exps. 3, 4)

Hypothesis 3 was tested in parallel field experiments 3 and 4 (May to August of 2019; Table 2), using the same general protocol described above, and running the same number of concurrent replicates in each test location. In both experiments, the treatment and the control box in each pair was baited with a piece of filter paper treated with

testosterone (750 ng) that was dissolved in ether (50 µL). In experiment 3, the treatment box in each pair was baited with brevicomin (1 mg) and thiazole (1 mg) formulated in mineral oil (10 mL) and contained in a 20-mL glass scintillation vial. In experiment 4, the treatment box in each pair was baited with 700 µL of the CPC formulation [3-methyl-2-pentanone (0.3 mg), 1-hexanol (0.3 mg), 2,3,5-trithiahexane (50 mg) in mineral oil (100 mL)] pipetted into a 0.5-dram glass vial. Effects of glassware or mineral oil potentially modulating attraction of mice to brevicomin and thiazole (Exp. 3), or to the CPCs (Exp. 4), were minimized by fitting treatment and control trap boxes in each experiment with the same glassware and volume of mineral oil.

3.3.5. (H4) 3-Methyl-2-pentanone does not contribute to pheromonal attraction of female house mice

Laboratory experiments (Exps. 5–8)

To determine the relative importance of each CPC, CPCs were tested in all binary and ternary combinations in laboratory experiments 5–8 (Table 2). The experimental set-up (Fig 1, c) consisted of a large circular galvanized steel arena (**12**) illuminated from above by a 7.5-W red bulb (Halco Lighting Technologies, Norcross, GA 17630071, USA) to facilitate observations and video recordings (Akaso EK7000) of the mouse's behavior and location in the arena. Two metal box-traps (**13a**, **13b**) (T. Eaton & Co. Inc., Twinsburg, OH 44087, USA) were placed in opposite quadrants of the arena 10 cm from the wall to serve as harborage. Both box-traps received a piece of filter paper (**11**; Whatman #1, Maidstone, England, 01622) to which (*i*) 750 ng testosterone in 50 µL ether and (*ii*) 3,4-dehydro-*exo*-brevicomin (1,317 ng) and 2-*sec*-butyl-4,5-dihydrothiazole (989 ng) in 50 µL ether were applied. The randomly assigned treatment box in each pair also received a piece of filter paper (**12**) treated with a ternary or a binary blend of the CPCs, each blend dissolved in ether (50 µL), as follows: Exp. 5: 1-hexanol (334 ng), 2,3,5-trithiahexane (1,585 ng); 3-methyl-2-pentanone (58 ng); Exp. 6: 1-hexanol (334 ng), 2,3,5-trithiahexane (1,585 ng); Exp. 7: 1-hexanol (334 ng), 3-methyl-2-pentanone (58 ng); and Exp. 8: 3-methyl-2-pentanone (58 ng), 2,3,5-trithiahexane (1,585 ng). The amounts of chemicals applied on filter paper were equivalent to those emanating from soiled bedding of one male mouse, on average, over the course of 24 h.

For each experimental replicate, a single female mouse was removed from her "home" cage and placed into a transportation container (**16**) which was then positioned

in the arena equidistant to both box-traps. The dorsal lid of the transportation container was then removed, and the container turned on its side such that its opening faced the arena wall, allowing the mouse to leave the container on her own accord and to explore the arena and the box-traps for 10 min. The arena quadrant the bioassay mouse entered first (with all four paws) was recorded as her first-choice response. Following completion of a bioassay replicate, the mouse was returned to her home cage, and the box-traps and the arena were cleaned with both a pet odor remover (A&H Pet Odor & Stain Remover, Walmart, CA) and 70% ethanol.

Each of these 11 mice was tested for her response to each of the four CPC blends, with the order of tests randomly assigned, and at least 30 days between bioassays.

3.3.6. Field experiment (Exp. 9)

With emerging evidence from laboratory experiments 5–8 that it is 1-hexanol and 2,3,5- trithiahexane that enhance the attractiveness of the sex pheromone blend of male house mice, and that the presence of 3-methyl-2-pentanone in a lure even reduces attraction of females (see Results), the final experiment (Table 2) was designed to substantiate these laboratory data in field settings. This field experiment followed the general field protocol described under hypothesis 2. Briefly, the experiment deployed 44 replicates of paired trap boxes (Fig 1, b), with 0.5-m spacing between the boxes in each pair, and circa 2 m between pairs. Each trap box was fitted with a Victor® snap trap set with a food bait [48]. Both the treatment and the control box in each pair received (*i*) a piece of filter paper treated with testosterone (750 ng) dissolved in ether (50 µL) and (*ii*) a synthetic blend of brevicomin (1 mg) and thiazole (1 mg) formulated in mineral oil (10 mL) and contained in a 20-mL glass scintillation vial. The randomly assigned treatment box in each replicate also received 700 µL of a CPC formulation [1-hexanol (0.3 mg), and 2,3,5-trithiahexane (50 mg) in 100 mL of mineral oil] presented in a 0.5-dram glass vial. The control box received 700 µL of mineral oil only, presented in a 0.5-dram glass vial. Whenever a mouse had been captured, its sex and age were recorded, a new trap box and snap trap were deployed, and the position of the treatment and the control box within a trap box pair was re-randomized after each capture.

3.3.7. Statistical analyses

For data analyses of hypothesis 1 (CPCs increase in abundance as male mice progress from juveniles to adults), we fit linear models to the mean amounts of each of the three CPCs (1-hexanol, 2,3,5-trithiahexane, 3-methyl-2-pentanone) and the previously known pheromone components thiazole and brevicomin as males matured from juveniles to adults. To avoid temporal pseudo-replication, we summarized the data across the three composite sample groups to give a single mean amount on each sampling date. Models initially included age (in days), age class (juveniles: < 33 days old; adults: ≥ 33 days old), and age and age class interaction, before non-significant terms were removed. As the fit of these models for 2,3,5-trithiahexane was suboptimal, the relationship between the mean amounts of 2,3,5-trithiahexane and the age of mice was presented as a non-linear sigmoid curve. We also ran paired t-tests comparing the mean amounts of each compound produced by juvenile and adult males. In this case, the data were summarized to give a single mean amount for each composite sample group across all sample dates for juveniles and adults.

For laboratory data analyses of hypothesis 2 (CPCs synergistically increase the blend attractiveness of brevicomin, thiazole and testosterone) and hypothesis 4 (3-methyl-2-pentanone does not contribute to pheromonal attraction of female house mice), we compared the proportion of mice entering first the treatment chamber in experiment 1, and the treatment quadrant in arena experiments 5–8, against a hypothetical 50:50 distribution, using a χ^2-test with continuity correction. χ^2-Tests were also applied for analyses of trap capture data in field experiments 2, 3 and 4, and 9 to test for proportions of captures deviating from a hypothetical 50:50 distribution, and to test for differences in proportion between male and female captures. All analyses were performed with R [52].

3.4. Results

3.4.1. (H1) CPCs increase in abundance as males progress from juveniles to adults

Headspace odorants of 24- to 30-day-old *M. musculus* males contained the CPCs at levels barely above detection threshold of the mass spectrometer (Fig 2, c, d,

e). The amounts of thiazole, one of the previously known pheromone components, were similarly low (Fig 2, a). As males progressed from 30 to 33 days of age, thiazole, 1-hexanol and 3-methyl-2-pentanone all markedly increased in abundance (thiazole: t = 6.42, df = 2, P = 0.0235; 1-hexanol: t = 13.46, df = 2, P = 0.0055; 3-methyl-2-pentanone: t = 6.42, df = 2, P = 0.0227; Fig 2, c, d, e). During the following 24 days (age 33–57), the amounts of these compounds remained relatively constant (slopes ≠ 0; thiazole: F = 0.50, P = 0.50; 1-hexanol: F = 0.90, P = 0.37; 3-methyl-2-pentanone: F = 0.46, P = 0.51). Throughout the study period (days 24–57), the relative abundance of 2,3,5-trithiahexane and brevicomin did not differ significantly between juvenile males and adult males (2,3,5-trithiahexane: t = 1.11, df = 2, P = 0.38; brevicomin: t = 3.08, df = 2, P = 0.09). Like other CPCs, brevicomin did increase markedly between days 30–33 (Fig 2, b) but then decreased over the next 24 days (slope = -303.67, F = 14.76, P = 0.0056). Unlike other CPCs, 2,3,5-trithiahexane increased gradually in a sinusoidal manner (Fig 2, e).

All analytical data combined support the hypothesis (H1) that the CPCs increase in abundance as males progress from juveniles to adults.

3.4.2. (H2) CPCs synergistically increase the blend attractiveness of brevicomin, thiazole and testosterone

Laboratory experiment (Exp. 1)

Seventeen adult females entered first the treatment chamber baited with both the ternary blend of brevicomin, thiazole and testosterone, and the CPCs, whereas only three females entered first the control chamber baited only with the ternary blend (χ^2 = 8.85; P = 0.0037; Fig 3), indicating a synergistic effect of the CPCs on pheromone blend attractiveness.

Field experiment (Exp. 2)

Treatment boxes baited with both the ternary blend and the CPCs captured 24 adult females, 3.4-times more than control boxes baited with just the ternary blend (χ^2 = 8.26; P = 0.0041; Fig 4), indicating – analogous to results of the laboratory experiment – a synergistic effect of the CPCs on pheromone blend attractiveness. Conversely, treatment boxes captured 5.5-times fewer adult males (2 *vs* 11) than control boxes (χ^2 = 4.92, P = 0.0265; Fig 4), revealing a deterrent effect of the CPCs on attraction of male

mice. The capture proportion of adult males and adult females differed significantly (χ^2 =12.13, P = 0.0005). Captures of a single juvenile female and four juvenile males (treatment *vs* control traps: 1 *vs* 3) were insufficient to warrant statistical analysis.

The combined data of laboratory experiments 1 and field experiment 2 support the hypothesis (H2) that CPCs synergistically increase the blend attractiveness of brevicomin, thiazole and testosterone.

3.4.3. (H3) CPCs, as effectively as thiazole and brevicomin, enhance the attractiveness of testosterone

Field experiments (Exps. 3, 4)

Traps baited with the ternary blend of brevicomin, thiazole and testosterone captured nine adult females, whereas traps baited with testosterone alone captured only one adult female (χ^2 = 4.9, *P* = 0.0269; Exp. 3, Fig 5, a), confirming the pheromonal activity of brevicomin and thiazole [10] in field settings. Numerically, captures of juvenile females (2), adult males (3), and juvenile males (1) were too low to warrant statistical analyses of data. In parallel field experiment 4, traps baited with testosterone and the CPCs captured two adult females and three adult males, whereas traps baited with testosterone alone captured four adult females (χ^2 = 0, *P* = 0.5), seven adult males (χ^2 = 0.9, *P* = 0.343), and one juvenile male (Exp. 4, Fig 5, b). While no data set in experiment 4 is statistically significant, it seems that the CPCs – even in the absence of brevicomin and thiazole – deter males. Interestingly, traps baited with the CPC lure in combination with testosterone captured 10 deer mice, *Peromyscus maniculatus,* eight of which were adult females (Fig 5, c).

The data of field experiments 3 and 4 do not support the hypothesis (H3) that the CPCs, as effectively as thiazole and brevicomin, enhance the attractiveness of testosterone.

3.4.4. (H4) 3-Methyl-2-pentanone does not contribute to pheromonal attraction of female house mice

Laboratory experiments (Exps. 5–8)

Female mice recognized the composition of CPC blends and responded accordingly (Fig 6). When the box-trap in the treatment quadrant of the arena (Fig 1, c) was baited with the 3-component CPC lure (1-hexanol, 2,3,5-trithiahexane, 3-methyl-2-pentanone), five females entered first the treatment quadrant and four females the control quadrant (Exp. 5: $\chi^2 = 0.11$; $P = 0.74$). However, when the CPC lure comprised only 1-hexanol and 2,3,5-trithiahexane, 10 females entered first the treatment quadrant and only one female entered the control quadrant (Exp. 6: $\chi^2 = 7.36$; $P = 0.0067$). When the CPC lure contained 1-hexanol and 3-methyl-2-pentanone, four females each entered first the treatment and the control quadrant (Exp. 7: $\chi^2 = 0$; $P = 1$). Finally, when the CPC lure contained 3-methyl-2-pentanone and 2,3,5-trithiahexane, three and seven females entered first the treatment and the control quadrant, respectively (Exp. 8: $\chi^2 = 1.6$; $P = 0.206$). That the 3-component CPC lure attracted C-57® strain mice in laboratory experiment 1, but not CD-1® strain mice in laboratory experiment 5, could have been due to the different strains of mice that were tested or due to differences in the experimental design or the response criterion that was recorded.

Field experiment (Exp. 9)

Treatment trap boxes baited with both the two CPCs 1-hexanol and 2,3,5-trithiahexane and the ternary blend of brevicomin, thiazole and testosterone captured 11-times more adult females and 5.3-times more juvenile females than control trap boxes baited with the ternary blend alone (adult females: $\chi^2 = 8.33$; $P = 0.0039$; juvenile females: $\chi^2 = 8.89$; $P = 0.0029$; Fig 7). Conversely, treatment trap boxes captured fewer adult males and fewer juvenile males than control trap boxes (adult males: 2 *vs* 6; $\chi^2 = 2.0$, $P = 0.157$; juvenile males: 0 *vs* 10; $\chi^2 = 8$, $P = 0.0047$).

The combined data of field experiments 2 and 9, and laboratory experiments 5–8, support the hypothesis (H4) that 3-methyl-2-pentanone does not contribute to pheromonal attraction of female house mice.

3.5. Discussion

Our data support the hypothesis that 1-hexanol and 2,3,5-trithiahexane are new sex attractant pheromone components of *M. musculus* males. Our conclusion is based on data showing that 1-hexanol and 2,3,5-trithiahexane (1) are distinctively more abundant in urine/feces headspace odorants of adult males than of adult females; (2) markedly increase in abundance as males sexually mature; and (3) in combination with the ternary blend of brevicomin, thiazole and testosterone attract significantly more adult wild females in field settings than the ternary blend alone.

Sexual maturity is an important trait of a prospective mate and seems to be broadcast by sex pheromone components. As males progressed from juveniles to sexually mature adults, 1-hexanol and thiazole rapidly increased in abundance (Fig 2), suggesting that these compounds, alone or in combination, divulge a male's sexual maturity. 2,3,5-Trithiahexane likely contributes to the message of "mature male scent", but its amount increased a few days later than that of other compounds and in a sigmoid- rather than single step-like form (Fig 2), resulting in statistically non-significant data. Brevicomin sharply increased in urine deposits of males > 33 days of age but then – surprisingly – declined steadily to a level not significantly different from that of juvenile males (Fig 2).

The message of sexual maturity likely also embodies testosterone because (*i*) testosterone titers in urine deposits of rodents increase with sexual maturation [44] and (*ii*) the biosynthesis of sex attractant pheromone components such as thiazole is testosterone-dependent. Moreover, testosterone titers may signal both "mature male" and "healthy male" because the level of testosterone in urine markings of healthy rodent males is significantly higher than that of diseased males [46].

The significant increase in thiazole abundance in urine/feces deposits of male mice as they sexually matured (Fig 2, a), coupled with the well-documented pheromonal activity of thiazole for attraction of female mice [9,10], implied that other constituents of male scent with similar increase in abundance in mature males, such as 1-hexanol, 2,3,5-trithiahexane and 3-methyl-2-pentanone, could also have a pheromonal function. In previous experiments, the blend of brevicomin and thiazole attracted females [9,10], and testosterone synergistically enhanced its attractiveness [8]. Here, we tested whether

1-hexanol, 2,3,5-trithiahexane and 3-methyl-2-pentanone further enhance the well-established pheromonal effect of brevicomin, thiazole and testosterone. The three CPCs tested in combination with the ternary blend of brevicomin, thiazole and testosterone enhanced the attractiveness of the ternary blend in a laboratory experiment (Fig 3), providing the first behavioral evidence for pheromonal activity of the CPCs.

As field experiments with wild rodents are the best measure to reliably assess the behavioral effect of candidate pheromone components [38], and because inbred laboratory strains of *M. musculus* may display behavioral responses divergent from those of their wild counterparts [53–55], we also tested the effect of the CPCs in a corresponding field experiment (Fig 4). Trap captures in this field experiment not only confirmed the synergistic pheromonal activity of the CPCs on attraction of *M. musculus* females (Fig 4), they also revealed a strong deterrent effect of the CPCs on wild *M. musculus* males. Sex-specific attraction of female mice, and deterrence of male mice, modulated by the CPCs in the sex attractant pheromone blend of *M. musculus* males are reminiscent of analogous behavioral effects expressed by the sex attractant pheromone blend of male brown rats [38]. Traps baited with synthetic sex pheromone of males captured 10-times more adult females and 4-times fewer adult males than unbaited control traps [38], demonstrating sex-specific pheromonal attractiveness and deterrence.

The strong behavioral responses of female and male *M. musculus* to the CPCs in the presence of brevicomin, thiazole and testosterone (Figs 3, 4) prompted us to explore whether the CPCs enhance attraction of females to testosterone even in the absence of brevicomin and thiazole. To study this question, we ran two parallel field experiments, each with a paired trap box design. All trap boxes in both experiments were baited with testosterone but the treatment box in each pair also received brevicomin and thiazole (Exp. 3) or the CPCs (Exp. 4). Unlike brevicomin and thiazole, the CPCs in combination with testosterone were not effective in attracting *M. musculus* females or strongly deterring males (Fig 5), indicating that their pheromonal activity hinges on the presence of brevicomin and thiazole.

Unexpectedly, traps baited with the CPC lure in experiment 4 also captured eight adult female deer mice (Fig 5) that likely responded to a sex pheromone component in that lure which was identical or similar to a sex pheromone component produced by

conspecific males. We hypothesized that this lure component was 3-methyl-2-pentanone. This ketone – as we have recently discovered – emanates not only from urine deposits of male house mice but also male deer mice (Varner et al., unpubl.). With 3-methyl-2-pentanone being a shared component in urine/feces odor profiles of male house and male deer mice, it follows that this ketone is the least likely constituent of the CPC blend to contribute to the attraction of female house mice. To test our prediction, we bioassayed the CPCs in all possible binary and ternary combinations in a large laboratory arena (Fig 6). In these experiments, the blend only of 1-hexanol and 2,3,5-trithiahexane attracted female mice, indicating that the presence of 3-methyl-2-pentanone is repelling to female mice or that the attraction of females hinges upon the presence of both 1-hexanol and 2,3,5-trithiahexane. That the ternary CPC blend (including 3-methyl-2-pentanone) was attractive in laboratory experiment 1 (Fig 3), but not in laboratory experiment 5 (Fig 6), may be attributed to (*i*) the different strains of mice that were bioassayed (Exp. 1: C-57®; Exp. 5: CD-1®), (*ii*) the contrasting experimental design used for testing (Fig 1, a, c), (*iii*) the different criteria (first chamber entered; first arena quadrant entered) scored as behavioral responses of females, and (*iv*) all of the above. That different strains of mice can exhibit different behavioral responses [56,57], or physiological responses [26,58], when presented with the same test stimulus is well documented. The proposed repellant effect of 3-methyl-2-pentanone was confirmed in the final field experiment of our study. Here, trap boxes baited with the two CPCs 1-hexanol and 2,3,5-trithiahexane and with the ternary blend of brevicomin, thiazole and testosterone captured 11-times more adult females and 5.3-times more juvenile females than control boxes baited with the ternary blend alone (Fig 7). Obviously, the effect of this two-component CPC blend was much stronger than that of the 3-component CPC blend which enhanced captures of females by only 3.4-fold (Fig 4).

The new pheromone components 1-hexanol and 2,3,5-trithiahexane discovered in this study engender at least two new research opportunities. First, it would be intriguing to study whether the currently known major urinary proteins (MUPs), which bind to and slowly release various pheromone components [25,59,60] including brevicomin and thiazole [61], also bind to and release these new components, or whether these components are ligands of other as yet unknown MUPs. Second, one or both of these new components may help elicit the complete Vandenbergh effect (accelerated puberty, shorter latency to vaginal opening, increased uterine weight,

behavioral attraction to male urine scent) in pre-pubertal female *M. musculus.* This question could be addressed by exposure of females to urine odor of mature males and to pheromone blends varying in the number of pheromone components.

Urine deposits of male mice reveal a plethora of information about the signaller, including his location, identity, and status [62]. Essentially, urine deposits are sexual advertisement and expression of competitive ability [23,63]. Pheromone components originating from urine deposits not only guide signal recipients to signal locations [64], they also induce learning [16]. The non-volatile sex pheromone component darcin promotes spatial preference and learning in females and rival males, allowing them to memorize signal locations [16]. Surprisingly, long-term memorization of pheromone locations is reinforced when two sensory modalities for signal perceptions are involved. Observations of coordinated sniffing and whisking during exploratory behavior [65,66] prompted investigations of the 'multimodal pheromonal learning' paradigm [67]. In this study, only female mice allowed to process information from both their olfactory and somatosensory whisker systems formed long-term memory of signal locations from male mice [67].

Incentive to incorporate pheromone technology for mice management is continually improving. Following the proof of concept that pheromone lures containing brevicomin and thiazole increase trap captures of wild female mice [10], testosterone was discovered as a sex steroid pheromone component and shown to synergistically enhance the attractiveness of thiazole and brevicomin [8]. The composition of brevicomin, thiazole, testosterone, 1-hexanol and 2,3,5-trithiahexane discovered in this study, is now a potent pheromone lure for attracting wild female mice. This lure, together with a proven-effective lure for attracting wild male mice [8,28], offers the opportunity for pheromone-based house mouse management.

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3.7. References

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3.8. Figures

Table 3.1. List of volatiles identified in the headspace of corncob bedding soiled by three separate groups of 15 female and 15 male house mice each, progressing from 24-day-old juveniles to 57-day-old adults (adapted from [28]; see methods for details). Bold-face compounds (3-methyl-2-pentanone, 1-hexanol, 2,3,5-trithiahexane, and 2-acetyl-pyrroline) were distinctively more abundant in headspace volatiles of males than of females. 2-Acetyl-pyrroline is toxic and thus was excluded from behavioural experiments.

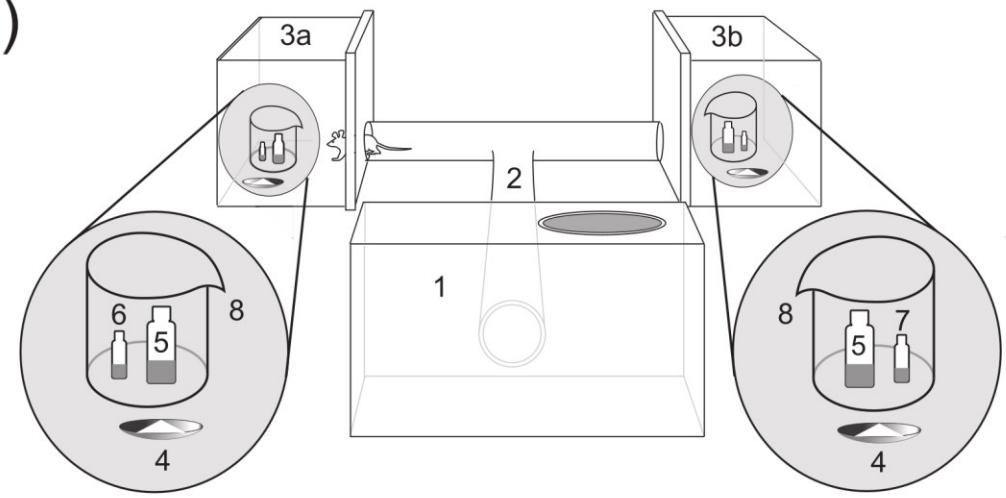
	Mean abundance (%)	
Compound[1]	Female	Male
2-pentanone	0.18	0.36
3-methyl-3-buten-1-ol	6.95	5.13
3-methyl-2-pentanone	**0.01**	**0.32**
1-pentanol	0.33	0.38
3-methyl-2-buten-1-ol	0.21	0.48
butyric acid	25.76	0.00
unknown	0.43	2.4
3-methylbutyric acid	4.28	0.00
2-methylbutyric acid	1.81	0.00
1-hexanol	**0.13**	**1.92**
4-heptanone	1.84	0.00
2-heptanone	32.07	40.53
*E*5-2-heptenone	5.59	4.51
2-acetyl-pyrroline	**0.12**	**0.92**
*E*3-2-heptenone	2.31	2.19
unknown	0.42	2.12
6-methyl-3-heptanone	7.39	5.41
dimethyl trisulfide	0.52	1.23
1-octen-3-ol	2.02	3.01
3,4-dehydro-*exo*-brevicomin	3.12	9.28
acetophenone	1.87	3.85
2-*sec*-butyl-4,5-dihydrothiazole	0.00	5.19
2,3,5-trithiahexane	**0.12**	**7.52**
2-undecanone	0.26	0.17
caryophyllene	0.29	0.32
geranyl acetone	0.30	0.63
farnesene	0.76	1.23
humulene	0.90	0.87

Table 3.2. List of stimuli, including the new candidate pheromone components (CPCs), tested in laboratory and field experiments with laboratory strain or wild house mice, *Mus musculus*

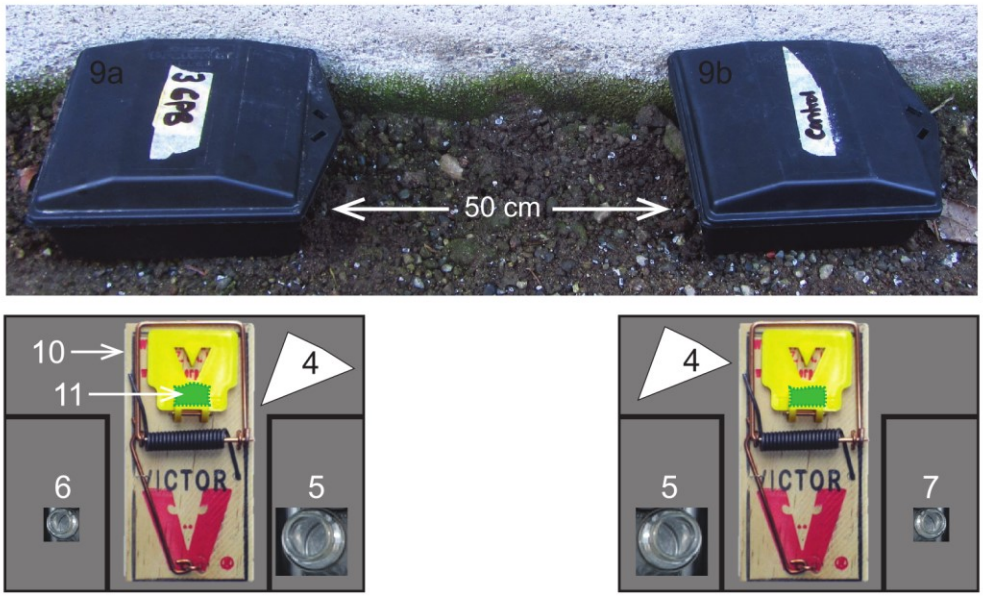
Exp. #	Stimulus 1	Stimulus 2
H2: CPCs synergistically increase the blend attractiveness of brevicomin1, thiazole2 and testosterone		
1 Lab	- 3 CPCs formulated in MO3,5 - Brevicomin + thiazole (1 mg each) in MO (10 mL)5 - Testosterone (750 ng) on filter paper	- MO - Brevicomin + thiazole (1 mg each) in MO (10 mL) - Testosterone (750 ng) on filter paper6,7
2 Field	- 3 CPCs formulated in MO - Brevicomin + thiazole (1 mg each) in MO (10 mL) - Testosterone (750 ng) on filter paper	- MO - Brevicomin + thiazole (1 mg each) in MO (10 mL) - Testosterone (750 ng) on filter paper
H3: CPCs, as effectively as thiazole and brevicomin, enhance the attractiveness of testosterone		
3 Field	- Brevicomin + thiazole (1 mg each) in MO (10 mL) - Testosterone (750 ng) on filter paper	- MO - Testosterone (750 ng) on filter paper
4 Field	- 3 CPCs formulated in MO - Testosterone (750 ng) on filter paper	- MO - Testosterone (750 ng) on filter paper
H4: 3-Methyl-2-pentanone does not contribute to pheromonal attraction of female house mice		
5 Lab	- 1-Hexanol (334 ng) + 2,3,5- trithiahexane (1,585 ng) + 3-methyl-2-pentanone (58 ng) on filter paper - Brevicomin (1,317 ng) + thiazole (989 ng) + testosterone (750 ng) on filter paper	- Filter paper - Brevicomin (1,317 ng) + thiazole (989 ng) + testosterone (750 ng) on filter paper
6 Lab	- 1-Hexanol (334 ng) + 2,3,5-trithiahexane (1,585 ng) on filter paper - Brevicomin (1,317 ng) + thiazole (989 ng) + testosterone (750 ng) on filter paper	- Filter paper - Brevicomin (1,317 ng) + thiazole (989 ng) + testosterone (750 ng) on filter paper
7 Lab	- 1-Hexanol (334 ng) + 3-methyl-2-pentanone (58 ng) on filter paper - Brevicomin (1,317 ng) + thiazole (989 ng) + testosterone (750 ng) on filter paper	- Filter paper - Brevicomin (1,317 ng) + thiazole (989 ng) + testosterone (750 ng) on filter paper
8 Lab	- 3-Methyl-2-pentanone (58 ng) + 2,3,5- trithiahexane (1,585 ng) on filter paper - Brevicomin (1,317 ng) + thiazole (989 ng) + testosterone (750 ng) on filter paper	- Filter paper - Brevicomin (1,317 ng) + thiazole (989 ng) + testosterone (750 ng) on filter paper
9 Field	- 2CPC formulation in MO4,5 - Brevicomin + thiazole (1 mg each) in MO (10 mL) - Testosterone (750 ng) on filter paper	- MO - Brevicomin + thiazole (1 mg each) in MO (10 mL) - Testosterone (750 ng) on filter paper

[1]brevicomin = 7-*exo*-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]-3-octene; [2]thiazole = 2-*sec*-butyl-4,5-dihydrothiazole; [3]3CPC: 700 µL of a CPC formulation [3-methyl-2-pentanone (0.3 mg), 1-hexanol (0.3 mg), and 2,3,5-trithiahexane (50 mg) in 100 mL of mineral oil (MO)]; [4]2CPC = 3CPC without 3-methyl-2-pentanone; [5]amounts and ratios of CPCs in MO formulations were adjusted until they generated a headspace odorant blend equivalent to that emanating from previously soiled bedding of one male mouse, on average, over the course of 24 h; [6]amounts of chemicals applied on filter paper were equivalent to those emanating from soiled bedding of one male mouse over the course of 24 h; [7]test chemicals were dissolved in either (50 µL), with the same volume of ether applied to control filter paper

(a)


3a
3b
2
1
6
5
8
4
8
7
5
4

(b)


9a
9b
50 cm
10
11
4
6
5
VICTOR
4
5
7
VICTOR

(c)

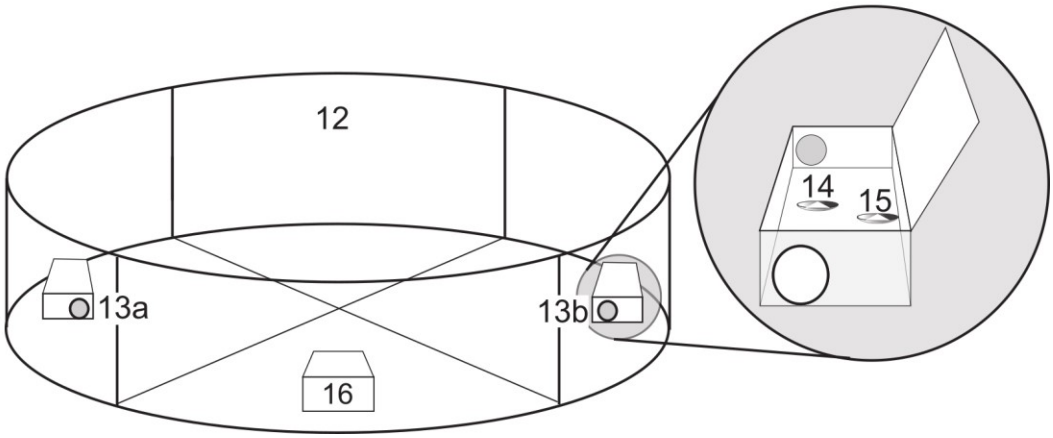

12
13a
13b
14
15
16

Figure 3.1. **Illustrations of laboratory and field experimental designs. Graphic and photographic illustrations of the experimental design used in laboratory and field experiments (adapted from [10,28]). (a) Olfactometer with release chamber (1; 50 cm × 20 cm × 30 cm), Pyrex glass T-tube (2; stem: 65 cm long, side arms: 45 cm long, all 10 cm diam.), and treatment and control chambers (3a, 3b; each 50 cm × 25 cm × 30 cm). Both chambers received a piece of filter paper baited with testosterone (4), and a 20-mL glass scintillation vial containing a synthetic blend of brevicomin and thiazole formulated in mineral oil (5). Whereas the treatment chamber received a 0.5-dram glass vial containing a mineral oil formulation of the candidate pheromone components (CPCs) 1-hexanol, 2,3,5-trithiahexane and 3-methyl-2-pentanone (6), the control chamber received a 0.5-dram glass vial containing mineral oil only (7). A 600-mL beaker (8) prevented mice from knocking over vials. (b) Schematic drawing of paired trap boxes (9a, 9b) deployed in field experiments, each box fitted with a Victor® snap trap (10) set with a food bait (11); treatment and control stimuli (4, 5, 6, 8) in this field experiment were the same as described in subpanel (a). (c) Circular galvanized steel arena (12; 60 × 200 cm) illuminated from above by a 7.5-W red bulb, two metal box-traps (13a, 13b; each 25 × 20 × 15 cm high), filter paper (14) treated with a ternary formulation of 3,4-dehydro-*exo*-brevicomin, 2-*sec*-butyl-4,5-dihydrothiazole, and testosterone; filter paper (15) treated with a ternary or binary blend of the CPCs; and the container (16, 20 × 37 × 14 cm high) for transportation of the bioassay mouse.**

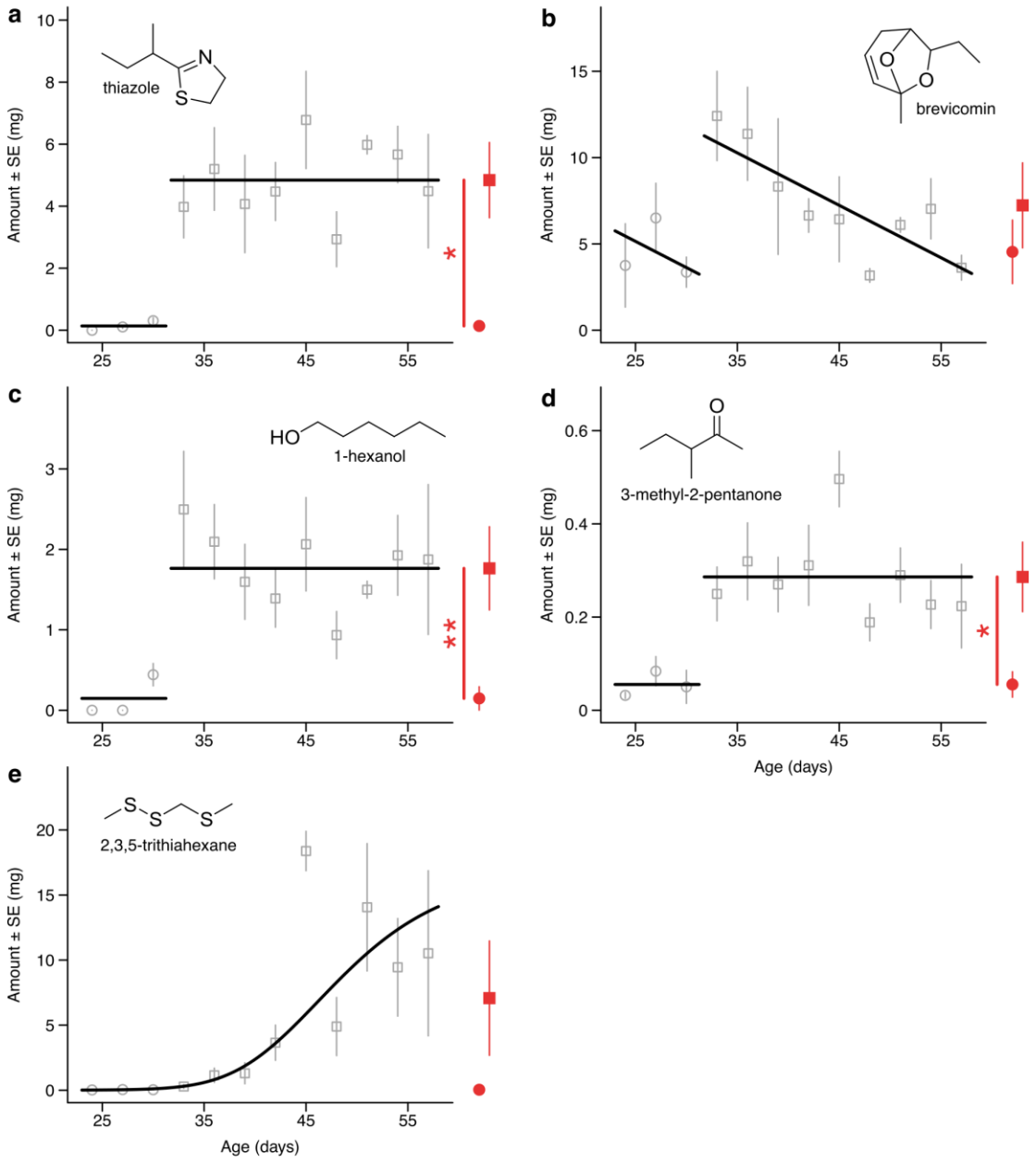

a
thiazole
N
S
Amount ± SE (mg)
b
brevicomin
O
O
c
HO
1-hexanol
d
O
3-methyl-2-pentanone
e
S
S
S
2,3,5-trithiahexane
Age (days)

Figure 3.2. **Quantitative changes in urine/feces odorants of male mice as they become adults. Changes in relative abundance of the previously known sex pheromone components 2-*sec*-butyl-4,5-dihydrothiazole (thiazole) (a) and 3,4-dehydro-*exo*-brevicomin (brevicomin) (b), and of the new candidate pheromone components (CPCs) 1-hexanol (c), 3-methyl-2-pentanone (d) and 2,3,5-trithiahexane (e) in headspace odorant extracts of urine- and feces-soiled bedding from laboratory-kept male house mice, *Mus musculus*, progressing from 24 to 57 days of age. Open symbols (± standard error) represent the mean amount of odorant from three sets of 15 males each (see Methods for details). Linear models for analyses of these means initially included age (in days), age class (juveniles: < 33 days old; adults: ≥ 33 days old), and age and age class interaction, before non-significant terms were removed; the relationship between the mean amounts of 2,3,5-trithiahexane and the age of mice was best presented by a non-linear sigmoid curve. Solid red points (± standard error) represent the mean amounts of each compound produced by groups of males as juveniles or as adults; these means were analyzed by paired *t*-tests, with the asterisk(s) indicating a significant difference (* $P < 0.05$; ** $P < 0.01$). Note the different scale on y-axes.**

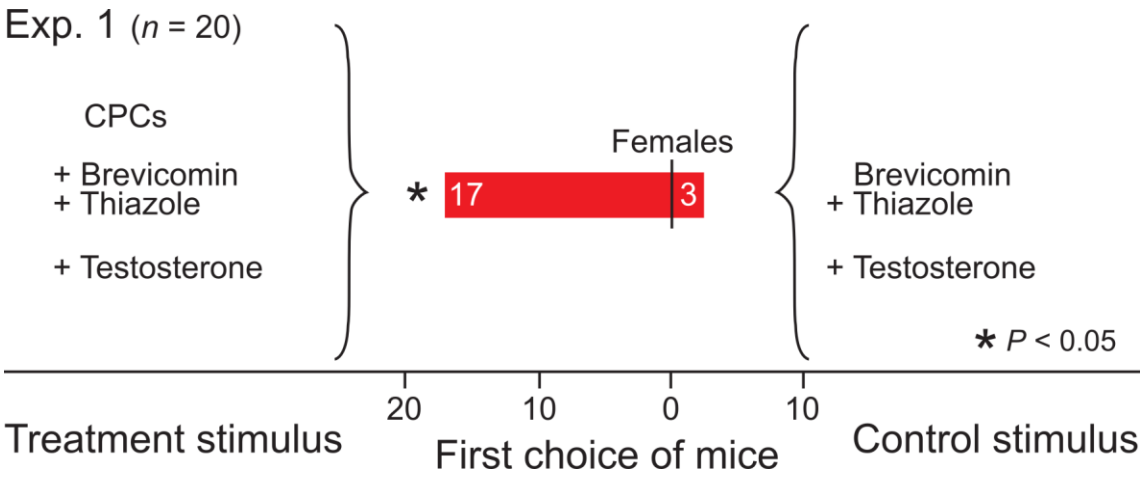


Figure 3.3. **Effect of pheromone blend on behavior of female mice in a laboratory experiment. First-choice entrance of female house mice, *Mus musculus*, into treatment or control chambers (Fig 1, a) baited with either the ternary blend of 3,4-dehydro-*exo*-brevicomin (brevicomin), 2-*sec*-butyl-4,5-dihydrothiazole (thiazole) and testosterone (all sex pheromone components of *M. musculus* males) or baited with the same ternary blend in combination with the candidate pheromone components (CPCs) 1-hexanol, 2,3,5-trithiahexane, and 3-methyl-2-pentanone. For composition, formulation, and dissemination of test stimuli see Table 2. The asterisk (*) indicates a significant preference for the treatment stimulus (χ^2-test; $P < 0.05$).**

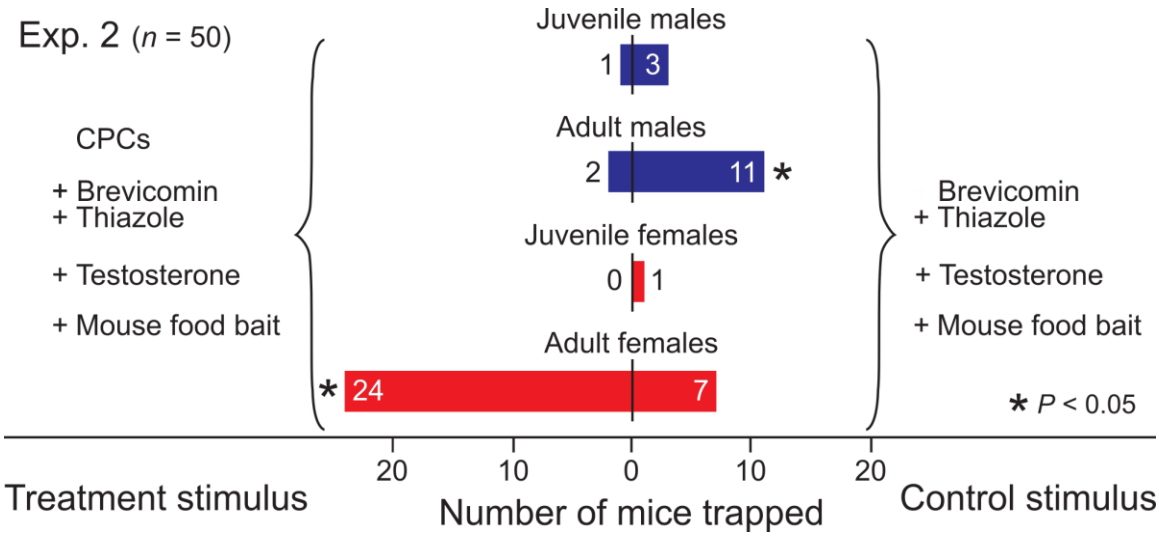
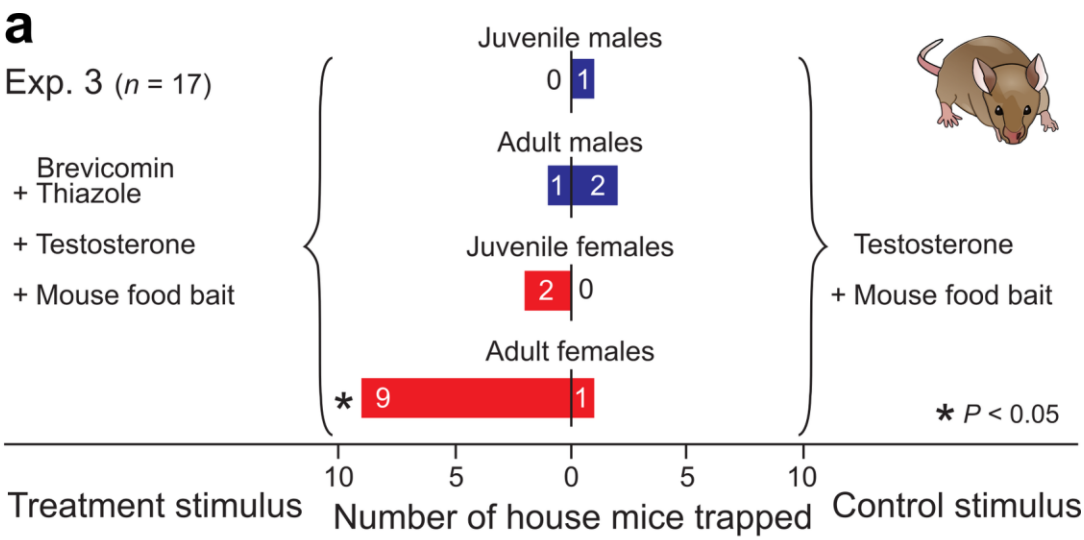


Figure 3.4. **Effect of pheromone blend on captures of house mice in a field experiment. Number of juvenile and adult male and female house mice, *Mus musculus*, field-captured in paired trap boxes (Fig 1, b) baited with either the ternary blend of 3,4-dehydro-*exo*-brevicomin (brevicomin), 2-*sec*-butyl-4,5-dihydrothiazole (thiazole) and testosterone (all sex pheromone components of *M. musculus* males) or baited with the same ternary blend in combination with the candidate pheromone components (CPCs) 1-hexanol, 2,3,5-trithiahexane, and 3-methyl-2-pentanone. For composition, formulation, and dissemination of test stimuli see Table 2. The asterisk (*) indicates a significant preference for the treatment stimulus (χ^2-test; $P < 0.05$).**


a
Exp. 3 (n = 17)
Brevicomin
+ Thiazole
+ Testosterone
+ Mouse food bait
Juvenile males
0 1
Adult males
1 2
Juvenile females
2 0
Adult females
* 9 1
Testosterone
+ Mouse food bait
* P < 0.05
10 5 0 5 10
Treatment stimulus
Number of house mice trapped
Control stimulus


b
Exp. 4 (n = 17)
CPCs
+ Testosterone
+ Mouse food bait
Juvenile males
0 1
Adult males
3 7
Juvenile females
0 0
Adult females
2 4
Testosterone
+ Mouse food bait
10 5 0 5 10
Treatment stimulus
Number of house mice trapped
Control stimulus

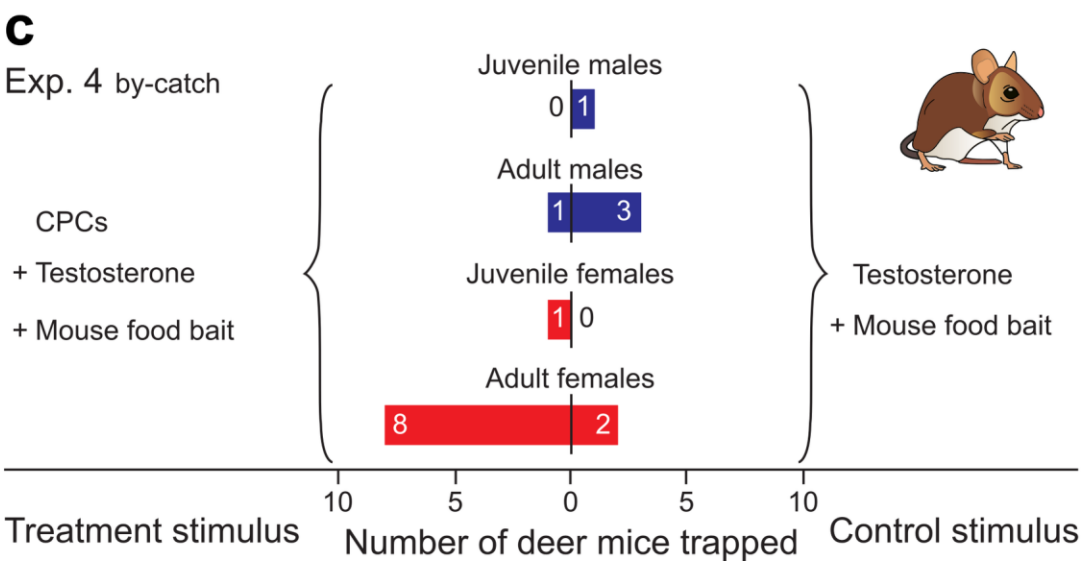

c
Exp. 4 by-catch
CPCs
+ Testosterone
+ Mouse food bait
Juvenile males
0 1
Adult males
1 3
Juvenile females
1 0
Adult females
8 2
Testosterone
+ Mouse food bait
10 5 0 5 10
Treatment stimulus
Number of deer mice trapped
Control stimulus

Figure 3.5. **Effect of pheromone blend on captures of house mice in field experiments. Number of juvenile and adult male and female house mice, *Mus musculus*, field-captured in parallel experiments 3 and 4 in paired trap boxes (Fig 1, b) baited with either testosterone alone or in combination with (*i*) the known male *M. musculus* sex pheromone components 3,4-dehydro-*exo*-brevicomin (brevicomin) and 2-*sec*-butyl-4,5-dihydrothiazole (thiazole) (Exp. 3) or (*ii*) the candidate pheromone components (CPCs) 1-hexanol, 2,3,5-trithiahexane, and 3-methyl-2-pentanone. For composition, formulation, and dissemination of test stimuli see Table 2. The asterisk (*) indicates a significant preference for the treatment stimulus (χ^2-test, $P < 0.05$).**

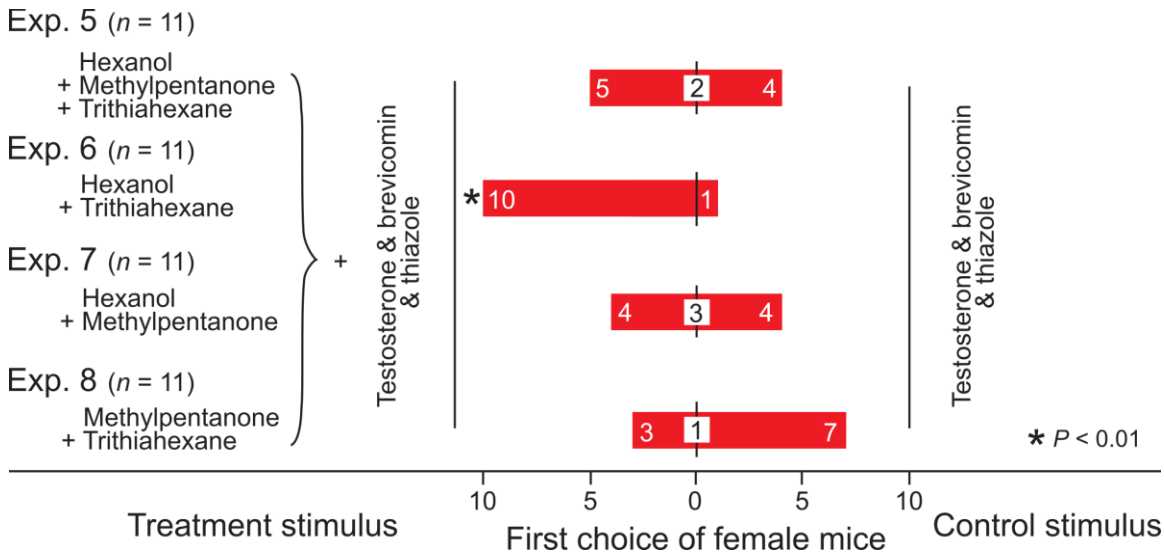


Figure 3.6. **Effect of odor blend on behavior of female mice in laboratory experiments. First choice of treatment or control arena quadrant (Fig 1, c) entered by female house mice, *Mus musculus*, in laboratory parallel experiments 5–8. The box-trap in both the treatment and the control quadrant received a piece of filter paper baited with a ternary blend of 3,4-dehydro-*exo*-brevicomin, 2-*sec*-butyl-4,5-dihydrothiazole and testosterone. The randomly assigned treatment box also received a piece of filter paper baited with a ternary or binary blend of the candidate pheromone components 1-hexanol, 2,3,5-trithiahexane, and 3-methyl-2-pentanone. For composition, formulation, and dissemination of test stimuli see Table 2. Numbers in white inserts within bars denote non-responding mice. The asterisk (*) indicates a significant preference for the test stimulus in experiment 6 (χ^2-test; $P < 0.01$).**

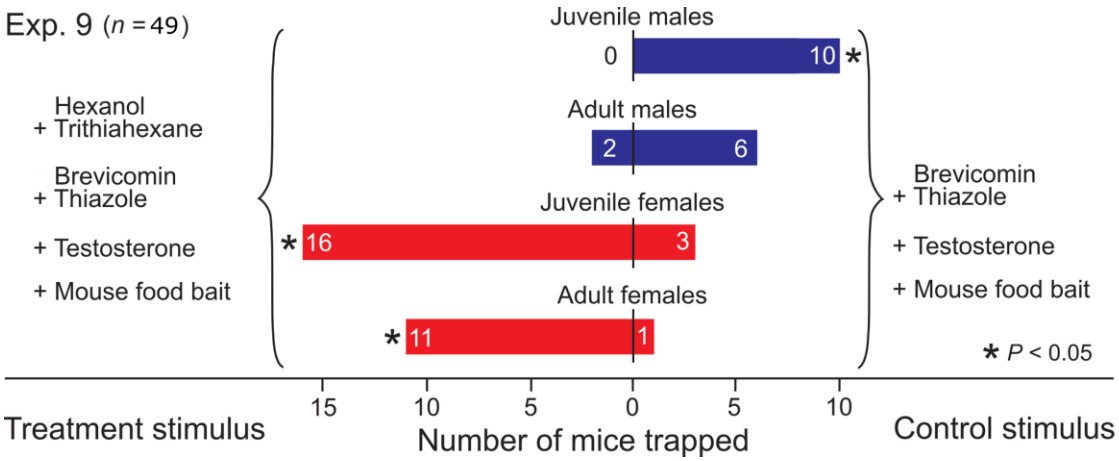


Figure 3.7. **Effect of pheromone blend on captures of house mice in a field experiment. Number of juvenile and adult male and female house mice, *Mus musculus*, field-captured in paired trap boxes baited with either the ternary blend of 3,4-dehydro-*exo*-brevicomin (brevicomin), 2-*sec*-butyl-4,5-dihydrothiazole (thiazole) and testosterone (all sex pheromone components of *M. musculus* males) or baited with the same ternary blend in combination with a binary blend of the new candidate pheromone components (CPCs) 1-hexanol and 2,3,5-trithiahexane. For composition, formulation, and dissemination of test stimuli see Table 2. The asterisk (*) indicates a significant preference for the treatment stimulus (χ^2-test, $P < 0.01$).**

Chapter 4.

Identification and field testing of sex attractant pheromone components of male deer mice, *Peromyscus maniculatus*

A very similar version of this chapter has been submitted as a manuscript to 'Scientific Reports' for review, with the following authors: Elana Varner, Regine Gries, Leah Purdey, Daniella Gofredo, Hanna Jackson, Alishba Bilal, Stephen Takács, Gerhard Gries

4.1. Abstract

Male deer mice, *Peromyscus maniculatus*, reportedly produce a sex pheromone that attracts female mice, but pheromone components have not yet been identified. Working with laboratory-strain and wild deer mice, our objectives were to (1) identify male-produced volatile sex pheromone components that attract female mice, and (2) determine whether the sex steroid testosterone enhances female attraction to volatile pheromone components. We captured headspace volatiles from urine and feces excreta of laboratory-kept male and female mice and analyzed headspace volatile extracts by gas chromatography-mass spectrometry. Among nine ketones present in headspace volatiles, one ketone (5-methyl-2-hexanone) was male-specific, and eight others (3-methyl-2-pentanone, 4-heptanone, 2-heptanone, 6-methyl-2-heptanone, 3-octanone, 2-octanone, 2-nonanone) were 2.6- to 5.6-times more abundant in male samples than female samples. In a field experiment with paired trap boxes, those baited with the ketone lure captured 3.4-times more females and 1.8-times fewer males than corresponding unbaited boxes. In a follow-up paired-trap field experiment, both trap boxes in each pair received the ketone lure while the treatment box also received synthetic testosterone. Testosterone-baited boxes captured 8-times more mature females, and 2.3-times more immature females than control boxes without testosterone. Conversely, 9-times more immature males were captured in boxes without testosterone, revealing a repellent effect of testosterone on males. The plethora of ketones in the odor profile of male Brown rats, *Rattus norvegicus*, and male deer mice, and the sex attractant function of ketones in both species, imply a conserved pathway for pheromone biosynthesis in these two murine rodent taxa.

4.2. Introduction

Macrosomatic rodents use their keen sense of smell during foraging[1,2] as well as intra- and interspecific communication[3] including predator avoidance[4]. Pheromone-based communication in rodents has been intensely studied, mostly with house mice, *Mus musculus*. Here, male-produced pheromone components have both physiological and behavioral effects on female mice. Physiological effects on females include estrus synchronization[5,6] and induction of spontaneous abortion by unfamiliar males[7,8]. Behavioral effects include attraction of females and deterrence of rival males. Sex pheromone components produced by males disseminate from their urine and feces deposits. Various compounds contribute to the pheromone signal or its delivery, such as peptides[9,10], major urinary proteins (MUPs)[11], the sex steroid testosterone[12], and the volatile sex attractant pheromone components 2-*sec*-butyl-4,5-dihydrothiazole (thiazole), 7-exo-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]-3-octene (= 3,4-dehydro-*exo*-brevicomin = brevicomin)[13], 1-hexanol and 2,3,5-trithiahexane[14]. There are also sex pheromone components produced by female house mice that attract males. These components comprise butyric acid, 2-methyl butyric acid, 4-heptanone, and the sex steroids progesterone and estradiol[12,15].

Deer mice, *Peromyscus maniculatus,* are nearly ubiquitous and likely the most common small mammal in North America[16]. They inhabit a wide variety of plant communities including grasslands, brushy areas, woodlands, and forests[17]. Deer mice are strictly nocturnal[18] and even limit their foraging activities during full moons to reduce predation risk[19]. Being nocturnal, deer mice rely on olfaction to navigate their environment. The seasonal reproductive activity of deer mice is linked to photoperiod. Days with decreasing photophase prompt females to delay the onset of sexual maturity and prompt males to lower the weight of their testes[20].

Like house mice, deer mice engage in pheromonal communication, with their pheromones causing both physiological and behavioral effects on signal recipients, similar to those described for house mice. Context-specifically, exposure of female deer mice to male pheromone may induce spontaneous abortion[21] and estrus-induction[22,23]. In the context of sexual communication, urine deposits of adult males attract females and repel rival males[24]. Exposure of juvenile females to male urine accelerates their puberty[25,26] but the urine from castrated males fails to elicit the effect[25], suggesting that

pheromone production is androgen-dependent, as shown in house mice[27]. Whereas testosterone is vital for pheromone production, testosterone itself is a sex pheromone component of both male house mice and male brown rats, *Rattus norvegicus*, and increases the attraction of females to sex attractant pheromone components[12]. As male deer mice excrete testosterone[28], it follows that testosterone may also be a pheromone component of male deer mice.

In a first study to identify pheromone components of deer mice, Ma et al. (1999) analyzed urine odor profiles of males and females. Whereas many compounds were identified, none was sex-specific, and none was tested for a pheromonal function. However, pheromone components are not necessarily sex-specific and may originate from sources other than urine. For example, brevicomin is produced by male house mice (see above) and together with thiazole strongly attracts females[29], but brevicomin is also produced by females – albeit at lower quantity[30]. Similarly, recent studies have shown that pheromone components disseminate from urine, feces and facial glands of the signaling sex[15,31].

Working with laboratory-strain and wild deer mice, in both laboratory and field experiments, our objectives (O) were (O1) to identify male-produced volatile sex pheromone components that attract female mice, and (O2) to determine whether the male sex steroid testosterone enhances female attraction to the sex attractant pheromone components.

4.3. Materials and Methods

4.3.1. Lab Animals

Deer mice, *Peromyscus maniculatus bairdii*, 2- to 3-month-old, were obtained from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA) and housed in Animal Care Services of Simon Fraser University (SFU). Upon arrival, females were housed in three groups of five (each cage: 45 × 23 × 15 cm), and each of 15 males was housed singly (each cage: 20 × 37 × 14 cm). Cages of females and males were lined with 450 g and 150 g, respectively, of corncob bedding (Anderson's Bed o'cobs, The Andersons Inc., Maumee, OH, USA) and fitted with a single Nalgene dome (Jaimeson's Pet Food Distributers, Richmond, BC, Canada).

Urine- and feces-soiled bedding was replaced with fresh bedding every two weeks. Rodent food (LabDiet® Certified Rodent Diet, LabDiet, St. Louis, MO, USA) and water were provided *ad libitum*. Mice were kept on a reversed photoperiod of 12L:12D in rooms maintained at about 50% relative humidity and 21 °C. At the time of behavioral laboratory experiments, female mice were 5- to 6-month-old and male mice were 11- to 12-month-old. The research protocol was approved and supported by the Animal Care Committee of Simon Fraser University (protocol #1295B-19) which abides by the Canadian Council on Animal Care guidelines. Following completion of all laboratory bioassays, mice were CO_2-euthanized. All methods were carried out in accordance with the relevant guidelines and regulations, including the ARRIVE guidelines.

4.3.2. Chemicals

All chemicals (% purity) were purchased from Sigma-Aldrich: 3-methyl-2-pentanone (99), 2-hexanone (98), 5-methyl-2-hexanone (99), 4-heptanone (98), 2-heptanone (99), 6-methyl-2-heptanone (>95), 3-octanone (98), 2-octanone 98), 2-nonanone (99).

4.3.3. (O1) Identify male-produced volatile sex pheromone components that attract female mice

Collection of urine and feces headspace volatiles from female and male deer mice.

For each experimental replicate (n = 5 for female and male mice), soiled bedding (see above) was collected from five group-housed females (450 g bedding) and three singly-housed males (3 × 150 g = 450 g of total bedding). The bedding was placed into separate Pyrex glass chambers (30 × 15 cm) connected to a Pyrex glass tube (15 cm × 5 mm OD) filled with the adsorbent Porapak Q (200 mg) which served as a volatile trap. Charcoal-filtered air was drawn through each chamber and the Porapak Q volatile trap at a flow of 1 $L \cdot min^{-1}$. After capturing urine and feces odorants on Porapak Q for 24 h, volatiles were desorbed with consecutive rinses of pentane (2 mL) and ether (2 mL), dodecyl acetate was added as an internal standard, and extracts were concentrated to 250 µL per sample.

Identification and quantification of urine and feces headspace volatiles from male and female deer mice.

Aliquots (2 µL) of Porapak Q extracts were analyzed on a Varian Saturn Ion Trap GC-MS fitted with a DB-5 MS GC column (30 m × 0.25 mm ID; Agilent Technologies Inc., Santa Clara, CA 95051, USA) using helium as the carrier gas (35 cm · s^{-1}) and running the following temperature program: 40 °C (5 min), 10 °C · min^{-1} until 280 °C (5 min). The injector port was set at 250 °C and the ion trap at 200 °C. Volatiles were identified by comparing their retention indices (relative to straight chain alkanes[32]) and mass spectra with those of authentic standards. Volatiles that were either male-specific (5-methyl-2-hexanone), or that were more abundant in headspace volatiles of males than females (2-hexanone, 3-octanone, 2-heptanone, 2-nonanone, 2-octanone, 6-methyl-2-heptanone, 3-methyl-2-pentanone, 4-heptanone) (see Results) were considered candidate pheromone components (CPCs). In total ion chromatograms, the amount of each ketone was quantified by comparing its area count with that of an internal standard (dodecyl acetate). These amounts were then divided by the number of mice in the sample (3 males or 5 females) to obtain the ketone amounts produced by a single deer mouse.

Effect of CPCs on behavioral response of female and male deer mice in laboratory experiments.

The effect of CPCs on attraction of female mice, and potential deterrence of male mice, was tested in laboratory experiments 1 and 2, using a large olfactometer (Fig. 1). The Pyrex glass olfactometer consisted of a central T-tube (all arms 30 cm long × 10 cm in diameter) interconnected to two lateral extension tubes and one ‘mouse release tube’ (each 30 cm long × 10 cm in diameter) via circular, quick-release metal clamps with inset rubber gaskets to achieve vacuum seal. The distal end of each lateral extension tube tapered to a 1-cm diameter, and via ground glass joint and metal clamp was attached to a stimulus chamber (12.5 cm long × 2.5 cm in diameter) which, in turn, was connected via glass joint and metal clamp to a charcoal filter. The tapered orifice of the ‘mouse release’ tube was attached via glass joint and metal clamp to a vacuum pump (Neptune Dyna-pump, Model 2 Dover, NJ, USA) that drew air through the olfactometer at a rate of 0.25 L/min. Each stimulus chamber was fitted with a piece of filter paper (approx. 6.5 cm^2; Whatman #1, 120 mm, Maidstone, England) that – by random assignment – received the treatment or control stimulus. The treatment stimulus consisted of an 8-µg

blend of CPCs [3-methyl-2-pentanone (0.06 µg), 2-hexanone (0.02 µg), 5-methyl-2-hexanone (0.04 µg), 4-heptanone (0.06 µg), 2-heptanone (1.6 µg), 6-methyl-2-heptanone (0.7 µg), 3-octanone (4.3 µg), 2-octanone (0.5 µg), 2-nonanone (1.4 µg)] formulated in mineral oil (20 µL) and applied on filter paper, whereas the control stimulus consisted of mineral oil alone (20 µL). The amounts and ratios of ketones in the CPC treatment blend were based on those released from urine and feces of the most prolific ketone-producing males during 24 hours of headspace volatile capture (Table 1). During the 10-min bioassay, the treatment formulation applied on filter paper released 5.2 ng of the ketone blend, 10-times less than released during the same time interval from urine- and feces-soiled bedding of one male at day 14 (the day of bedding replacement).

For each replicate, a mouse was removed from its home cage and transported to the olfactometer in an amber Plexiglass cylinder (100 mm long × 50 mm diameter; Bio-Serve's Mouse Tunnels™, Bio-Serve Inc., Flemington, NJ 08822, USA) with two press fit caps (53 mm diameter × 6 mm depth). This type of transportation was deemed helpful to minimize stress on the mouse[33,34]. The transportation cylinder was placed into the disconnected release tube and stabilized with tape. Once the release tube was re-connected to the T-tube, a twine affixed to the distal cap of the cylinder was threaded through the tapered orifice of the release tube. After pulling the twine, and thus allowing the mouse to enter the olfactometer on its own accord, the twine was cut and the release tube attached to the vacuum pump. Recordings started when a mouse had left the transportation cylinder, and a response was recorded when the mouse, with all four paws, entered a lateral extension tube.

All replicates were run under dim red light to facilitate observations[30] and were terminated after 10 min. Following each replicate, a male mouse was returned to his home cage and a female mouse was placed in a new group-holding cage to ensure that she was tested only once. The olfactometer was cleaned with Saber ® (Wood Wyant, Victoriaville, QC G6P 7E3, CA) followed by 70% ethanol.

Effect of a CPC trap lure on captures of deer mice in field settings.

Experiment 3 was run in three deer mouse-infested premises in the Greater Vancouver and Abbotsford areas of British Columbia, Canada, between September 2019 and April 2021. Each replicate (n = 36) consisted of paired trap boxes (PROTECTA® Mouse, Bell Laboratories Inc. Madison, WI 53704, USA), with 0.5-m

spacing between the boxes in each pair (Fig. 2a), and at least 2 m between pairs. Replicates were placed along the interior and exterior walls of buildings. Each trap box was fitted with a Victor® snap trap (M325 M7 Pro mouse Woodstream Co., Lititz, PA 175543, USA) baited with a food bait[35] which prompted feeding and thereby capture of responding mice. Traps within each replicate received a 20-mL glass scintillation vial containing either a 4-mg CPC blend [3-methyl-2-pentanone (0.03 mg), 2-hexanone (0.01 mg), 5-methyl-2-hexanone (0.02 mg), 4-heptanone (0.03 mg), 2-heptanone (0.8 mg), 6-methyl-2-heptanone (0.03 mg), 3-octanone (2.1 mg), 2-octanone (0.3 mg), 2-nonanone (0.7 mg)] formulated in mineral oil (10 mL) or an unscented mineral oil control (10 mL) (Fig. 2b). The treatment CPC blend on day 1 of its formulation released 3-times more of the ketones than the urine- and feces-soiled bedding of the most prolific ketone-producing deer mouse males. Considering further that the release of the synthetic ketones on day 5 had declined by 30% and that lures were replaced in 7-day intervals (see below), the release rate of the field-tested lure was well within biological relevance.

Traps were checked twice each week and the food bait and pheromone lure were replaced once a week. Whenever a mouse had been captured, its sex and maturity were recorded, a new trap box and snap trap were deployed, and the position of the treatment and the control box within a trap box pair was re-randomized. Sex and sexual maturity of the captured mouse were determined based on ano-genital distance[36] and genitalia development, such as visibly discernable testes of sexually mature males[37] and vaginal opening of sexually mature females[38].

4.3.4. (O2) Determine whether the male sex steroid testosterone enhances female attraction to the sex attractant pheromone components

The ability of testosterone to enhance captures of female mice in CPC-baited traps was tested between November 2021 and January 2022 in experiment 4, which was run on deer mouse-infested commercial and private premises in the Greater Vancouver area of British Columbia, Canada. Each replicate (n = 40) consisted of paired trap boxes set up and serviced as described for experiment 3. Both trap boxes in each pair were baited with the CPC lure (see Exp. 3) and the treatment box also received a piece of filter paper (Whatman #1, 120 mm, Maidstone, England, 01622) to which testosterone (750 ng) dissolved in acetonitrile (50 µL) was applied (Fig. 2c). This dose

represents 1.5-times the amount of testosterone present in 1 g of male deer mouse feces[28]. The filter paper in the control trap was treated with 50 µL of acetonitrile.

4.3.5. Statistical Analysis

First-choice response data of mice in laboratory experiments 1 and 2, and capture data of mice in field experiments 3 and 4 were analyzed by a χ^2 test. The quantities of each ketone in five samples of males and five samples of females were compared by χ^2 tests.

4.4. Results

4.4.1. (O1) Identification of male deer mouse volatile sex attractant pheromone components

Identification of urine and feces headspace volatiles from male and female deer mice.

Comparative GC-MS analyses of the headspace volatiles emanating from urine- and feces-soiled bedding of adult male and female deer mice revealed various groups of organic compounds including ketones, acids, and alcohols. Among nine ketones, one ketone (5-methyl-2-hexanone) was male-specific, and eight others (2-hexanone, 3-methyl-2-pentanone, 4-heptanone, 2-heptanone, 6-methyl-2-heptanone, 3-octanone, 2-octanone, 2-nonanone] were 2.6- to 5.6-times more abundant in samples of males than in samples of females (Fig. 3, Table 1). All of these nine ketones were deemed candidate pheromone components (CPCs).

Effect of CPCs on behavioral responses of female and male deer mice in laboratory experiments.

When adult females (n = 12) in a laboratory olfactometer (Fig. 1) were offered a choice between a CPC lure and a solvent control stimulus, 10 females entered first the lateral olfactometer arm leading to the CPC lure and two females entered first the lateral arm leading to the solvent control (Exp. 1: $\chi^2 = 0.021$, $P < 0.05$, Fig. 4). When adult males (n = 10) were offered the same choice as females, eight entered first the CPC treatment arm and two the control arm (Exp. 2: $\chi^2 = 3.6$, $P > 0.05$; Fig. 4).

Effect of a CPC trap lure on captures of deer mice in field settings.

CPC trap lures increased field captures of female deer mice. CPC-baited traps captured 17 mature female deer mice, whereas unbaited paired control traps captured only five females (Exp. 3: $\chi^2 = 5.26$, $P < 0.05$, Fig. 5). Conversely, CPC-baited traps and unbaited paired control traps captured five and nine mature male deer mice, respectively (Exp. 3: $\chi^2 = 1.14$, $P > 0.05$; Fig. 5). There were no captures of immature male or female deer mice in this experiment.

4.4.2. (O2) Testosterone enhances attraction of females to sex attractant pheromone components

Testosterone increased captures of female deer mice in CPC-baited traps. Traps baited with both the CPC-lure and testosterone captured eight mature and 14 immature female deer mice, whereas traps baited only with the CPC lure captured one mature and six immature female deer mice (Exp. 4: mature females: $\chi^2 = 5.44$, $P < 0.05$; immature females: $\chi^2 = 3.2$, $P > 0.05$; Fig. 6). Conversely, traps baited with both the CPC-lure and testosterone captured one mature and one immature male deer mouse, whereas traps baited only with the CPC lure captured no mature and nine immature male deer mice (Exp. 4: immature males: $\chi^2 = 6.4$, $P > 0.05$; Fig. 6). Capture of only one mature male mouse in this experiment did not warrant statistical analysis.

4.5. Discussion

Our data support the hypotheses that male deer mice produce sex attractant pheromone components that attract conspecific females and that the sex steroid testosterone enhances female attraction to these sex attractant pheromone components. Below, we elaborate on our data.

Our study was inspired by three main considerations. First, there are intriguing similarities in the pheromone system of deer mice and house mice. Both rodent species produce pheromones that induce analogous physiological and behavioral effects on signal recipients. Second, many of the house mouse pheromone components that matter in the context of sexual communication have already been identified. The procedures to collect, identify and synthesize these components are well documented and provide an ‘analytical’ road map for the identification of deer mouse pheromone

components. Third, despite previous attempts, not a single deer mouse pheromone component has been identified to date.

In light of all the progress made with the identification of the male house mouse sex pheromone and its dissemination[5–14], we focussed our study on the identification of sex attractant pheromone components produced by male deer mice. As sex attractant pheromone components may originate from either urine or feces deposits of the signalling sex, as shown with house mouse females[15], we took an inclusive approach and collected headspace volatiles from bedding soiled with both urine and feces of male deer mice. Moreover, while previous studies have frozen urine for subsequent analysis[6], we captured headspace volatiles exclusively from urine and feces excreta that were never subjected to freezing because temporary freezing of urine may alter signal characteristics, as shown with house mice[39]. Freezing urine from female mice reduced its efficacy for eliciting ultrasonic vocalizations from male mice[39]. Finally, as sex pheromone components are typically deemed sex-specific, or at least more abundant in the signalling sex, we compared urine/feces headspace volatiles from males and females.

As expected, based on a previous study[40], the urine/feces headspace volatiles of male and female deer mice were complex, comprising many groups of organic compounds including ketones, acids, and alcohols. Among these groups, ketones stood out because of their relative abundance and chemical diversity. One ketone (5-methyl-2-hexanone) was male-specific (Fig. 3) and eight others in combination (2-hexanone, 3-methyl-2-pentanone, 4-heptanone, 2-heptanone, 6-methyl-2-heptanone, 3-octanone, 2-octanone, 2-nonanone) were, on average, significantly more abundant in samples of males than of females (Table 1). These data, coupled with findings that male Brown rats produce a blend of seven ketones that attract females[41], made us hypothesize that some, or all, of the male deer mouse ketones may serve as sex attractant pheromone components. To test our hypothesis, we prepared a synthetic blend of all nine ketones and tested its effect on attraction of deer mice in a T-tube olfactometer (Fig. 1). As predicted, female mice preferred the ketone blend to a control stimulus (Fig. 4) and – surprisingly – male mice exhibited a similar preference, but the sample size was too low to show a statistically significant effect. Despite these positive T-tube bioassay data obtained with laboratory-strain deer mice, it was imperative to obtain definitive proof in a field experiment that the synthetic ketone blend is indeed attractive to *wild* female deer

mice. In this field experiment, traps baited with the ketone blend captured 3.4-times more females than unbaited control traps (Fig. 5), indicating that some or all ketones in this blend served as sex attractant pheromone components. Conversely, in contrast to T-tube bioassay data, male deer mice were not attracted to the ketone lure, with numerically more males being captured in unbaited control traps than in ketone-baited traps (Fig. 5).

Our data demonstrate that the sex steroid testosterone is a major sex pheromone component of male deer mice. When added to the ketone blend as a trap lure component, testosterone increased the blend's attractiveness to female deer mice, while concurrently deterring immature males. Traps baited with lures comprising both the ketone blend and testosterone captured 8-times more mature females and 2.3 times more immature females than traps baited with the ketone blend alone (Fig. 6). Conversely, nine out of 10 immature males captured avoided traps with testosterone as a trap lure component (Fig. 6). With captures of 40 deer mice in this experiment, the capture of only a single mature male is not likely a reflection of low population size. Rather, it may indicate that mature males sensed the ketone blend and testosterone in the surroundings of trap pairs and avoided the area which – naturally – may be an adaptive behavior to avoid encounters with a territorial male. Following house mice and Brown rats[12], deer mice are now the third rodent species shown to use a combination of volatile sex attractant pheromone components and less volatile sex steroid pheromone components.

Our findings that the ketone blend attracted males of laboratory-strain but not wild deer mice (Figs. 4, 5) are reminiscent of analogous reports that males of laboratory-strain but not wild Brown rats were attracted to a synthetic lure of male rat sex pheromone components[41]. All data combined support previous conclusions that domesticated rodents in laboratory settings behave differently than their wild counterparts[42–44] and that field data are essential to unravel the pheromone communication system of wild rodents.

Our laboratory and field data in combination support the conclusion that the ketone blend contains essential sex attractant pheromone components produced by male deer mice. We selected these components based on their specificity and relatively greater abundance in urine/feces odors of males than females (Fig. 3). Conceivably,

however, there is plasticity and redundancy in the ketone pheromone blend in that some components may be attractive on their own while others may be omittable without affecting the blend's attractiveness. For example, 3-methyl-2-pentanone as a single representative of the ketone blend attracted female deer mice in a trapping experiment with house mice[14]. Moreover, some components of the ketone blend may have the exclusive function of suppressing attraction of heterospecific rodents, while other ketones may have the dual function of attracting mates while deterring heterospecifics. These types of signal functions have been demonstrated in odor/pheromone blends of insects including several species of moths[45–47].

With ever expanding knowledge about murine rodent sex pheromones, opportunities arise to study pheromone blends with respect to (shared) biosynthetic pathways, species-specificity of sex pheromones, and phylogenetic relatedness of species[3,48,49]. The presence of 2- and 4-heptanone in urine/feces odors of male Brown rats and male deer mice[14,41, this study], and of 3-methyl-2-pentanone in urine/feces odors of male house mice and male deer mice[14, this study], implies a shared biosynthetic pathway for these ketones. Pheromones of similar structure and shared biosynthetic pathways for pheromones and odorants are well-documented in closely related insect taxa[49,50] but – to our knowledge – had not yet been reported for mammals. The plethora of ketones in the odor profile of male Brown rats and male deer mice, and the sex attractant function of ketones in both species, further imply that Brown rats and deer mice – phylogenetically – are more closely related than deer mice and house mice. With testosterone being a sex pheromone component of male Brown rats, house mice and deer mice[12, this study], there is distinct overlap in their pheromone blend. In male Brown rats and house mice, pheromone specificity is achieved through the volatile sex attractant pheromone components[3] that markedly differ between these two species. The same type of mechanism may separate communication channels of deer mice and house mice, and of deer mice and brown rats.

In conclusion, we report data showing that the sex pheromone of male deer mice comprises some or all of nine volatile ketones and the less volatile sex steroid testosterone. Based on the composition of this blend, deer mice – phylogenetically – seem more closely related to Brown rats than to house mice. As deer mice can be significant urban and agricultural pests[51], commercial development of this blend, or a less complex version thereof, as a trap lure seems warranted.

4.6. Acknowledgements

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4.7. References

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4.8. Figures

Table 4.1. Quantitative comparison of nine ketones in headspace volatiles of urine and feces excreta from single male and female deer mice.

	Mean (± SE) and [top] amounts (ng)[1] of ketones in headspace volatile extract			
Compounds	Males	Females	Male-female differential	P-value[2]
3-Methyl-2-pentanone	57.58 (7.155) [63]	10.33 (5.42)	5.6	0.00001
2-Hexanone	21.38 (10.73) [20]	7.36 (4.53)	2.9	0.009
5-Methyl-2-hexanone	9.50 (9.167) [46]	0.00 (0)	N/A	0.0021
4-Heptanone	21.68 (11.74) [63]	7.47 (5.03)	2.9	0.0084
2-Heptanone	550.36 (251.82) [1,536]	111.13 (55.05)	5.0	0.00001
6-Methyl-2-heptanone	17.38 (12.62) [67]	4.44 (3.70)	3.9	0.0056
3-Octanone	1,235.18 (775.71) [4,294]	234.92 (163.35)	5.35	0.00001
2-Octanone	153.52 (102.71) [553]	59.47 (53.86)	2.6	0.00001
2-Nonanone	404.87 (247.66) [1,371]	80.46 (59.69)	5.0	0.00001
Total ketones	1,976.45 [8,013]	515.58	3.8	

[1]Ketones were present in headspace volatiles of bedding (450 g) soiled with feces and urine from three laboratory-kept male deer mice or five female deer mice during two weeks. Headspace volatiles of such soiled bedding were collected over 24 h and quantified in total ion chromatogram analyses (Fig. 3). The amount of each ketone was derived by comparing its area count with that of an internal standard (dodecyl acetate). These amounts were then divided by the number of mice in the sample (5 males or 3 females) to obtain the ketone amounts produced by a single mouse. There were 5 samples each of males and females. [2]The quantities of each ketone in 5 samples of males and 5 samples of females were compared by χ^2 tests.

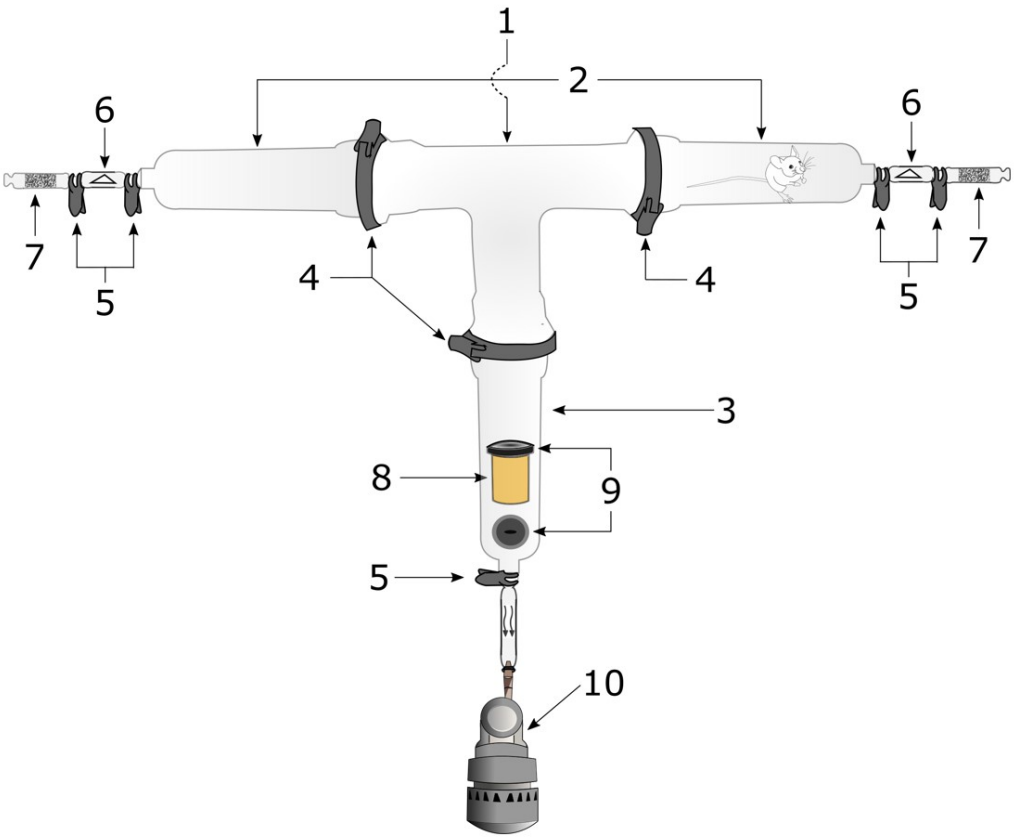

Figure 4.1. **Graphical illustration of the olfactometer designed and deployed for laboratory experiments 1 and 2. The central T-tube (1; arms: 30 cm long × 10 cm diameter) was connected to two lateral tubes (2) and a release tube (3) (all 30 cm long × 10 cm diameter) via circular metal clamps (4). The two lateral tubes, in turn, were each attached via ground glass joint and metal clamp (5) to a stimulus chamber (6; 12.5 cm long × 2.5 cm in diameter) which, in turn, was connected to a charcoal filter (7) to purify incoming air. Each stimulus chamber (6) received a piece of filter paper (approx. 6.5 cm^2) treated with a test stimulus or a control stimulus. For each replicate, an amber Plexi-glass cylinder (8; 100 mm long × 50 mm diameter) with press-fit caps (9; each 53 mm diameter × 6 mm depth) housing a mouse was placed inside the (disconnected) release tube and stabilized with tape. A piece of twine attached to the distal cap of the cylinder was threaded through the tapered orifice of the release tube, and once the release tube was re-connected to the T-tube, the twine was pulled to remove the cap, thus releasing the mouse. The twine was then trimmed back to facilitate connection of the release tube to a vacuum pump (10) that drew air through the olfactometer at 0.25 L/min.**

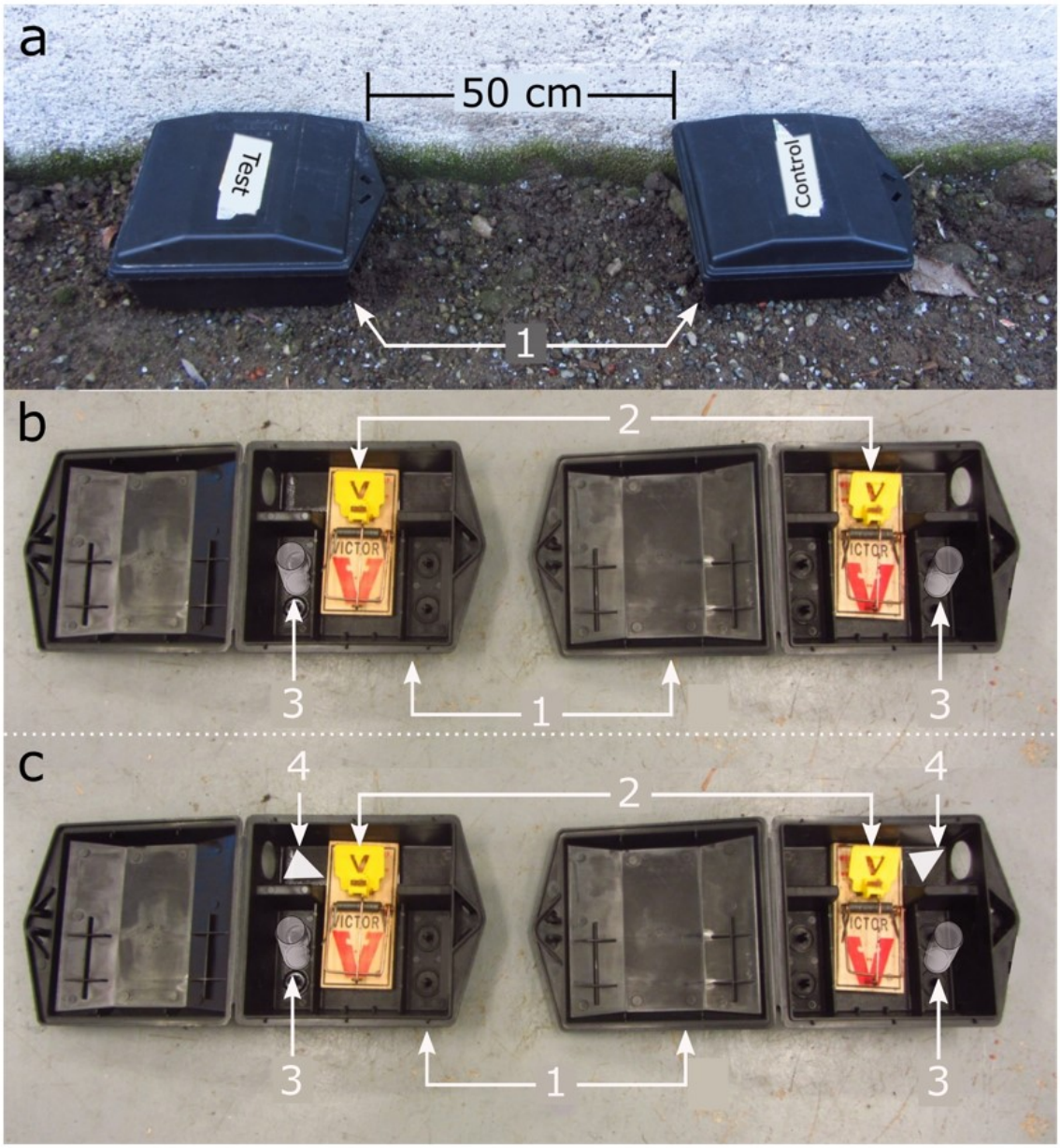

Figure 4.2. **Photographs illustrating (a) the experimental paired-trap design deployed in field experiments, and (b, c) details of the food bait and pheromone lure tested in randomly assigned treatment and control traps, as follows: 1 = trap box, 2 = snap trap with food bait[34], 3 = glass scintillation vial (20 mL) containing a blend of candidate sex attractant pheromone components formulated in mineral oil (10 mL) or mineral oil alone (10 mL; control); and 4 = piece of filter paper treated either with testosterone (750 ng) dissolved in acetonitrile (50 µL) or with acetonitrile alone (50 µL; control).**

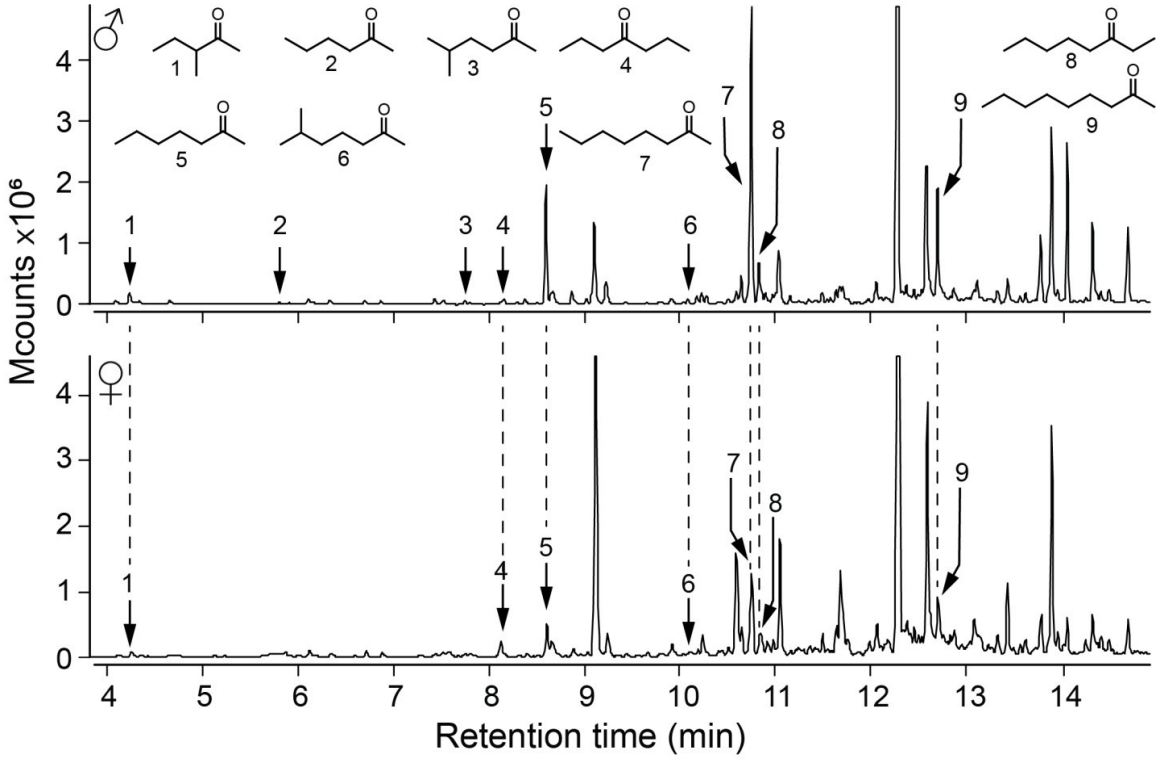

Figure 4.3. **Total ion chromatograms [Varian Saturn Ion Trap GC-MS; DB-5 MS GC column; temperature program: 40 °C (5 min), 10 °C · min^{-1} to 280 °C (5 min)] of headspace volatile extracts obtained from bedding soiled with urine and feces from three male deer mice (top trace) and five female deer mice (bottom trace) (see Methods for details). One ketone [5-methyl-2-hexanone (3)] was male-specific, and eight ketones [3-methyl-2-pentanone (1), 2-hexanone (2), 4-heptanone (4), 2-heptanone (5), 6-methyl-2-heptanone (6), 3-octanone (7), 2-octanone (8), 2-nonanone (9)] were 2.6- to 5.6-times more abundant, on average, in samples of three males each (n = 5) than in samples of five females each (n = 5) (see Table 1). Note: 2-hexanone (2) was not present in this particular female sample but was detectable in other samples.**

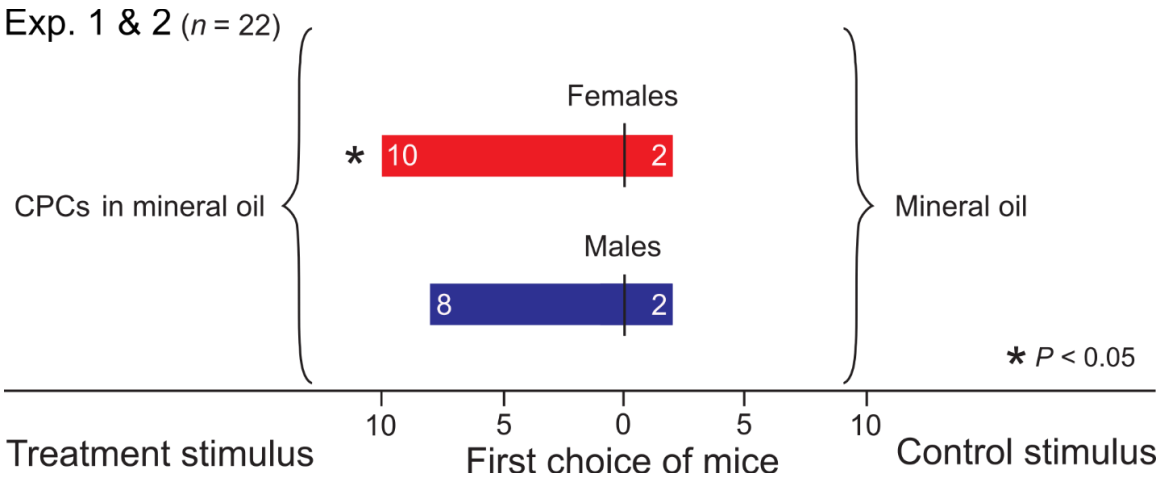

Figure 4.4. **First-choice entrance of female and male deer mice, *Peromyscus maniculatus*, into lateral arms of a T-tube olfactometer (Fig. 1), each arm leading to a stimulus chamber fitted with a piece of filter paper that was treated either with an 8-µg blend of candidate pheromone components (CPCs) [3-methyl-2-pentanone (0.06 µg), 2-hexanone (0.02 µg), 5-methyl-2-hexanone (0.04 µg), 4-heptanone (0.06 µg), 2-heptanone (1.6 µg), 6-methyl-2-heptanone (0.07 µg), 3-octanone (4.3 µg), 2-octanone (0.5 µg), 2-nonanone (1.4 µg)] formulated in mineral oil (20 µL) or with a mineral oil (20 µL) control stimulus. The asterisk (*) indicates a significant preference for the test stimulus (χ^2-test; *P* < 0.05). Note: The CPC formulation applied on filter paper released 5.2 ng of the ketone blend during the 10-min bioassay.**

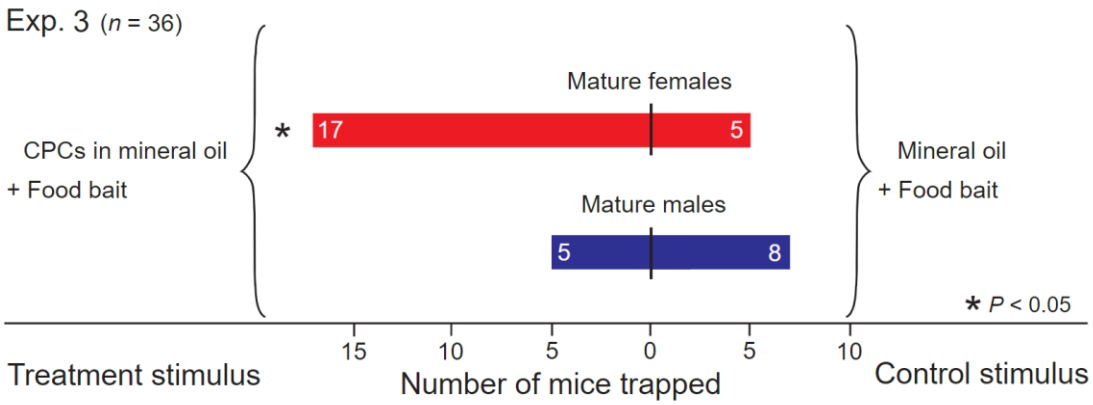

Figure 4.5. **Captures of mature female and male deer mice, *Peromyscus maniculatus,* in paired traps (n = 36) (Fig. 2a) in experiment 3. Both boxes in each pair received a glass scintillation vial (20 mL; Fig. 2b) containing either a 4-mg blend of candidate pheromone components (CPCs) [3-methyl-2-pentanone (0.03 mg), 2-hexanone (0.01 mg), 5-methyl-2-hexanone (0.02 mg, 4-heptanone (0.03 mg), 2-heptanone (0.8 mg), 6-methyl-2-heptanone (0.03 mg), 3-octanone (2.1 mg), 2-octanone (0.3 mg), 2-nonanone (0.7 mg)] formulated in mineral oil (10 mL) or a mineral oil (10 mL) control stimulus. The asterisk denotes significantly more captures of females in traps baited with the CPC lure (χ^2-test using Yate's correction for continuity, $P < 0.05$). Note: The synthetic lure on day 1 of its formulation released 6-times more of the ketones than the urine- and feces-soiled bedding of one male deer mouse. Considering that the ketone release declined on subsequent days and that lures were replaced in 7-day intervals (see below), the release rate of the field-tested lure was well within biological relevance.**

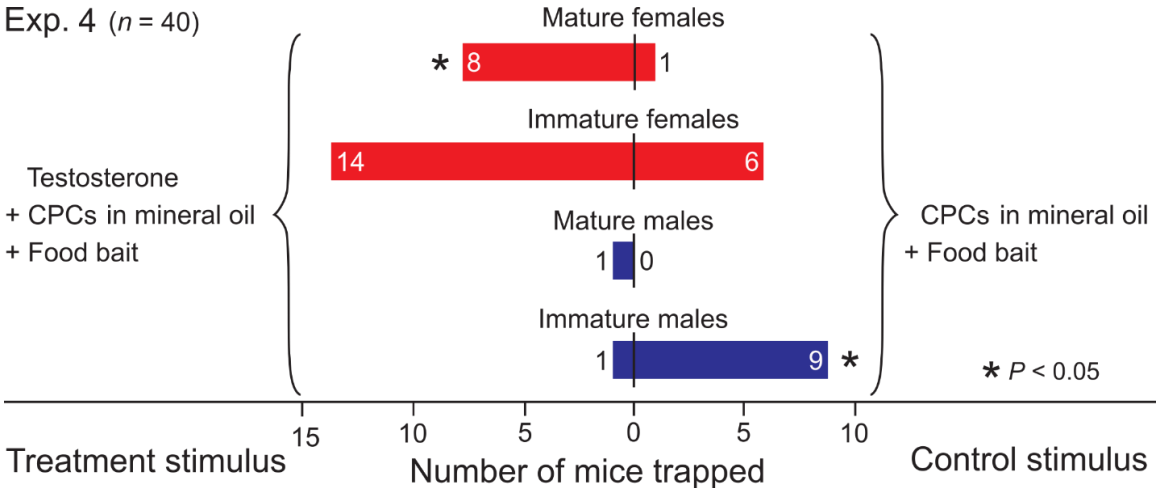

Figure 4.6. **Captures of mature and immature female and male deer mice, *Peromyscus maniculatus,* in paired traps (n = 40) (Fig. 2a) in experiment 4. Both boxes in each pair received a glass scintillation vial (20-mL) containing the 4-mg blend of candidate pheromone components (CPCs) [3-methyl-2-pentanone (0.03 mg), 2-hexanone (0.01 mg), 5-methyl-2-hexanone (0.02 mg, 4-heptanone (0.03 mg), 2-heptanone (0.8 mg), 6-methyl-2-heptanone (0.03 mg), 3-octanone (2.1 mg), 2-octanone (0.3 mg) 2-nonanone (0.7 mg)] formulated in mineral oil (10 mL). The amounts and ratios of the CPCs in this mineral oil formulation were carefully adjusted until they generated a headspace odorant blend equivalent to that emanating from previously soiled bedding of one male mouse over the course of 24 h. The treatment box in each pair also received a piece of filter paper (Fig. 2c) treated with testosterone (750 ng) dissolved in acetonitrile (50 µL), whereas the filter paper in the corresponding control trap received acetonitrile only (50 µL). The asterisks denote significantly more captures of mature females, and fewer captures of immature males, in testosterone-baited traps (χ^2-test using Yate's correction for continuity, $P < 0.05$). Note: (1) The synthetic lure on day 1 of its formulation released 6-times more of the ketones than the urine- and feces-soiled bedding of one male deer mouse. Considering that the ketone release declined on subsequent days and that lures were replaced in 7-day intervals (see below), the release rate of the field-tested lure was well within biological relevance. (2) The amount of testosterone represents 1.5-times the amount of testosterone present in 1 g of male deer mouse feces[28].**

Chapter 5.

Brown rats and house mice eavesdrop on each other's volatile sex pheromone components

A very similar version of this chapter has been published: Elana Varner, Hanna Jackson, Manveer Mahal, Stephen Takács, Regine Gries, Gerhard Gries (2020) Scientific Reports 10:1-9.

5.1. Abstract

Mammalian pheromones often linger in the environment and thus are particularly susceptible to interceptive eavesdropping, commonly understood as a one-way dyadic interaction, where prey sense and respond to the scent of a predator. Here, we tested the "counterespionage" hypothesis that predator and prey co-opt each other's pheromone as a cue to locate prey or evade predation. We worked with wild brown rats (predator of mice) and wild house mice (prey of brown rats) as model species, testing their responses to pheromone-baited traps at infested field sites. The treatment trap in each of two trap pairs per replicate received sex attractant pheromone components (including testosterone) of male mice or male rats, whereas corresponding control traps received only testosterone, a pheromone component shared between mouse and rat males. Trap pairs disseminating male rat pheromone components captured 3.05 times fewer mice than trap pairs disseminating male mouse pheromone components, and no female mice were captured in rat pheromone-baited traps, indicating predator aversion. Indiscriminate captures of rats in trap pairs disseminating male rat or male mouse pheromone components, and fewer captures of rats in male mouse pheromone traps than in (testosterone-only) control traps indicate that rats do eavesdrop on the male mouse sex pheromone but do not exploit the information for mouse prey location. The counterespionage hypothesis is supported by trap catch data of both mice and rats but only the mice data are in keeping with our predictions for motive of the counterespionage.

5.2. Introduction

Functional roles of mammalian pheromones have routinely been investigated in an intraspecific context, such as territorial marking, sexual signaling and health status conveyance[1]. Yet, closely related species in mammalian communities often use similar communication signals[2] which facilitates bi-directional (interspecific) olfactory communication[3] and lowers the relative cost of maintaining sensory receptors[4]. This concept appears to apply to olfactory communication signals of sympatric murine rodents, including the brown rat, *Rattus norvegicus,* and the house mouse, *Mus musculus,* because there is overlap in pheromone components of female mice and female rats[5,6]. Native to the plains of Asia[7,8], brown rats and house mice co-evolved in a predator-prey relationship, with rats preying on mice[9,10]. Both of these macrosmatic rodents are prolific scent markers[11,12] that rely on their sense of smell during mostly nocturnal activity bouts. Within each species, respective urine marks offer a wealth of information about the signaler, including its age[13], health[14], breeding status[15,16], dominance[17], kinship and individual identity[18,19]. Moreover, rat odor elicits an innate avoidance behavior in mice[20,21].

Urine marks of rats and mice also disseminate sex attractant pheromone components. Although rats and mice share some pheromone components (e.g., testosterone, progesterone, estradiol)[22], the more volatile sex attractant pheromone components of males differ markedly. The ketone blend in urine marks of male brown rats (2-heptanone, 4-heptanone, 3-ethyl-2-heptanone, 2-octanone, 2-nonanone, 4-nonanone[6]) bears no resemblance to pheromone components emanating from urine marks of male house mice (3,4-dehydro-*exo*-brevicomin; 2-*sec*-butyl-4,5-dihydrothiazole[23,24]).

While acoustic and visual signals have a fleeting presence, odors and specifically pheromones often linger in the environment[25,26]. This makes pheromones particularly susceptible to inter-species exploitation[12,26,27] which is well known in insects[28–31] but has hardly been studied in mammals[4,32–35]. Studies on mammalian prey eavesdropping on the communication of their predators have focused on audio and visual communication signals[36,37]. Only two studies have demonstrated that rodents recognize the presence of predators based on their major urinary proteins and lacrimal proteins[10,38]. Compared to these high molecular-weight proteins, volatile sex attractant pheromone components

contrive long-range mate attraction[39] and thus are particularly susceptible to interspecies-eavesdropping[36].

Intercepting scent communication in vertebrate communities has long been studied, or viewed, as a one-way dyadic interaction, with prey sensing predator scent[25]. For instance, feline and canine odors elicit stereotyped fear and avoidance responses in rodents[34]. However, expanded views of auditory and visual communication systems now portray a multi-directional eavesdropper community network[25,36]. For example, mustelid, canid and felid predators exploit mammalian prey scent to locate prey[12,40], imposing significant costs on chemical signaling in the prey species[41–43]. Whether vertebrate predator-prey interactions are informed and guided by bi-directional (mutual) eavesdropping, or "counterespionage", on scent signals is entirely unknown, as are the underlying mechanisms.

Scent marks disseminate a myriad of odorants, only a few of which are pheromones, and hardly any pheromones are known to date. When prey avoided locations scent-marked by predators[20,34,44], and predators responded to scent marks of prey[12], these animals may simply have recognized generic prey and predator scent without necessarily eavesdropping on pheromone signals of target prey or predator foe. Testing the concept of mutual eavesdropping by predator and prey on each other's pheromones is contingent upon pheromone identification and the availability of synthetic pheromone. When synthetic volatile sex attractant pheromone components of both brown rats (predator of mice[9]) and house mice (prey of rats[10]) became available[6,22–24,45,46], the stage was set for testing the counterespionage hypothesis that mice co-opt rat pheromone components as cues to avoid rat predation, and rats co-opt mouse pheromone components as cues to facilitate mouse prey location. Testing these hypotheses, we were cognizant that the natural sex pheromone of mice and rats comprises additional constituents (e.g., urinal and lacrimal proteins[10,38]) which – expense-wise – could not be included in our synthetic pheromone lure, and that these constituents as well as non-pheromonal odors[47] may amplify any counterespionage evidence demonstrated in our study.

5.3. Results

5.3.1. Hypothesis 1: mice co-opt rat pheromone as a cue to avoid rat predation

In mouse-infested sites, trap pairs (see Fig. 1 for the general experimental design) baited with synthetic sex pheromone components of male rats captured 3.05 times fewer mice than trap pairs baited with synthetic pheromone components of male mice ($\chi^2 = 19.75$, $P < 0.0001$) (Fig. 2, top), suggesting that mice avoided macro-locations indicative of rat presence. Moreover, traps baited with male mouse pheromone components captured 15-times more adult female mice and 2.4-times more juvenile female mice than control traps baited with testosterone alone (adult females: $\chi^2 = 10.56$, $P < 0.01$; juvenile females: $\chi^2 = 5.30$, $P < 0.05$) (Fig. 3, bottom), confirming a synergistic effect of testosterone, brevicomin and thiazole on attraction of female mice[22]. Captures of adult male mice (2) and juvenile male mice (6) were insufficient to warrant statistical analysis. Conversely, traps baited with male rat pheromone components failed to capture a single female mouse, whereas corresponding (testosterone-only) control traps captured one adult female mouse and 13 juvenile female mice ($\chi^2 = 11.01$, $P < 0.01$) (Fig. 3, top), further indicating recognition and avoidance of micro-locations indicative of rat presence. Captures of adult male mice (2) and juvenile male mice (4) in traps baited with male rat lures were insufficient for statistical analyses.

5.3.2. Hypothesis 2: rats co-opt mouse pheromone as a cue to facilitate mouse-prey location

In rat-infested sites, trap pairs baited with synthetic male mouse pheromone components captured as many rats as trap pairs baited with synthetic male rat pheromone components ($\chi^2 = 0.01$, $P > 0.05$) (Fig. 2, bottom), revealing that foraging rats did not actively seek macro-locations indicative of mouse prey. On the contrary, traps baited with male mouse pheromone captured significantly fewer male and female rats than (testosterone-only) control traps ($\chi^2 = 5.30$, $P < 0.05$) (Fig. 4, top). Traps baited with a blend of male rat pheromone components – expectedly – captured significantly more females and significantly fewer males than (testosterone-only) control traps (females: $\chi^2 = 4.08$, $P < 0.05$; males: $\chi^2 = 9.48$, $P < 0.01$) (Fig. 4, bottom), confirming the

reported attractiveness and deterrence of male rat pheromone components to female and male rats, respectively[6].

5.4. Discussion

Our data support the “counterespionage” hypothesis. Mice and rats did eavesdrop on each other’s sex pheromone but they used the information they gleaned in a way only partially in keeping with our predictions for motive. This is the first evidence for bi-directional interspecific recognition of sex pheromones within a guild of mammals and between mammalian prey and predator. Our data also reveal that the sex attractant pheromone components of male mice (brevicomin and thiazole) and male rats (ketone blend) are underlying mechanisms that impart species-specificity to pheromonal communication between these murine rodents.

We deemed field experiments with pheromone-baited traps the most effective way to test our “counterespionage” hypothesis that predator and prey co-opt each other’s pheromone as a cue to locate prey or evade predation. We considered trap captures of wild male and female mice, and wild male and female rats, an excellent means to reveal attraction or deterrence of these murine rodents to their own pheromone and that of their mouse prey or rat foe. For future studies, however, we plan on video recording the behavior of rats and mice near select trap boxes to (*i*) reveal subtleties of behavioral responses indicative of attraction or fear according to the lure presented, and (*ii*) document the number of rodents that are approaching trap boxes but are not getting captured, indicating the proportion of the population that generates the data. Testing wild rodents in their natural environments was imperative because domesticated rodents in laboratory settings are known to behave differently than their wild counterpart[48–50]. As mice and rats typically do not share the same habitat[51], we needed to run experiments in locations infested with either mice or rats.

As predicted, female house mice co-opted the sex pheromone of male rats as a cue indicative of rat presence and potential predation risk by rats. Female mice largely avoided locations of paired traps disseminating synthetic male rat pheromone (Fig. 2), and not one single mouse female entered a trap box baited with rat sex pheromone (Fig. 3). These results are not surprising given that predator avoidance behavior is critical to the survival of mice, whereas rats do not avoid the odors of their predators, at least not

when collecting food in relatively safe and familiar habitats[52]. Recognizing scent marks of predators such as rats and cats enables mice to detect and avoid locations frequented by these predators, or to adjust their temporal foraging pattern accordingly[53]. Sensory neurons in the vomeronasal organs of mice detect specific major urinary proteins in urine scent marks of rats and cats which ultimately cause avoidance responses by mice[10,54,55]. Similarly, a lacrimal protein of rats (rat CRPI) decreases locomotion of mice and lowers their body temperature and heart rate[38]. However, all behavioral responses by mice in these studies to urinary and lacrimal proteins of rats were recorded in the confines of very small laboratory bioassay arenas where even “heavy” proteins could invoke behavior-modifying effects. Our field data obtained with populations of wild mice and rats conclusively show that the volatile sex attractant pheromone components of male rats have a long-distance aversion effect (Fig. 2) and a short-distance avoidance effect (Fig. 3) on female mice.

The hypothesis that rats co-opt the sex pheromone of male mice as a cue to locate mouse prey was not supported by our data. In rat-infested sites, locations of paired traps disseminating synthetic male mouse pheromone did not yield more captures of foraging rats than locations of paired traps disseminating synthetic male rat pheromone (Fig. 2). Remarkably, both male and female rats recognized the male mouse sex pheromone, and many stayed away from trap boxes, or “burrows”, apparently occupied by a male mouse (Fig. 4). While female rats may have simply recognized the “message” of an inappropriate (heterospecific) mate, the aversion responses of male rats can only be explained in a context other than sexual communication and mate recognition. Irrespective, rats did not exploit male mouse pheromone to locate mouse prey. Rather, they showed the propensity to avoid encounters with potential male mouse prey. There are several explanations for this seemingly peculiar behavior. First, brown rats are omnivores and only opportunistic predators of mice, which are not a primary food source for rats in the urban and industrial settings where we trapped. Second, all of our trapping sites had an abundant and constant supply of food other than live mouse prey, making rats not reliant on predation success for survival. Third (and perhaps least likely), brown rats may have traded the nutritional benefits of a proteinaceous male mouse meal for not risking injury during predation bouts.

Our study has shown that mammalian pheromones, comparable to auditory or visual communication signals, are under surveillance by a network of eavesdroppers.

“Designed” for incessant information flow, rodent sex pheromone components and their delivery systems are particularly susceptible to eavesdropping on these signals by illicit recipients, such as predators or prey. Major urinary proteins in urine scent marks of mice and rats serve as dissemination conduits for the volatile sex attractant pheromone components[56–58]. These delivery systems are so sophisticated that they even have inherent timestamps, informing the signal recipient of how recently the message was placed[59]. The functional role of mouse and rat major urinary proteins could not be assessed in our field study, but we surmise that these proteins would have contributed to the behavioral effects prompted by the sex *attractant* pheromone components.

Our findings that brown rats and house mice recognize each other’s sex pheromone engender exciting new research opportunities, particularly in conservation ecology. The long-distance aversion effect of brown rat pheromone components on house mice (Figs. 2, 3) could be used as a means to expel mice from biodiverse hotspots in island communities, where rat control has prompted harmful outbreaks of mice[60]. The tactic of exploiting predator scent for pest control[35,61] was successful in various wildlife conservation projects[62–64] but sourcing of scent directly from predators is impractical and would not be necessary if synthetic rat pheromone was used for mice manipulation. The failure of some studies to achieve repellent effects with predator odors for pest control[34] has likely multiple reasons, one of which being insuffcient longevity of predator urine or feces odors. Slow-release formulations of synthetic pheromone components, possibly presented in combination with some non-pheromonal predator odors[47], may not only prolong the effect of predator scent on prey but make this tactic more affordable than sourcing of scent directly from predators.

If synthetic mouse sex pheromones were experimentally shown to attract feral cats, synthetic mouse pheromone lures could be developed for capturing, and subsequent neutering of feral cats that otherwise would continue to reproduce prolifically, extending their already devastating impact on bird populations[65]. The same pheromone lures could be deployed for trapping feral cats that have invaded, or were deliberately introduced to, island communities where they now threaten seabird colonies[66] and many endemic reptiles[67]. If the eavesdroppers’ network were to include other mesopredators of murine rodents such as the red fox, *Vulpes vulpes*, or striped skunk, *Mephitis mephitis*, then synthetic rodent pheromone could be used to help eliminate diseases from mesopredator populations. For example, adding synthetic

rodent pheromone to baits laced with oral rabies vaccine[68] would likely make these baits olfactorily more apparent to foraging predators and thus expedite bait location and disease elimination.

5.5. Materials and methods

5.5.1. General design of field experiments

Parallel field experiments for trapping house mice and brown rats were run between March – June 2017 and October 2016 – November 2019 in mouse-infested sites (Exps. 1, 2; 81 paired trap boxes each for mice and rats) and in rat-infested sites (Exps. 3, 4; 76 paired trap boxes each for mice and rats) in the Fraser Valley of British Columbia, Canada. The four sites infested with rats (inferred by the presence of 0.6- to 1.3-cm long fecal pellets with pointed ends) included a food production facility, a food bank, and two recycling centers, whereas the two sites infested with mice (inferred by the presence of 0.6-cm long fecal pellets with blunt ends) included a duck farm and a bird sanctuary. Based on fecal pellet evidence, all sites were exclusively infested with either rats or mice. Population densities in these sites were likely weak to moderate based on infrequent rodent sightings, the amount of feces present, and the time needed to generate the trap catch data. In all sites, rodents had steady access to animal or human food and were exposed to predation by feral cats and owls. Mouse-infested sites had been used in previous research projects with mice[5,22,46,69] but were not used for one year prior to the onset of our study. All sites were subject to rodent control measures mainly in the form of poison bait stations.

In each site, experimental replicates for mice and rats were set up along interior or exterior walls of buildings (Fig. 1). Each replicate consisted of two sets of paired trap boxes (PROTECTA Mouse or Rat, Bell Laboratories Inc., Madison, WI 53704, USA), with 0.5-m spacing between the boxes in each pair, and at least 2 m between pairs (Fig. 1). Each trap box contained a Victor snap trap (M325 M7 Pro mouse or M326 M7 Pro rat Woodstream Co., Lititz, PA 175543, USA) that was set with a food bait[69] which prompted feeding and thus capture of responding mice or rats. Twice or 3-times every week, traps were checked, and food baits and pheromone lures (see below) replaced. Captured rodents were assessed for their age (juvenile or adult) based on genitalia development[70], and for their sex based on ano-genital distance[71] or PCR genotyping carried out on DNA

extracted from ear or tail clips[72]. Whenever a mouse or a rat had been captured, a new trap box and snap trap were deployed. This procedure ensured that the odor of captured mice or rats did not affect future captures. The position of the treatment and the control trap box within a trap box pair was re-randomized after each capture. The research protocol was approved and supported by the Animal Care Committee of Simon Fraser University (protocol #1159B-15 and #1295B-19) which abides by the Canadian Council on Animal Care guidelines.

5.5.2. Synthetic sex pheromone components tested

Both the treatment and the control trap box in each trap box pair received testosterone, a pheromone component of low volatility shared between house mouse and brown rat males[22]. Adding the volatile sex attractant pheromone components of either male mice or male rats (see below) to testosterone, we could then test whether these components impart species-specificity to the sex pheromone blend and enable cross-recognition of predator or prey communication signals. This plain experimental design was guided by recent studies already showing that: (1) synthetic testosterone on its own tested *versus* an unbaited control strongly attracts female mice and female rats[22]; (2) traps baited with synthetic sex attractant pheromone components of male mice (brevicomin & thiazole; see below), or of male rats (ketone blend; see below), capture significantly more female mice[46], and more female rats[6], than unbaited control traps; and (3) synthetic trap lures containing both testosterone (or androstenone) and sex attractant pheromone components of male rats or male mice synergistically attract more female rats[22], and more female mice[22,73], than partial pheromone lures containing either the sex steroid or the sex attractant pheromone components. As the more complete pheromone lure for mice and rats is clearly more effective than partial pheromone lures, there is no need for testing it further *versus* unbaited controls.

Testosterone was dissolved in acetonitrile (50 µl) and applied to a piece of filter paper at the biologically relevant dose of 750 ng (about five times the amount of testosterone a single male mouse discharged with urine during one day)[22]. The treatment box in each pair received synthetic sex attractant pheromone components of either male house mice [3,4-dehydro-*exo*-7-ethyl-5-methyl-6.8-dioxabicyclo[3.2.1]octane (= 3,4-dehydro-*exo*-brevicomin = brevicomin); 2-*sec*-butyl-4,5-dihydrothiazole (= thiazole)] or male brown rats (2-heptanone, 4-heptanone, 3-ethyl-2-heptanone, 2-

octanone, 2-nonanone, 4-nonanone). The house mouse pheromone components brevicomin and thiazole were each formulated at 1 mg in mineral oil (10 ml) and contained in a 20-ml glass scintillation vial (VWR International, LLC Randor, PA 19087, USA). This formulation afforded the release of brevicomin and thiazole at rates of 180 ng h^{-1} and 75 ng h^{-1}, respectively, very similar to the hourly release rates of these two compounds from bedding material soiled by laboratory-kept male mice[46]. The sex attractant pheromone components of male brown rats were formulated as a 1-mg blend at the same ratio [2-heptanone (100), 4-heptanone (10), 3-ethyl-2-heptanone (10), 2-octanone (1), 2-nonanone (1), 4-nonanone (10)]) as found in headspace odorants of male rat urine, and afforded release rates comparable to those from soiled bedding material of laboratory-kept rats[6]. The potential of glassware or mineral oil to modulate the effects of brevicomin and thiazole or the blend of ketones was minimized by fitting treatment and control trap boxes in each trap pair with the same glassware and volume of mineral oil.

5.5.3. Statistical analyses

We analyzed all data with R 3.5.0[74]. For each of experiments 1-4, we compared the proportion of captures in treatment and control traps against a theoretical 50:50 distribution, using a χ^2-test with Yate's correction for continuity. We also used paired χ^2-tests to compare total captures of mice and of rats in traps baited with synthetic pheromone components of male mice or male rats in mouse- and rat-infested sites.

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5.8. Figures

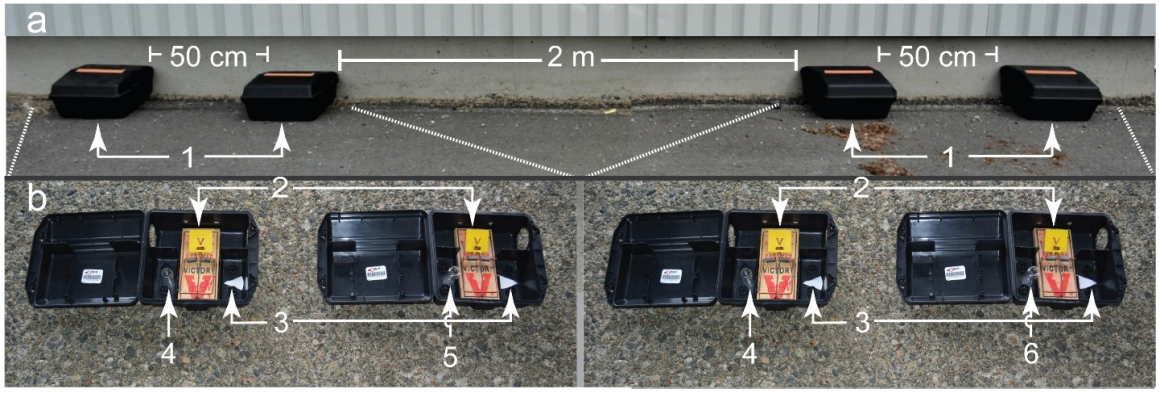


Figure 5.1. **Photographs illustrating (a) the double-set, paired trap box design of an experimental replicate, and (b) details of snap trap, food bait and pheromone lure. Each experimental replicate (n = 157) consisted of two pairs of large trap boxes (placed in rat-infested sites), or two pairs of small trap boxes (placed in mouse-infested sites; not shown in this figure), for capturing rats and mice, respectively, with 0.5-m spacing between the boxes in each pair, and at least 2 m between pairs. Numbers refer to: 1 = trap box; 2 = snap trap with food bait[69] for capturing (killing) responding rodents; 3 = filter paper treated with synthetic testosterone (a pheromone component shared by male brown rats and male house mice); 4–6 = a 20-ml glass scintillation vial containing plain mineral oil (4; control stimulus) or mineral oil laced with sex attractant pheromone components of either male house mice (5) or male brown rats (6). Note: the smaller trap boxes for mice (not shown here) were fitted with glass scintillation vials reduced in height (cut to size).**

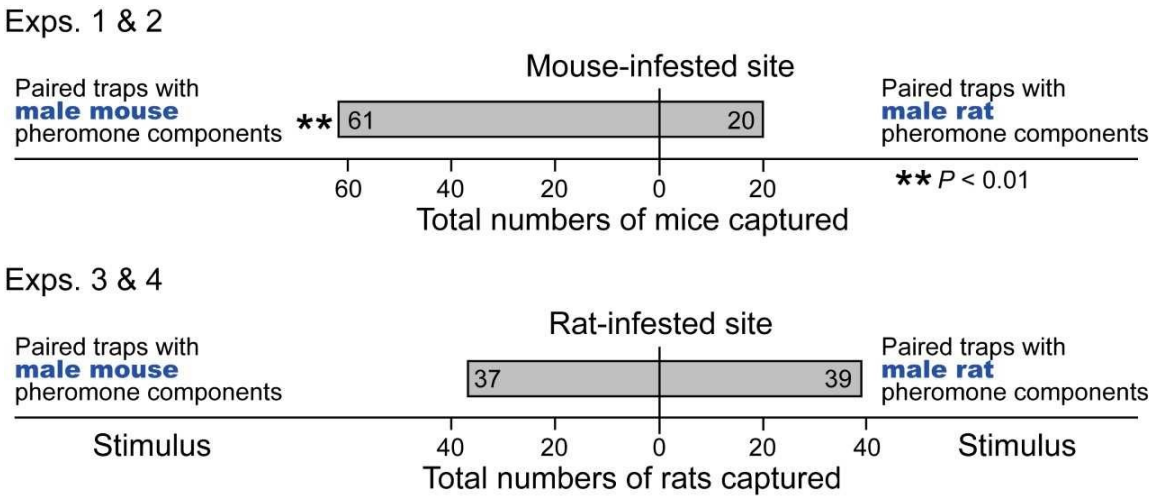
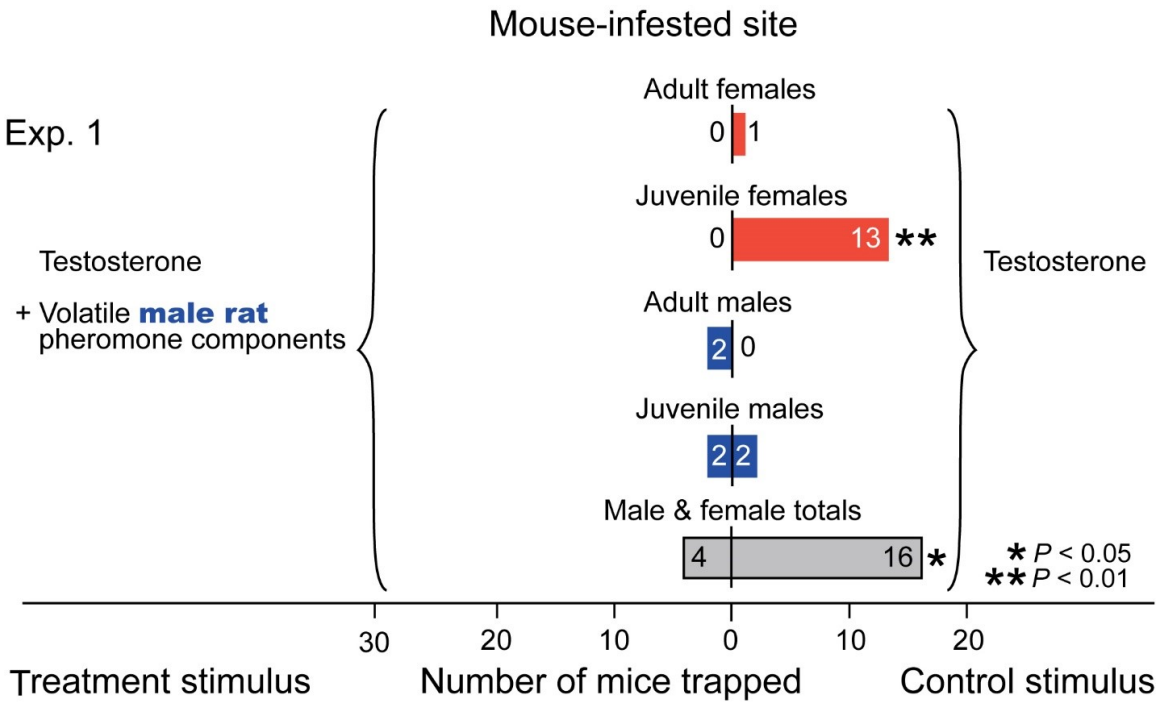


Figure 5.2. **Trap catch data revealing that house mice are averse to macro-locations (trap box pairs; see Fig. 1) indicative of brown rat presence. The treatment trap in each pair received the volatile synthetic sex attractant pheromone components of male house mice (testosterone, 3,4-dehydro-*exo*-brevicomin, 2-*sec*-butyl-4,5-dihydrothiazole) or brown rats (testosterone, 2-heptanone, 4-heptanone, 3-ethyl-2-heptanone, 2-octanone, 2-nonanone, 4-nonanone), whereas corresponding control trap boxes received testosterone only. Trap pair locations with rat pheromone components captured 3.05 times fewer mice than trap pair locations with mouse pheromone components, whereas trap pair locations with rat or mouse pheromone components captured equal numbers of rats, revealing predator-aversion behavior by mice and no evidence for prey-seeking behavior by rats. The asterisks indicate a significant difference in the number of mice captured in paired traps (χ^2-tests with Yate's correction for continuity compared against a theoretical 50:50 distribution, ** $P < 0.01$).**


Mouse-infested site
Exp. 1
Testosterone
+ Volatile male rat pheromone components
Adult females
0
1
Juvenile females
0
13
**
Adult males
2
0
Juvenile males
2
2
Male & female totals
4
16
*
Testosterone
* P < 0.05
** P < 0.01
30
20
10
0
10
20
Treatment stimulus
Number of mice trapped
Control stimulus

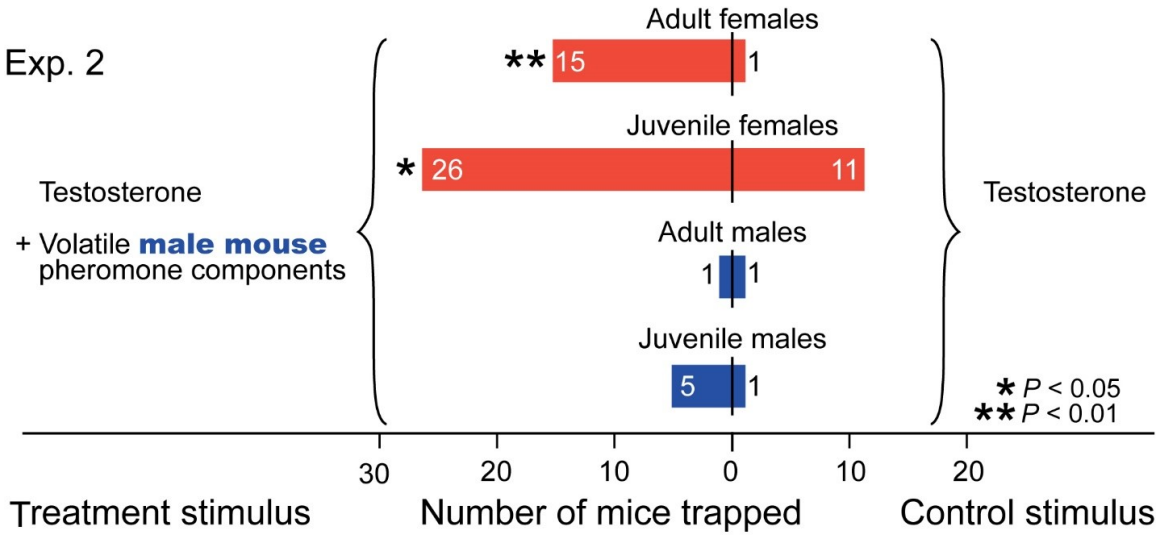
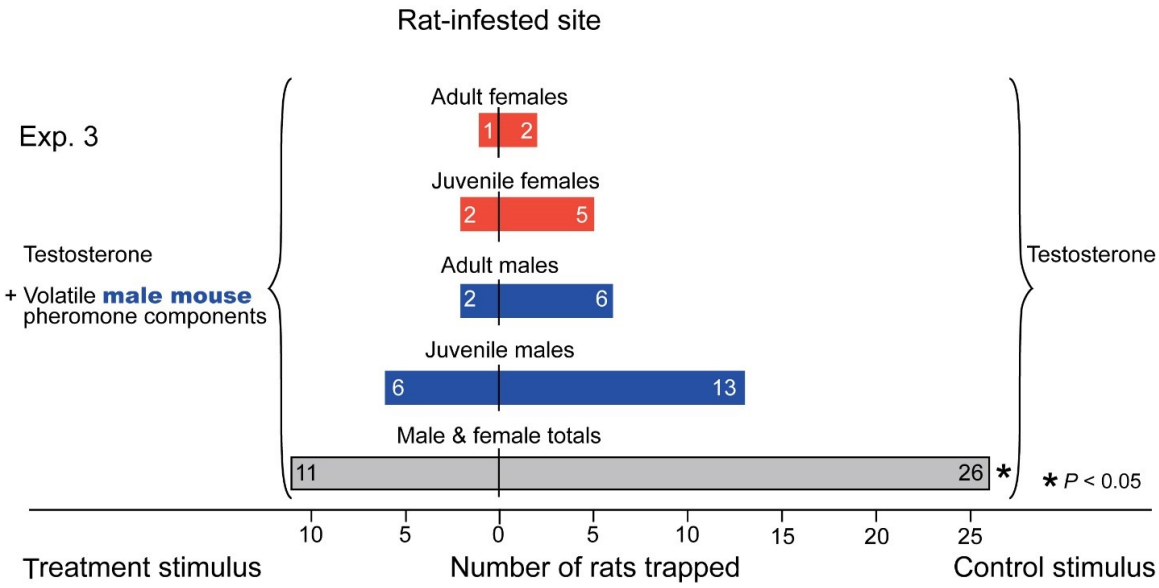

Exp. 2
Testosterone
+ Volatile male mouse pheromone components
Adult females
**
15
1
Juvenile females
*
26
11
Adult males
1
1
Juvenile males
5
1
Testosterone
* P < 0.05
** P < 0.01
30
20
10
0
10
20
Treatment stimulus
Number of mice trapped
Control stimulus

Figure 5.3. **Trap catch data revealing that female house mice stay away from and seek micro-locations (specific trap boxes) indicative of male brown rat and male house mouse presence, respectively. The treatment trap in each pair received the volatile synthetic sex attractant pheromone components of male house mice (testosterone, 3,4-dehydro-*exo*-brevicomin, 2-*sec*-butyl-4,5-dihydrothiazole) or male brown rats (testosterone, 2-heptanone, 4-heptanone, 3-ethyl-2-heptanone, 2-octanone, 2-nonanone, 4-nonanone), whereas corresponding control trap boxes received testosterone only. The asterisks indicate a significant difference in the number of mice captured in treatment and control traps (χ^2-tests with Yate's correction for continuity compared against a theoretical 50:50 distribution; * $P < 0.05$, ** $P < 0.01$).**


Rat-infested site
Exp. 3
Testosterone
+ Volatile male mouse pheromone components
Adult females
1
2
Juvenile females
2
5
Adult males
2
6
Juvenile males
6
13
Male & female totals
11
26
*
Testosterone
* P < 0.05
10
5
0
5
10
15
20
25
Treatment stimulus
Number of rats trapped
Control stimulus

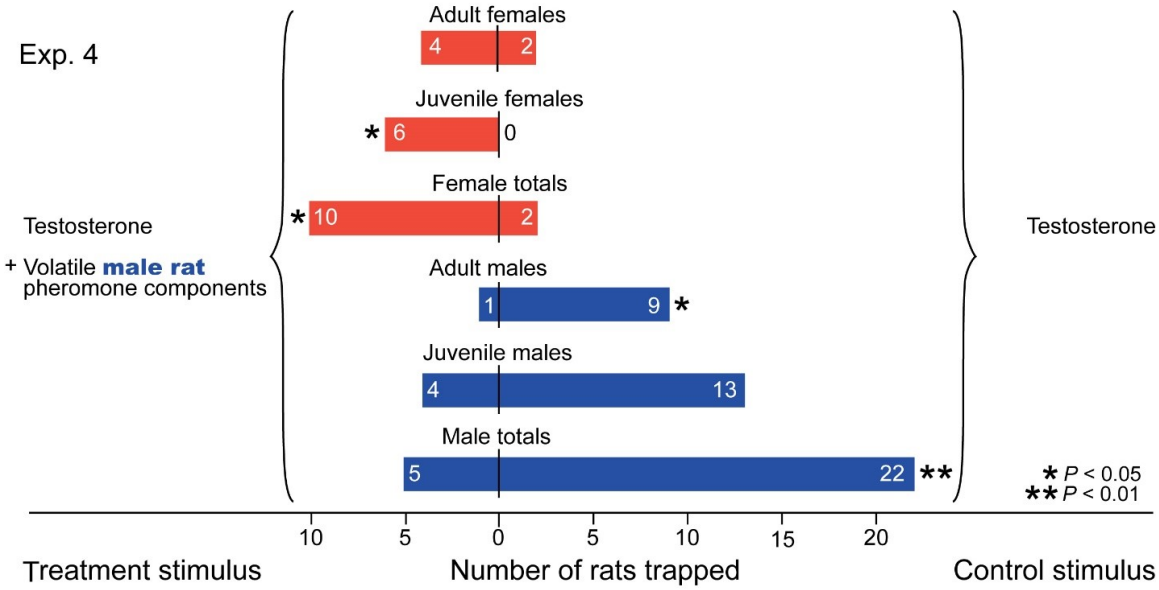

Exp. 4
Testosterone
+ Volatile male rat pheromone components
Adult females
4
2
Juvenile females
*
6
0
Female totals
*
10
2
Adult males
1
9
*
Juvenile males
4
13
Male totals
5
22
**
Testosterone
* P < 0.05
** P < 0.01
10
5
0
5
10
15
20
Treatment stimulus
Number of rats trapped
Control stimulus

Figure 5.4. **Trap catch data revealing that brown rats stay away from micro-locations (specific trap boxes) indicative of male mouse presence, and that female and male brown rats seek and avoid micro-locations indicative of prospective mates and rival males, respectively. The treatment trap in each pair received the volatile synthetic sex attractant pheromone components of male house mice (testosterone, 3,4-dehydro-*exo*-brevicomin, 2-*sec*-butyl-4,5-dihydrothiazole) or male brown rats (testosterone, 2-heptanone, 4-heptanone, 3-ethyl-2-heptanone, 2-octanone, 2-nonanone, 4-nonanone), whereas corresponding control trap boxes received testosterone only. The asterisks indicate a significant difference in the number of rats captured in treatment and control traps (χ^2-tests with Yate's correction for continuity compared against a theoretical 50:50 distribution; * $P < 0.05$, ** $P < 0.01$).**

Chapter 6.

Attraction of domestic and feral cats to prey-derived pheromone and sound lures

A very similar version of this chapter submitted as a manuscript to 'Biological Invasions' for review, with the following authors: Elana Varner, Kendal Singleton, Daniela Gofredo, Amy Tran, Jinah Hong, James Parker, Regine Gries, Stephen Takács, Gerhard Gries

6.1. Abstract

Free-ranging domestic and feral cats, *Felis catus,* are considered the world's worst invasive predators. The success of trap-neuter-release (TNR) programs for control of feral cat populations relies on cat captures. However, feral cats are not easily trapped. We predicted that trap lures presenting prey signals or cues would trigger the cats' predation drive and thus improve cat capture rates. Working with domestic and feral cats in both field and animal-shelter experiments, we specifically tested the hypotheses that (H1) prey-derived pheromone and sound lures, in combination, attract and help capture cats, and (H2) the pheromone lure or the sound lure on its own mediates cat attraction. We prepared pheromone lures that contained synthetic sex attractant and sex steroid pheromone components of male and female house mice, *Mus musculus*, and male deer mice, *Peromyscus maniculatus*. We also assembled a sound lure comprising playback recordings of house mouse vocalizations and Brown rat, *Rattus norvegicus*, pasta-chewing sounds. When singly-tested cats in animal shelters were offered a choice between two mouse-look-alike toys, one of which was baited with pheromone and sound lures and the other left unbaited, 12 out of 14 cats tested contacted or closely approached first the baited toy. In field settings, 12 feral cats fully entered live traps baited with pheromone and sound lures, whereas three cats entered paired unbaited control traps. To tease apart the effect of pheromone and sound lures on cat attraction, shelter cats were then presented with two mouse toys that were either pheromone-baited and scentless, or that were sound-baited and silent. Nine out of 11 cats tested contacted or closely approached first the pheromone-baited toys, but cats did not exhibit a similar preference for sound-baited mouse toys. This is the first evidence that cats are attracted to sex pheromone components of mice. Although prey sound on its own did not

prompt behavioural responses by cats, it seemed to entice entry of feral cats into pheromone-baited live traps. Our study provides proof of concept that rodent pheromone and sound lures can be developed to expedite captures of feral cats in TNR programs.

6.2. Introduction

Free-ranging domestic and feral cats, *Felis catus,* have caused population declines and extinctions of multiple prey species (Bonnaud et al. 2011), and are considered the world's worst invasive predators (Lowe et al. 2000). Annually, they kill an estimated 1.3–4 billion birds and 6.3–22.3 billion mammals in the U.S. alone (Loss et al. 2013). Ground-nesting birds are particularly susceptible to feral cat predation, with endangered species and populations suffering irrevocable damage (Loss et al. 2013). Free-ranging domestic and feral cats also impose sub-lethal effects on birds, reducing their fecundity, and aggravating their vulnerability to other predators (Bonnington et al. 2013).

Control measures for feral cats are chosen dependent upon environmental circumstances and public opinion (Wolf et al. 2022). Lethal control (trapping-and-killing) of feral cats on mainland has widely failed (Fancourt et al. 2021; Wolf et al. 2022). Trapping-and-killing temporarily reduces the number of cats in any habitat but over a longer term may lead to an upsurge of feral cat populations by disturbing established territories and by increasing the number of migrating cats (Lazenby et al. 2014). As a result, animal control professionals need to keep trapping and killing new cats. In this endless feedback loop, animal control resources can be wasted and cat management objectives are not being met. Animal welfare concerns coupled with public opinion have prompted operational implementation and expansion of trap-neuter-release (TNR) programs, instead of lethal programs, to control feral cat populations. After being humanely trapped, cats are taken to veterinary clinics, where they are neutered or spayed, marked, and often vaccinated before being released (Aeluro et al. 2021, 2022). According to case-studies in urban areas, TNR programs have high long-term success rates (Spehar and Wolf 2018; Wolf et al. 2022), with cat populations reduced up to 99% over 16 years (Spehar and Wolf 2020). For feral cat management, TNR programs are commonly used in urban centers (Aeluro et al. 2021; Debrot et al. 2022), whereas trap-and-kill methods are favored in more rural communities (Loyd et al. 2010).

While there is still much debate about the tactic that is most prudent and humane for feral cat control (Crawford et al. 2019; Wolf et al. 2019; Calver et al. 2020; Read et al. 2020), the success of both trap-and-kill and trap-neuter-release programs hinges upon cat captures. However, feral cats are often trap-shy and not easily trapped (Short et al. 2002; Vantassel 2013; Dutcher et al. 2021) or re-trapped (Buckmaster 2011). Food lures such as fresh or canned fish (Veitch 1985; Clapperton et al. 1994) quickly spoil and do not appeal to felids that generally prefer to feed on freshly killed prey rather than prey carrion (Bradshaw et al. 1996). Catnip and matatabi trap lures are somewhat effective but still fail to prompt the cats' strong predation drive which would improve capture rates (Clapperton et al. 1994; Nogales et al. 2004). As obligate carnivores (Bradshaw et al. 1996) and generalist predators, cats prey on murine rodents, birds, rabbits, amphibians, reptiles, and even invertebrates (Liberg, 1984; Kutt, 2012; Krauze-Gryz et al., 2016). With acute hearing (Tavolga et al. 1991) and a keen sense of vision (Bradshaw 2012), cats are formidable hunters (Brown and Bradshaw 2012). They have the broadest hearing range of all mammals (48–85,000 Hz; measured at 70 dB sound pressure), and thus are extremely capable of locating (Tavolga et al. 1991) and detecting both low- and high-frequency prey sounds (Heffner and Heffner 2007), well beyond the cats' own vocalization range (Brown and Bradshaw 2012). This wide hearing range is thought to facilitate detection of prey vocalizations (Musolf and Penn 2012) as well as rodent foraging/feeding sounds (e.g., scratching, scurrying, rustling, chewing) (Takagi et al. 2016).

Cats have relatively large eyeballs that endow a great light-gathering capacity (Bradshaw 2012). Their relatively wide field of view and great range of peripheral vision (Long et al. 2010; Bradshaw 2012; Abdai et al. 2022) further facilitate prey detection. With rapid and conjugate movement of both eyes, they can readily track fast-moving rodent and bird prey (Brown and Bradshaw 2012). Moreover, with comparatively many rod cells in their eyes, cats have low-light sensitivity (Brown and Bradshaw 2012) which facilitates hunting of crepuscular prey.

Whereas acoustic and visual prey cues have a fleeting presence, prey odors – and specifically prey pheromones – often linger in the environment (Banks et al. 2016; Roitberg 2018). Volatile sex attractant pheromone components of rodents contrive long-range mate attraction (Musso et al. 2017) and thus are particularly susceptible to eavesdropping by predators. Although cats are believed to primarily exploit visual and

auditory cues of prey (Brown and Bradshaw 2012; Moseby and McGregor 2022), their well-developed vomeronasal organ and accessory olfactory bulb (Salazar and Sánchez-Quinteiro 2011) may play key roles in detecting prey pheromones and informing foraging behavior.

Mice are the most abundant small mammals in North America (Joyner et al. 1997) and frequent prey of feral cats (Széles et al. 2018; Rendall et al. 2022). We predicted that foraging cats encounter mouse prey not by random chance, but exploit both pheromonal and auditory communication signals of mice as prey-location cues. Male house mice, *Mus musculus*, e.g., are prolific scent markers that deposit urine to communicate information about their identity (Barnard and Fitzsimons 1988), age (Osada et al. 2008), social dominance (Jones and Nowell 1973), reproductive status (Hurst 1989) and health (Kavaliers et al. 2005). Moreover, house mice produce sophisticated sonic and ultrasonic vocalizations (Musolf and Penn 2012; Kowalski et al. 2021; Takács et al., 2021), with male courtship songs rivaling the complexity of bird songs (Marler and Slabbekoorn 2004; Musolf and Penn 2012). Cats recognize scent marks of mice, visiting mouse-scented plots faster and in greater number than unscented control plots (Hughes et al. 2010) but it is not yet known whether cats sense mouse pheromones as prey-location cues. Cats are attracted to bird songs (Moseby et al. 2004) but their potential orientation to mouse vocalization signals or to rodent foraging and feeding sounds have not yet been explored. Murine rodents gnaw and consume hard food items, including bones (Pokines 2015), seeds and grains (National Research Council 1995), and in the process produce audible chewing sounds (EV & KS, person. observ.) which may serve as prey location cues for hunting cats.

With multiple sex attractant pheromone components of house mice, *Mus musculus*, and deer mice, *Peromyscus maniculatus,* known and available (Jemiolo et al. 1985; Novotny et al. 1985; Schwende et al. 1986; Musso et al. 2017; Takács et al. 2017; Varner et al. 2018, 2022), and with house mouse vocalization signals and Brown rat, *Rattus norvegicus,* chewing sounds readily recordable from laboratory- or home-kept mice and rats, it has become possible to test whether communication signals and foraging cues of murine rodents are sensed by cats and guide their foraging decision. Working with domestic and feral cats in both field and animal-shelter experiments, we tested the hypotheses that (H1) prey-derived pheromone and sound lures, in

combination, attract and help capture domestic and feral cats, and (H2) the pheromone lure or the sound lure on its own mediates cat attraction.

6.3. Methods and materials

6.3.1. Ethics statement

The research protocol was approved and supported by the Animal Care Committee of Simon Fraser University (protocol #1282B-18) which abides by the Canadian Council on Animal Care guidelines.

6.3.2. Animals

House mice

House mice (CD-1®) were available from another project (1284B-18) and were on loan for this project. They were originally obtained from Charles River Laboratories International Inc. (Saint-Constant, QC, Canada) and cared for by Animal Care Services of Simon Fraser University (SFU). Mice were accommodated in a cage (50 × 40 × 20 cm) lined with corncob bedding (Anderson's Bed o'cobs, The Andersons Inc., Maumee, OH, USA) and enriched with Nalgene toys and running wheels (Jaimesons Pet Food Distributers, Richmond, BC, Canada). Males were singly housed, and females were housed in groups of five. Rodent food (LabDiet® Certified Rodent Diet, LabDiet, St. Louis, MO, USA) and water were provided *ad libitum*.

Shelter cats

All cats tested in bioassays were previously spayed or neutered, physically healthy, and 9–108 months old. The animals were housed at three separate animal shelters in the Greater Vancouver area and had been at 'their' shelter for > 1 month.

Pet rats

Two 24-month-old female Brown rats, *Rattus norvegicus*, kept as pet rats were used to record chewing and shuffling sounds. We used rats instead of mice to record foraging and chewing sounds anticipating that rat-produced sounds would to be louder and more distinct than mouse-produced sounds.

6.3.3. Preparation of sound lure

Recordings of house mouse vocalizations and Brown rat chewing sounds

To obtain vocalizations by house mice, one adult male and one adult female of indeterminate reproductive state were placed in a cage (60 × 40 × 20 cm) and allowed to interact but were separated when mounting behavior was imminent. A microphone (Sony F-V100 Omni-Directional Dynamic Vocal Microphone; frequency response: 100 Hz to 10 kHz; Best Buy Canada Ltd., Burnaby, BC, CA) was positioned above the centre of the cage and sonic frequency components of their vocalizations were recorded (44,100 Hz mono) using Audacity 2.3.2 (copyright © 1999-2018, Build: MSVC 19.16.27027.01) to generate .wav files. Following recordings, the male and female were returned to their home cages. Recordings were saved to a Dell desktop computer (Dell, Round Rock, TX, USA). Recorded sounds were analyzed for duration, frequency, intermittency and relative intensity using Audacity Analyzer (plot spectrum).

To record chewing sound, a pet Brown rat was placed in a cardboard box (35 × 30 × 45 cm) and offered dry pasta for food. A Sony F-V100 microphone (see above) connected to a Dell desktop computer was inserted through the top of the closed box to record the sound of the rat chewing the pasta. The sound intensity (decibel level) was measured using a Sound Meter App on a LG G5 Android Device (Sound Meter, Splend Apps, Google Play).

Playback file of sound recordings and selection of playback devices

Track recordings of mouse vocalizations and rat chewing sounds, together with intermittent silent periods, were then combined into a single mono track 4.5-min .wav sound file (Fig. 1), using ‘Audacity track, mix and render’. This sound file was looped (automatically rerun) and continuously played back during cat bioassays. Realizing the difficulty of accurately emitting ultrasonic sounds in field and animal-shelter experiments, the sound file contained components only in the sonic range (0–22 kHz). The sound intensity of playback recordings during all cat bioassays was standardized and adjusted (Sound Meter, Splend Apps, Google Play) to the same sound level measured during recordings.

In animal shelter experiments 1 and 4, the combined sound file was emitted through an earbud headphone (sound range: 20 Hz – 20 kHz; Classic in-ear

Headphones, Miniso, Richmond, BC, CA) connected to an iPhone 6, with the audio quality enhanced by an amplifier (Boomcloud 360 Boomstick, BoomCloud 360 Inc., Palo Alto, CA, USA). In the field experiment, the sound file was emitted through two speakers (sound range: 80 Hz – 18 kHz; MIFA A1 Bluetooth speakers, Best Buy Canada Ltd., Burnaby, BC, CA) remotely connected to an iPhone 6. One speaker was placed at the trap entrance and the other in the back of the trap.

Preparation of synthetic house mouse and deer mouse pheromone components

The mouse pheromone trap lure consisted of synthetic sex pheromone components of male house mice [3,4-dehydro-*exo*-brevicomin (brevicomin), 2-*sec*-butyl-4,5-dihydrothiazole (thiazole) (Jemiolo et al. 1985; Novotny et al. 1985), 1-hexanol, 2,3,5-trithiahexane (Varner et al. 2022); testosterone (Takács et al. 2017)], of female house mice [butyric acid, 2-methylbutyric acid, 4-heptanone (Varner et al. 2018); progesterone, estradiol (Takács et al. 2017)], and of male deer mice [3-methyl-2-pentanone, testosterone (Varner et al., unpubl.)] The volatile pheromone components of the blend (all except the sex steroids testosterone, progesterone and estradiol) were formulated to match the headspace volatile blend emanating from urine/feces excreta of one male and one female house mouse, respectively, on average over the course of 24 h (Varner et al. 2018, 2022). To this end, we first prepared three 10-mL solutions of mineral oil. Solution #1 contained butyric acid (140 mg), 2-methyl butyric acid (14 mg), and 4-heptanone (1 mg); solution #2 contained 3-methyl-2-pentanone (0.03 mg), 1-hexanol (0.03 mg), and 2,3,5-trithiahexane (5 mg); and solution #3 contained brevicomin (1 mg) and thiazole (1 mg). Then, aliquots of solutions #1 (30 µL), #2 (2 mL), and #3 (8 mL) were combined in a stock solution (10.03 mL). For bioassays in cat shelters, a 100-µL aliquot of this stock solution was applied onto a cotton ball (Richmond Dental, Charlotte, NC, USA) inside a mouse-shaped toy (Fig. 2a), and for field experiments with feral cats (Fig. 3), a 500-µL aliquot was applied onto a cotton roll (10 × 25 mm; Richmond Dental, Charlotte, NC, USA) inside a glass vial. For dissemination of the less volatile sex steroid pheromone components, a 50-µL ether solution of testosterone (750 ng), or of progesterone (250 ng) and estradiol (125 ng), were applied on filter paper (Whatman #1, 120 mm, Maidstone, ENG).

6.3.4. (H1) Prey-derived pheromone and sound in combination attract cats

Experiment in animal shelters

Experiment 1 (Table 1) tested the ability of prey-derived pheromone and sound lures, offered in combination, to attract domestic cats. The experiment was run between September and November 2019 in three animal rescue shelters in the Greater Vancouver area of British Columbia, Canada. In each replicate (n = 14), a single cat was offered a choice between two mouse toys, one of which was baited with both the pheromone lure and the sound lure, and the other remained odorless and silent (Fig. 2a, b).

In preparation for cat bioassays, mouse-look-alike 'toys' (12 cm long, 3 cm wide, 4.8 cm tall; Fig. 2a) were assembled from two pieces of black 100-% polyester felt (each 12 × 3 × 4.8 cm) (Creatology™, Michaels, North Vancouver, BC, CA) that were hot-glued together and filled with two medium-sized cotton balls (Fisher Scientific, Houston, TX, USA). The toys were suspended via headphone cable (treatment) or cotton string (control) from stands placed 60 cm apart from one another (Fig. 2b) at the back of an enclosure (floor: 1 × 1.2 m). A small posterior opening in each toy allowed insertions of treatment or control stimuli. To test the effect of mouse pheromones, the treatment toy received a cotton ball treated with the volatile pheromone blend in mineral oil and two pieces of filter paper treated with either testosterone, or progesterone and estradiol, dissolved in ether. The corresponding control toy received a cotton ball treated with an equivalent volume of mineral oil and two pieces of filter paper each treated with an equivalent volume of ether. For delivery of the playback sound stimulus (see above), the treatment toy was fitted with a concealed earbud headphone (Classic in-ear Headphones) attached to a custom-made holder inside the toy and connected to an iPhone 6 (Fig. 2b). Potential electromagnetism from the iPhone that may possibly have affected the cats' responses was not accounted for in this pilot experiment but was controlled for in follow-up experiment 4 (see below).

To initiate a bioassay, a randomly selected cat was placed at the entrance of the enclosure and allowed 10 min to respond. The cat's first choice of toy – the toy it contacted first or sniffed first at close range (< 10 cm) – was observed and video recorded (AKASO EK7000 camera, Amazon.com). Following the completion of a

bioassay, the enclosure door was opened and the cat was returned to the main area of the animal shelter to roam freely with the other cats. Moreover, treatment and control stands were sanitized with 70% ethanol and Saber® (Wood Wyant, Victoriaville, QC, CA) and their position within the enclosure was randomized. Treatment and control mouse toys were discarded and replaced with new ones.

Experiment in field settings

The effect of rodent pheromone and sound as trap lures on captures of feral cats was field-tested in experiment 2 (Table 1) at two livestock farms with active feral cat populations and a feral cat rescue sanctuary in the lower mainland of British Columbia, Canada, between May and October 2019. For each replicate (n = 15), cats were offered a choice between two Live Catch Traps (#299-3683-6; 81.2 × 30.4 × 25.4 cm; Canadian Tire, Burnaby, BC, CA) covered with a white cotton towel and placed 1 m apart, with entrances facing each other (Fig. 3). The traps were open but not set to allow entry without capture of responding cats.

The treatment trap received the mouse pheromone lure. Specifically, a 500-µL aliquot of the pheromone stock solution in mineral oil (see above) was applied onto a braided cotton roll (10 × 25 mm; Richmond Dental, Charlotte, NC, USA) inside an open 4-mL vial (15 × 45 mm, 1 dram; Fisher Scientific, Fair Lane, NJ, USA), whereas the cotton roll in the control trap was treated with 500 µL of mineral oil. The treatment trap also received two pieces of filter paper treated with male or female mouse steroid pheromone components (see above) dissolved in ether, whereas the control trap received two pieces of filter paper treated with an ether control. Treatment and control stimuli were placed in an open-top treatment and control box (each 15.5 × 7.5 × 10.5 cm) respectively, covered with white cotton fabric and positioned in the back of traps (Fig. 3b).

The treatment trap also received the playback sound lure (Fig. 1) emitted through one speaker (MIFA A1 Bluetooth speakers, Best Buy Canada Ltd., Burnaby, BC, CA) at the trap entrance and another speaker in the back of the trap, both speakers were remotely controlled via an iPhone 6. The speakers were connected to a motion sensor (Safe House 49-7048, InterTAN Canada Ltd., Barrie, ON, CA) that detected a cat's presence at the trap entrance and then triggered sound emission from the speaker in the back, instead of the front, of the trap. The front speaker was visually concealed by an

open plastic box (13 × 5 × 10.5 cm) covered with white cotton cloth, whereas the back speaker resided in the same box containing the pheromone stimuli. For visual symmetry, the control trap was fitted with an identical box at the entrance, but the box did not contain a speaker.

The behavior of feral cats was video-recorded by two cameras (AKASO EK7000, GoPro Hero 3, Amazon.com), each camera positioned 3 m from, and pointing towards, one trap. Bioassay replicates were run for 20 min but were terminated sooner when a cat had fully entered a trap with all four paws, equivalent to a successful capture. If no cat entered within 20 minutes, the pheromone stimuli were replaced, and the traps were moved and set up at a new location. If a cat had entered a trap, both traps were submerged in a 1% bleach solution and were rinsed before being deployed in another replicate. In between bioassay days, the towels covering traps were machine-washed, and the traps were cleaned in an industrial sink using Sparkleen™ (Fisher Scientific, Fair Lane, NJ, USA).

6.3.5. (H2) The pheromone lure or the sound lure on its own mediates cat attraction

Hypothesis 2 was tested in two parallel experiments (Exps. 3, 4) that followed the same general protocol, including the preparation of test stimuli, as described above for experiment 1. The experiments were run at three animal rescue shelters in the Greater Vancouver area between January and March 2020, and July and October 2021.

Experiment 3 (n = 11) tested the effect of pheromone alone on the cats' responses. Cats were offered a choice between two mouse toys that were, or were not (control), pheromone-baited (Table 1). Experiment 4 (n = 12) tested the effect of the sound lure alone on the cats' responses. Cats were offered a choice between two mouse toys that were, or were not (control), fitted with an earbud headphone (connected to an iPhone 6), which emitted the playback sound (Fig. 1; Table 1). To ensure that cats responded to playback sound rather than to electromagnetism associated with the iPhone, a second iPhone was assigned to the control mouse and set to airplane mode, thus generating some electromagnetism. For visual symmetry of the experimental design, both iPhones were hidden under the base of the paired ring stands (Fig. 2b) during bioassay replicates.

6.3.6. Statistical analysis

A chi-squared test was used to analyze the effect of treatment versus control stimuli in each of experiments 1-4.

6.4. Results

6.4.1. (H1) Prey-derived pheromone and sound lures, in combination, attract and help capture cats

Experiment in animal shelters

When shelter cats were offered a choice between (*i*) a treatment mouse toy baited with both the pheromone lure and the sound lure and (*ii*) an odorless and silent control mouse toy (Fig. 2b), 12 cats contacted or closely (< 10 cm) approached first the treatment toy, whereas two cats contacted or closely approached first the control toy (χ^2 = 7.14, *P* = 0.0075; Fig. 4, Exp. 1). These responses by shelter cats indicate recognition of either rodent-derived pheromone and/or sound signals or sound cues.

Experiment in field settings

When feral cats in a field experiment were offered a choice between a treatment trap baited with both the pheromone lure and the sound lure and an unbaited control trap (Fig. 3), 12 cats entered the treatment trap, whereas three cats entered the control trap (χ^2 = 5.4, P = 0.020, Fig. 5, Exp. 2). These responses by feral cats indicate recognition of either rodent-derived pheromone and/or sound signals or cues.

6.4.2. (H2) The pheromone lure or the sound lure on its own mediates cat attraction

When shelter cats were offered a choice between a treatment mouse toy baited with the pheromone lure and an odorless control mouse toy (Fig. 2b), nine cats contacted or closely (< 10 cm) approached first the treatment toy, whereas two cats contacted or closely approached first the control toy (χ^2 = 4.45, *P* = 0.035; Fig. 6, Exp. 3). Conversely, when cats were offered a choice between a treatment mouse toy baited with the sound lure and a silent control mouse toy (Fig. 2b) – with electronic devices under both toys turned on and producing an electromagnetic field – seven cats

contacted or closely approached first the treatment toy, whereas five cats contacted or closely approached first the control toy ($\chi^2 = 0.33$, $P = 0.57$, Fig. 6, Exp. 4). The combined data of experiment 3 and 4 indicate that shelter cats recognized the pheromone signals of mice but did not respond to sound signals or cues of rodent prey.

6.5. Discussion

Our data support the hypotheses that prey-derived pheromone and sound lures, in combination, attract and help capture domestic and feral cats, and that mouse pheromones on their own mediate cat attraction. Conversely, our data do not support the hypothesis that rodent-derived sound, in the absence of prey pheromone, attracts cats. This surprising latter result contrasts conventional assertion that foraging domestic cats rely primarily on their visual and auditory senses to locate and capture prey (Brown and Bradshaw 2012; Mayes et al. 2015).

Our findings that cats are attracted to pheromone signals of mouse prey support the concept that predator-prey interactions, like those between cats and mice (Takahashi 2014; Széles et al. 2018; Rendall et al. 2022) or between rats and mice (Karli 1956; Papes et al. 2010), are informed and guided by mutual eavesdropping, or 'counterespionage,' on specific chemical signals (e.g., (Takahashi 2014; Parsons et al. 2018; Varner et al. 2020). Intercepting scent communication in vertebrate communities was conventionally viewed as a one-way dyadic interaction, with prey sensing predator odor (Banks et al. 2016), and predator odor causing aversion behavior in prey (Pérez-Gómez et al. 2015). Our study reveals that the cats' well-developed vomeronasal organ and accessory olfactory bulb (Salazar and Sánchez-Quinteiro 2011) are capable of sensing mouse pheromones and informing the cats' hunting behavior. This implies that it might have been the pheromone components of mice that prompted cats to more quickly and in greater numbers visit mouse-scented plots than untreated control plots (Hughes et al. 2010). Cats apparently take advantage of the prolific scent marking behavior of male house mice that are driven to advertise their location (Sheehan et al. 2019), social dominance (Novotny et al. 1990), and reproductive fitness (Zala et al. 2004). The degree of their scent-marking behavior, however, seems to be a dangerous trade-off between their need to attract females and to avoid attention of (cat) predators. In the perceived presence of predators, male house mice significantly lower or alter their scent-marking behavior (Roberts 2001; Arakawa et al. 2008). The semiochemical (message bearing

chemical) in the cats' odor that evokes fear and avoidance behavior by mice is a major urinary protein (Papes et al. 2010), homologous to the MUP pheromone component of mice that induces aggressive behavior (Papes et al. 2010; Chamero et al. 2011). Interestingly, the cats' fear-evoking MUP is also present in odor profiles of snakes and rats (Papes et al. 2010; Miessler de Andrade Carvalho et al. 2020) which also prey on mice. Mice, as well, avoid Brown rats by eavesdropping on the rats' volatile sex attractant pheromone components (Varner et al. 2020).

Mice innately recognize and fear cat scent (Pérez-Gómez et al. 2015) but is not yet clear whether cats innately recognize mouse pheromones, or learn to associate mouse pheromones with mouse prey. An animal's experience can modulate the organisation and function of its olfactory system (Rochefort et al. 2002) and cats are able to learn and associate specific prey odor cues with prey (Mayes et al. 2015). The shelter cats that we bioassayed and that responded to mouse pheromones may, or may not, have been mouse-prey experienced. To conclusively show that cats innately recognize mouse pheromones, the response of naïve cats (i.e., not previously exposed to mouse prey) would need to be tested.

The ineffectiveness of the rodent sound lure on its own to attract shelter cats was perplexing. The sound lure containing mouse vocalizations as well as rat feeding sounds (Fig. 1) was expected to be highly appealing to cats. However, the ultrasonic signal components in vocalizations of mice (Holy and Guo 2005; Musolf and Penn 2012; Takács et al., 2021) could not – for technical reasons – be transmitted by the sound speakers, a deficiency which may have rendered the sound lure less appealing. Alternatively, the sound lure in the shelter cat experiment was presented out of context. We envision that cat predation on mice ensues in a sequence of steps. First, cats are attracted by mouse odor or pheromones to an area inhabited or frequented by mice. Cats then use mouse vocalizations or mouse foraging sounds to home in on the micro-location of a prospective prey that may still be obscured by vegetation or litter. Finally, when a mouse becomes visible or produces foraging sound, the cat strikes. Foraging steps one and/or two, of course, may be skipped if a cat fortuitously comes across a mouse. In our shelter cat experiment, the two mouse-look-alike toys were already in full view and the guiding function of the sound may already have been ineffective. The guiding function of prey sound, however, may have been instrumental for capturing feral cats in field settings. Pheromone and sound lures of the live trap triggered classical

hunting behavior by cats. Homing in on treatment traps, cats slowed their approach, hunched down, and directed their gaze and ears. They frequently sniffed around the rear of the live trap where the mouse pheromone lure was located, and they often sat next to, or on top of, that section of the trap, presumably adopting a sit-and-wait hunting tactic. One cat even swatted at the towel covering the treatment trap and another licked its lips. However, to lure cats into traps we relied on our prey sound file emitted from separate speakers positioned at the trap front and back. When a motion detector sensed a cat at the trap entrance, the detector shifted the emission of the sound file from the front speaker to the back speaker, thereby enticing the cat to enter the trap. We have no definitive experimental proof that it was this shift in prey sound emission from the front to the back of the trap which prompted full entry of cats into traps. However, our observations and video recordings of the cats' behavior support the interpretation that the cats simply followed the prey sound when they entered the trap. With bird song lures proven effective in attracting feral cats (Moseby et al. 2004), there is every reason to infer that our rodent sound lure also contributed to the capture of feral cats. Whether the mouse vocalizations, the rat chewing sounds, or both played essential roles for triggering the cats' responses remains unknown.

Our study is the first to report that cats sense and orient toward mouse pheromones. The cats' behavioral responses indicate that they co-opt the sex pheromones of mice as cues to locate mouse prey. Similarly, cats spent more time investigating soiled bedding of brown rats which has a strong odor profile, including greater abundance of ketone pheromone components (Takács et al. 2016a), than soiled bedding with a weaker odor profile (Zhang et al. 2016). Unlike cats, however, brown rats, which also prey on mice (Karli 1956; Papes et al. 2010), do not exploit mouse pheromone as prey location cues (Varner et al. 2020). Rats, as omnivores and only opportunistic predators of mice (Samuels 2009), may not have had sufficient selection pressure to innately learn the communication signals of an infrequent prey as prey location cues. Cats, in turn, as obligatory carnivores (Bradshaw et al. 1996), with mice as a staple food in their diet (Széles et al. 2018; Rendall et al. 2022), greatly benefit from co-opting mouse pheromones as prey location cues.

Our study provides proof of concept that rodent-derived pheromone and sound lures can be designed and developed to expedite captures of domestic and feral cats. As we did not know the relative contribution of prey pheromone and prey sound to the

overall attractiveness of the lure, we designed a bimodal lure complex that offered both prey pheromone and prey sound. Theoretically, this type of bimodal lure should be more effective than any monomodal lure. Sex pheromones of mice contrive long-range attraction of mates (Roberts et al. 2010; Musso et al. 2017) and – as kairomones – would mediate long-range attraction of predators. Low-intensity rodent sound, in contrast, does not travel far and would draw the attention only of a predator that is already nearby. On the other hand, kairomones at high concentrations near the source may cause sensory overload (Suckling 2000) and disorient, rather than guide, a foraging predator. Unlike pheromones, intermittent prey sound, as presented by our sound playback file, avoids sensory habituation and allows a cat predator to pinpoint the microlocation of a prospective prey.

Whereas a bimodal trap lure is likely more effective for attracting and capturing cats than a monomodal pheromone or sound lure, a bimodal lure will also incur higher purchasing costs. These costs, however, could be kept to a minimum by formulating a pheromone lure with only key pheromone components and by presenting a less complex sound file emitted from a piezoelectric transducer (Takács et al. 2016b). Concerns that the pheromone lures would require frequent replacements are not warranted. Insect pheromones formulated for monitoring and control of pest insect population last weeks or even months (Suckling and Karg 2000; Weinzierl et al. 2005) and rodent pheromone lures could be developed with similar efficacy and longevity. Indeed, the very same pheromone lures for attracting cats could also be deployed for expeditious captures of mice in pest management programs (Clapperton et al. 2017; Wyatt 2017).

Our rodent pheromone and sound lures for cat attraction and trap captures appeal to and trigger the cats' strong predation drive. The same or similar prey cues could be developed for enhanced environmental enrichment of pet cats which is an important welfare factor for the circa 94.2 million cats in the US alone (Dutcher et al. 2021). A mouse toy baited with mouse pheromones would appeal to the predatory instinct of cats and enable them to express hunting-like activities (Herron and Buffington 2010). For free-ranging cats, 'pseudo' hunting or handling of pheromone-scented mouse toys would satiate the cats' hunting instinct and curtail their drive to hunt wild mice or wildlife (Cecchetti et al. 2021).

In conclusion, we have shown that rodent-derived pheromone and sound lures, in combination, are effective in luring feral cats into traps. Reducing the complexity of the pheromone blend and delivering a simplified sound file through a piezoelectric device would save manufacturing and purchasing expenses. Coupling trap lure technology with a cat-capture alert app (Chad et al. 2010) would make trapping of feral cats time- and cost-efficient. Applying the rodent pheromone lures not only in TNR programs for feral cat management, but also for rodent pest control and as enhanced environmental enrichment for pet cats should provide ample incentive for commercial lure production.

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6.8. Figures

Table 6.1. List of mouse pheromone and rodent sound stimuli assigned to mouse-look-alike toys (Fig. 2) and tested in animal shelter and field experiments with shelter and feral cats, *Felis catus*

Exp. # (Location)	Stimulus 1	Stimulus 2
Hypothesis 1: Prey-derived pheromone and sound, in combination, attract cats		
1 (Shelter)	Mouse pheromone lure: - Volatile pheromone blend[1,2] in MO (100 µL) on cotton ball - Testosterone (750 ng)[3,4] in ether (50 µL) on filter paper - Progesterone (250 ng) and estradiol (125 ng)[3,4] in ether (50 µL) on filter paper Rodent sound with EMF	Corresponding solvent control: - MO (100 µL) - Ether on filter paper (50 µL) - Ether on filter paper (50 µL) Silence without EMF
2 (Field)	Mouse pheromone lure: - Volatile pheromone blend[1,2] in MO (500 µL) on cotton wick in glass vial - Testosterone (750 ng)[3,4] in ether (50 µL) on filter paper - Progesterone (250 ng) and estradiol (125 ng)[3,4] in ether (50 µL) on filter paper Rodent sound with EMF	Corresponding solvent control: - MO (500 µL) on cotton wick in glass vial - Ether on filter paper (50 µL) - Ether on filter paper (50 µL) Silence without EMF
Hypothesis 2: The pheromone lure or the sound lure on its own mediates cat attraction		
3 (Shelter)	Mouse pheromone lure: - Volatile mouse pheromones[1,2] in MO (100 µL) on cotton ball - Testosterone (750 ng)[3,4] in ether (50 µL) on filter paper - Progesterone (250 ng) and estradiol (125 ng)[3,4] in ether (50 µL) on filter paper	Corresponding solvent control: - MO (100 µL) - Ether on filter paper (50 µL) - Ether on filter paper (50 µL)
4 (Shelter)	Rodent sound with EMF	Silence with EMF

[1]Volatile pheromone lure: stock solution contained 30 µL of solution #1 [butyric acid (140 mg), 2-methyl butyric acid (14 mg), and 4-heptanone (1 mg) in mineral oil (MO, 10 mL)], 2 mL of solution #2 [3-methyl-2-pentanone (0.03 mg), 1-hexanol (0.03 mg), and 2,3,5-trithiahexane (5 mg) in MO (10 mL)], and 8 mL of solution #3 [brevicomin (7-*exo*-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]-3-octene) and thiazole (2-*sec*-butyl-4,5-dihydrothiazole) (1 mg each) in MO (10 mL)]; [2]The volatile pheromone components of the blend (all except the sex steroids testosterone, progesterone and estradiol) were formulated to match the headspace volatile blend emanating from urine/feces excreta of one male and one female house mouse, respectively, on average over the course of 24 h (Varner et al. 2018, 2022); [3]amounts of chemicals applied on filter paper were equivalent to those emanating from soiled bedding of one male mouse over the course of 24 h; [4]test chemicals were dissolved in either (50 µL), with the same volume of ether applied to control filter paper.

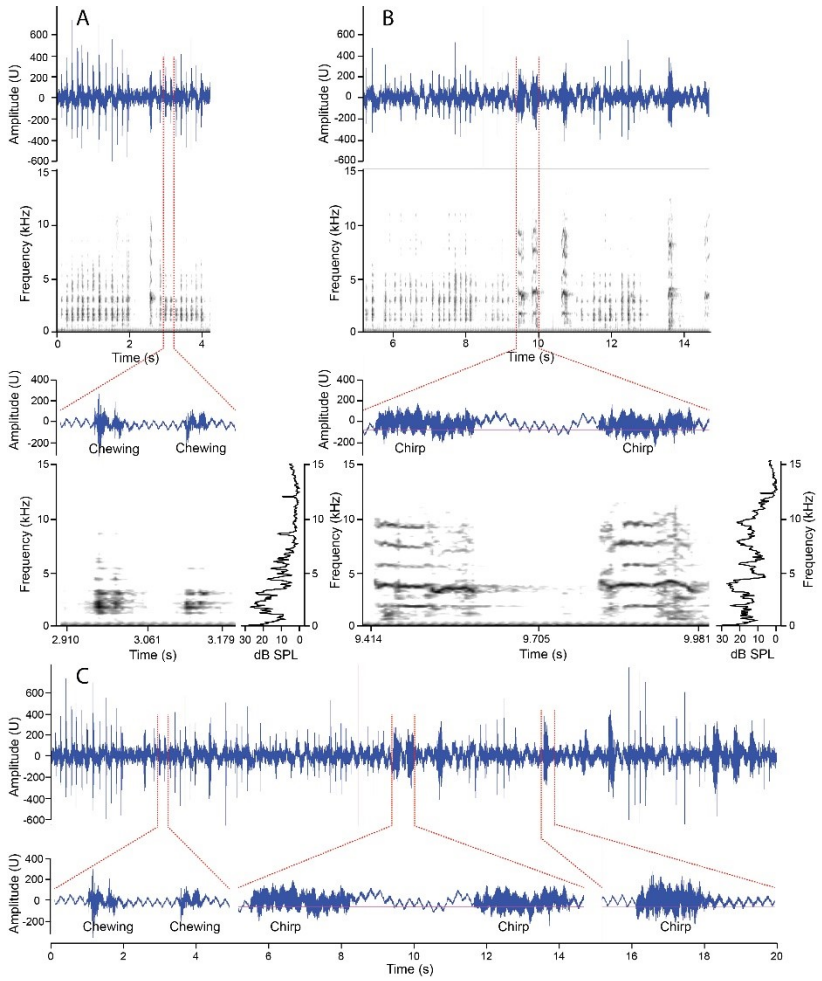

Figure 6.1. **Excerpts of a 4-min playback sound file, comprising vocalizations (chirps) of a male-female house mouse, *Mus musculus*, pair, and chewing sounds of a Brown rat, *Rattus norvegicus*, eating dry pasta. (A) top: 4-s excerpt of the waveform (amplitude; time domain) and sonogram (time-frequency domain) related to the grinding sounds of chewing ('chewing'); bottom: magnified 200-ms interval revealing details of the waveform, sonogram and power spectrum (frequency domain) of two adjacent chewing events. (B) top: 8-s excerpt of the waveform (amplitude; time domain) and sonogram (power spectrum: time-frequency domain) related to mouse chirps ('chirp'); bottom: magnified 500-ms interval revealing details of waveform, sonogram and power spectrum (frequency domain) of two adjacent chirp events. (C) top: 20-s excerpt (combined waveform recordings) of the 4-min playback sound file which was looped (automatically rerun) every 4 min during bioassays; bottom: magnified examples of chewing and chirping sounds. All amplitude deviations from the 60-Hz constant background (silence) are mouse vocalization or rat chewing sounds.**

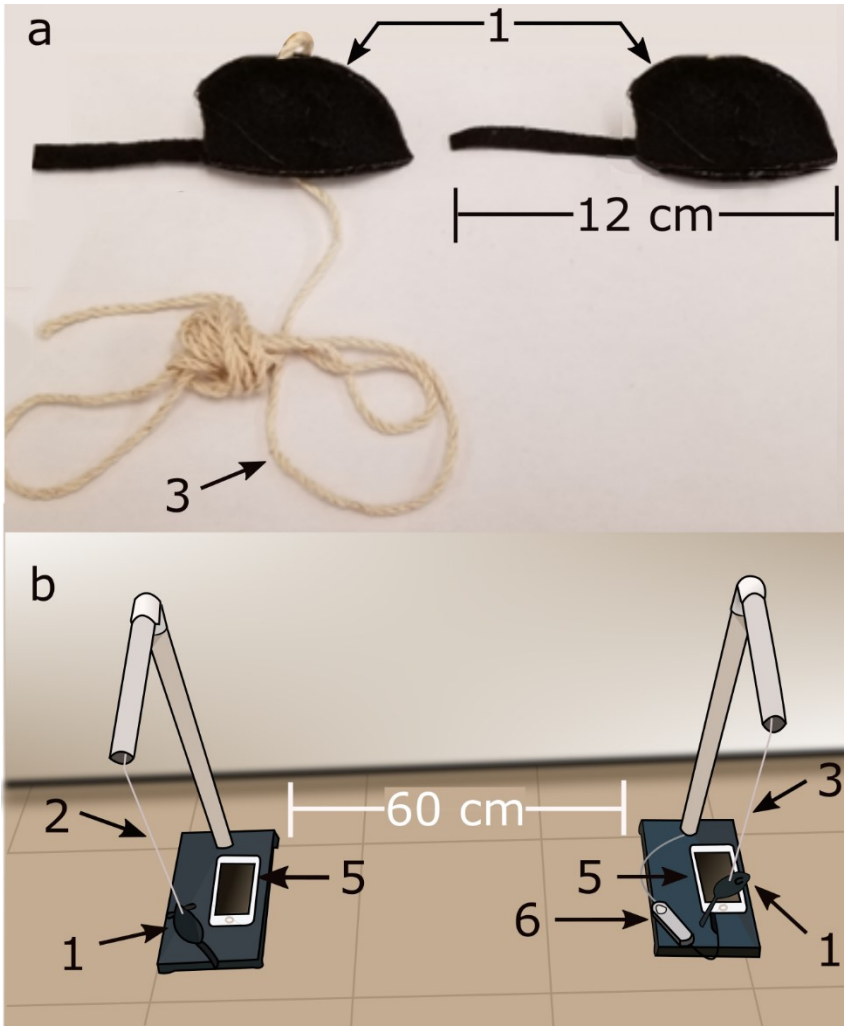

Figure 6.2. **Graphical illustrations of (a) mouse-look-alike 'toys' (1; 12 cm long, 3 cm wide, 4.8 cm tall); and (b) the design of two-choice experiments for testing behavioral responses of cats, *Felis catus*, to treatment or control mouse toys. Experiment-dependent, the toys were suspended via earbud headphone cable (2) or string (3) (each 93-cm long) from stands (4; vertical stem: 60 cm high; cross bar: 30 cm long) placed 60 cm apart from each other. The earbuds were nestled invisibly within the treatment toy and was connected to an iPhone (5) that emitted the playback sound file (see Fig. 1c), with the audio quality enhanced by an amplifier (6). The control stimulus (in experiment 4) also included an iPhone which was turned on but did not play any sound file. The iPhones and amplifier are shown here for illustrative purposes only but were concealed during bioassays. The pheromone lures (Table 1) in the treatment toy are also not shown.**

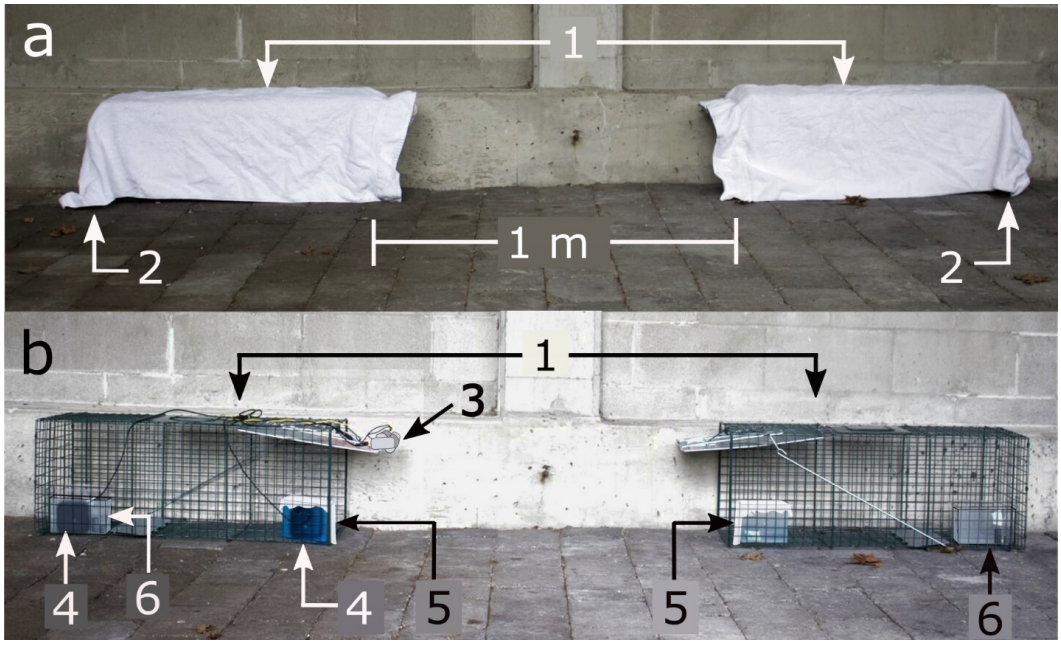

Figure 6.3. **Photographs illustrating the field experimental design for testing the entry of feral cats, *Felis catus*, into live traps (1) baited with rodent pheromone lures (Table 1) and a sound lure (Fig. 1c), or left unbaited. Traps were spaced 1 m apart with their entrances facing each other and were covered with white cloth (2). The randomly assigned treatment trap was fitted with a motion sensor (3) connected to a bluetooth speaker (4) both at the front/entrance and rear of the trap. The speaker at the front resided in a fabric-covered open box (5) which – for visual symmetry – was also present in the control trap. The speaker in the rear of the trap was placed in a top-open box (6) that also contained the pheromone lures. The same box (but with unscented mineral oil and ether rather than pheromones lures) was present in the control trap. When the motion sensor detected a cat's presence at the trap entrance, it shifted emission of the sound playback file (Fig. 1c) from the speaker at the front to the speaker at the rear of the trap, thereby enticing the cat to enter the trap.**

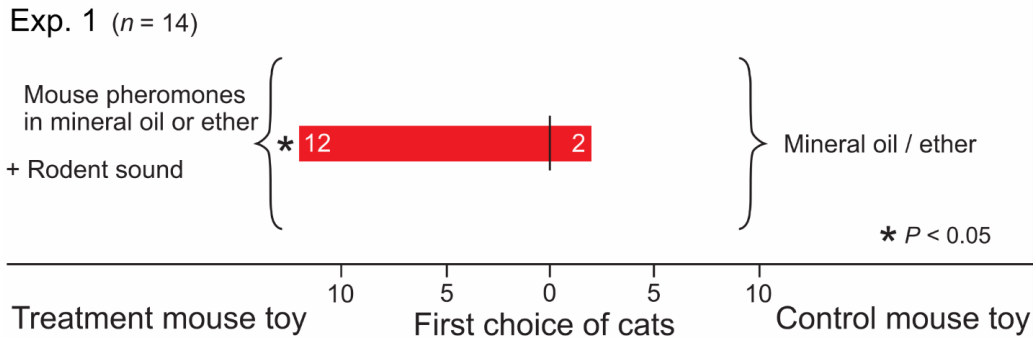

Figure 6.4. **Responses by domestic cats, *Felis catus,* in animal shelters when offered a choice (Fig. 2b) between (*i*) a treatment mouse toy baited with mouse pheromones lures (see Table 1) and a rodent sound lure [playback sound file (Fig. 1c) emitted from an earbud speaker] and (*ii*) a control mouse toy left unbaited. Significantly more cats contacted or closely (< 10 cm) approached first the treatment toy than the control toy. The asterisk (*) indicates a significant preference for the treatment mouse toy (χ^2-test; $P < 0.05$).**

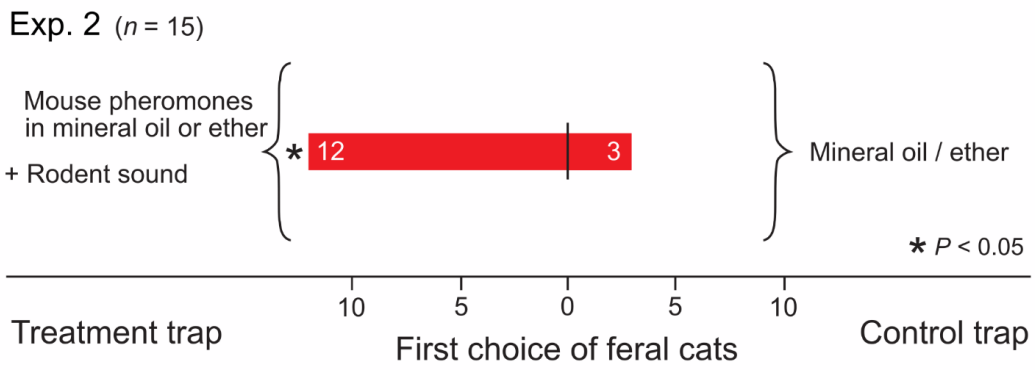

Figure 6.5. **Entry by feral cats, *Felis catus,* into live traps (Fig. 3) placed at livestock farms with active feral cat populations and a feral cat rescue sanctuary in the lower mainland of British Columbia, Canada, between May and October 2019. Treatment traps were baited with mouse pheromone lures and a rodent sound lure [playback sound file (Fig. 1c) emitted from an earbud speaker] (Table 1), whereas control traps were left unbaited. Significantly more cats entered treatment than control traps. The asterisk (*) indicates a significant preference for the treatment trap (χ^2-test; $P < 0.05$).**

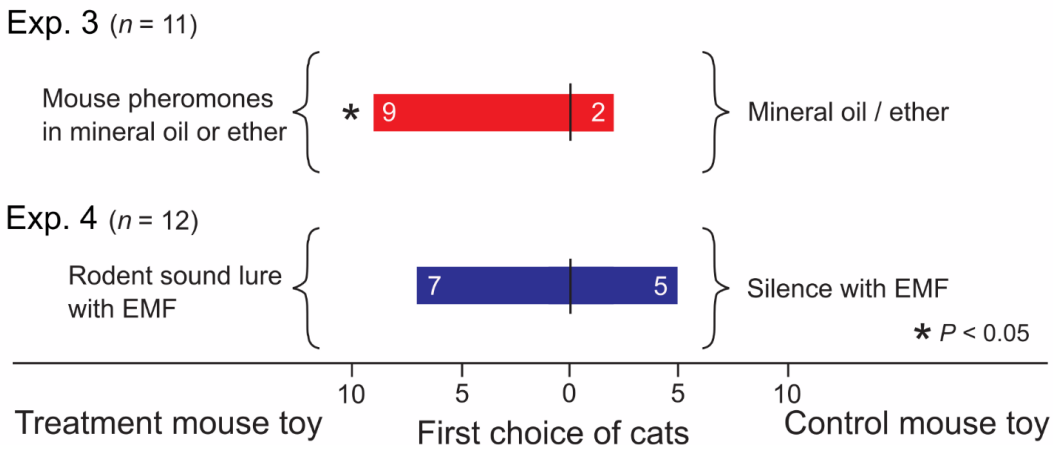

Figure 6.6. **Responses by domestic cats, *Felis catus*, when offered in parallel animal-shelter experiments 3 and 4 a choice (Fig. 2b) between (*i*) a treatment mouse toy baited with pheromone lures and an unbaited control mouse toy (Exp. 3) (Table 1), and (*ii*) a treatment mouse toy baited with a rodent sound lure [playback sound file (Fig. 1c) emitted from an earbud speaker] and a silent control mouse toy (Exp. 4) (Table 1). Significantly more cats contacted or closely (< 10 cm) approached first the treatment toy than the control toy in experiment 3 (χ^2-test; $P < 0.05$), but not in experiment 4 (χ^2-test; $P > 0.05$). Note: In experiment 4, iPhones (producing a weak electromagnetic field) were hidden under the base of each ring stand (Fig. 2) during bioassay replicates.

Chapter 7.

Rodent odor bait – a new bumble bee conservation tool to enhance nest box occupancy

A very similar version of this chapter has been submitted to 'Insect Conservation and Diversity' for review, with the following authors: Elana Varner, Kayla Mark, Hanna Jackson, Kendal Singleton, Laura Luo, Sarah Johnson, Regine Gries, Gerhard Gries

7.1. Abstract

Bumble bee conservation focuses on supplementing floral resources. Yet, nesting site availability is linked to bumble bee abundance. Artificial nest boxes have low occupancy (10%) and thus are impractical for conservation use. As queen bumble bees reportedly establish colonies in abandoned rodent burrows, we hypothesized (1) that queen bumble bees sense, and behaviorally respond to, rodent odor, and (2) that lures of synthetic rodent odor can guide spring queens to nest boxes.

We collected headspace odorants from bedding soiled with urine and feces of house mice, *Mus musculus*, and identified the 10 odorants that elicited responses from queen bumble bee antennae. To field-test attraction of queens to mouse excreta odorants, we tree-mounted paired nest boxes in florally rich locations, and assigned clean and soiled bedding, respectively, to one box in each pair. Queens established colonies in 17 mouse-scented boxes and in six unscented boxes. This 43% occupancy rate of mouse-scented boxes represents a significant improvement over the 10% occupancy rate common for unscented boxes. In a further field experiment, we baited one box in each pair with a *synthetic* mouse odor lure and found that queens established colonies in 13 baited boxes and in six unbaited control boxes. Specifically, *Bombus mixtus* established seven colonies in baited boxes and only one colony in an unbaited box.

With this proof-of-concept that synthetic lures can guide queens to nest boxes, we anticipate that bumble bee conservation programs will soon be able to offer both expanded floral resources and baited nest boxes readily detectable by queens.

7.2. Introduction

Globally, populations of many bumble bees, *Bombus* spp., are declining (Cameron *et al.*, 2011), in part due to habitat loss from agricultural intensification (Williams & Osborne, 2009). Current bumble bee conservation efforts focus on supplementing floral resources but often neglect providing nest sites (Lye *et al.*, 2011). Widely available nest sites, however, are a key ecological requisite for bumble bees (Tscharntke *et al.*, 1998) because of their rather narrow foraging range (Lye *et al.*, 2009). As scarceness of nest sites results in small population sizes (McFrederick & LeBuhn, 2006), offering artificial nest boxes is an obvious conservation goal. However, nest boxes tend to have low occupancy (Fussell & Corbet, 1992; Kells & Goulson 2003; Lye *et al.*, 2011) even in the absence of natural nest sites (McFrederick & LeBuhn, 2006), thus rendering nest boxes impractical for bumble bee conservation and scientific studies of bumble bee nest-site preferences (Svensson & Lundberg, 1977; Richards, 1978; Fussell & Corbet, 1992). Inferring nest-site preferences by watching nest site-seeking queens (Kells & Goulson, 2003; Lye *et al.*, 2009; O'Connor *et al.*, 2017) is time- and work-intensive and limits the amount of data that can be collected.

Both the design of nest boxes and the provisioning of insulation material address the bumble bees' need for nesting in a dry and well-insulated cavity (Donovan & Wier, 1978) but the cues queen bumble bees exploit to locate such nest sites are poorly understood (Barron *et al.*, 2000).

Despite the pervasive presumption that bumble bees nest in abandoned rodent burrows (Svensson & Lundberg, 1977; Fussell & Corbet, 1992; Colla *et al.*, 2014), it remains unknown or inconclusive whether queens selectively seek rodent burrows as nest sites (Sladen, 1912; Hobbs *et al.*, 1960; Barron *et al.*, 2000). In an observational study, bumble bees favored nest sites with grasses from 'field mice' (taxonomic name not provided) (Frison, 1918), and in a controlled experiment bumble bees selected burrows with experimentally induced previous occupancy by mice (Fye & Medler, 1954). However, the results obtained by Fye & Medler (1954) could not be substantiated in a repetition of this experiment (Hobbs *et al.*, 1960).

Bumble bees have an exquisite sense of smell (Sprayberry, 2018), and semiochemicals (message bearing chemicals) play an important role in their life history,

mediating – among others – nest entrance marking (Foster & Gamboa, 2010), nestmate recognition (Gamboa *et al.*, 1987), and floral foraging (Leonard *et al.*, 2010). It is entirely conceivable that rodent-derived semiochemicals, such as urine, feces and fur odorants emanating from burrows, guide Spring queens to rodent burrows. Nest site-searching queens exhibit a characteristic zig-zag flight with an overall forward motion (Kells & Goulson, 2003), reminiscent of odor-tracking behavior in many animal taxa (Svensson *et al.*, 2014). This flight pattern suggests that queens exploit rodent semiochemicals to locate the often visually obscured entrance holes of rodent burrows.

But even if we were to experimentally demonstrate that queen bumble bees exploit rodent semiochemicals for locating nest sites, such a finding would contribute little to bumble bee conservation. To optimize the likelihood that queens detect, and adopt, artificial nest boxes for starting their colony, these nest boxes would need to be baited with synthetic lures of rodent semiochemicals. Yet, research in chemical ecology for conservation purposes is lagging. While synthetic semiochemicals are widely used to control pest insects, very few semiochemicals have been developed for biodiversity and conservation studies such as the application of pheromones to detect the presence of endangered species and to delineate their distribution range (Larsson, 2016). As our study offered a unique opportunity to apply chemical ecology research for the purpose of bumble bee conservation, we decided to not only test whether queen bumble bees exploit rodent semiochemicals for nest site location but – if so shown – to also identify these semiochemicals for nest box lure development.

Here we tested two hypotheses: (H1) queen bumble bees searching for nest sites in spring antennally sense, and behaviorally respond to, natural rodent odor, and (H2) lures of synthetic rodent odor can be used to guide queen bumble bees to nest boxes. As the source of rodent odor, we selected house mice, *Mus musculus*, because (*i*) house mice are prevalent in urban environments (Pocock *et al.*, 2005) where bumble bees thrive (Samuelson *et al.*, 2018), and (*ii*) many house mouse odorants (sex attractant pheromone components) have already been identified and field-tested (Novotny *et al.*, 1985; Musso *et al.,* 2017; Takács *et al.*, 2017; Varner *et al.*, 2019, 2020, unpublished), thus expediting the development of a synthetic lure as a bumble bee guide to nest boxes.

7.3. Materials and methods

7.3.1. Experimental house mice as odor sources

As odor sources, CD-1® female and male house mice between 2- to 3-month-old were purchased from Charles River Laboratories International Inc. (Saint-Constant, QC, CA). Upon arrival, females were housed in cages (45 × 23 × 15 cm) in groups of five, and males were housed singly in cages (20 × 37 × 14 cm), all cages lined with corncob bedding (Anderson's Bed o'cobs, The Andersons Inc., Maumee, OH, USA) and fitted with a Nalgene dome (Jaimesons Pet Food Distributers, Richmond, BC, CA). Rodent food (LabDiet® Certified Rodent Diet, LabDiet, St. Louis, MO, USA) and water were provided *ad libitum*. Staff of Animal Care Services at Simon Fraser University (SFU) provided care for the mice in accordance with the Canadian Council on Animal Care guidelines and experimental protocols approved by SFU's Animal Care Committee (protocol #1159B-15-21).

7.3.2. (H1) Queen bumble bees sense, and behaviorally respond to, natural rodent odor

Acquisition of headspace odorants emanating from soiled mouse bedding

The procedure was previously detailed (Varner *et al*., 2019) and is only outlined here. Briefly, corncob bedding (100 g per mouse) soiled over the course of three days with urine, feces, shed fur and skin cells of singly-housed males, or of five group-housed females, was collected and the material from several cages combined and mixed. One-hundred gram aliquots of male- or of female-soiled bedding was then placed into separate Pyrex glass chambers (30 × 15 cm), each connected to a Pyrex glass tube (15 cm × 15 mm OD) filled with Porapak Q (200 mg) to trap headspace odorants. After drawing charcoal-filtered air through each chamber and the Porapak Q odorant trap at 1 L min^{-1} for 24 h, Porapak Q-trapped odorants were desorbed in sequence with pentane and ether (2 mL each), dodecyl acetate was added as an internal standard to extracts, and each extract was concentrated to 250 µL. Clean bedding (100 g; control) was subjected to the same procedure.

Gas chromatographic-electroantennographic detection (GC-EAD) analyses of mouse odorants

To test whether queen bumble bees can sense mouse odorants, we captured a wild queen of *Bombus vosnesenskii* and obtained a queen of *B. impatiens* from a commercially supplied colony. We also captured and tested wild bumble bee workers of *B. vosnesenskii*, *B. mixtus*, and *B. flavifrons*. We analyzed 2-µL aliquots of Porapak Q extracts (described above) by GC-EAD and GC-mass spectrometry (MS), with procedures and equipment previously detailed (Gries *et al*., 2002). Briefly, the GC-EAD set-up employed a Hewlett-Packard 5890 gas chromatograph (GC) fitted with a DB-5 GC column (30 m × 0.32 mm I.D., film thickness 0.25 µm; J & W Scientific, Folsom, CA, USA). Helium served as the carrier gas (35 cm · s^{-1}) with the following temperature program: 40 °C for 1 min, increasing 10 °C · min^{-1} to 280 °C. The injector port and flame ionization detector (FID) were set at 260 °C. For each GC-EAD recordings (n = 9), we carefully dislodged an antenna from the head of a queen or worker bumble bee and suspended it between two glass capillary electrodes (1.0 × 0.58 × 100 mm; A-M Systems, Carlsborg, WA, USA) adapted to accommodate a bumble bee antenna and filled with a saline solution.

Analysis of soiled bedding headspace odorants by GC-MS

Odorants in the headspace of soiled mouse bedding that consistently elicited responses from queen and worker bumble bee antennae were analyzed on a Varian Saturn 2000 Ion Trap GC-MS operated in full-scan electron impact mode and fitted with a DB-5 MS column (30 m × 0.25 mm I.D., film thickness 0.25 µm), with helium as carrier gas (35 cm · s^{-1}). The injector port and ion trap were set at 250 °C and 200 °C, respectively, and the column oven program was as follows: 50 °C for 5 min, then 10 °C · min^{-1} to 280 °C, held for 10 min. To identify antennally-active odorants (see Results), we compared their retention indices (relative to aliphatic alkanes; Van den Dool & Kratz, 1963) and mass spectra with those of authentic standards.

Purchase and syntheses of soiled bedding headspace odorants

Authentic standards were obtained from various sources: 2-heptanone, 4-heptanone, 1-hexanol, 1-octen-3-ol and acetophenone: Sigma-Aldrich, St. Louis, MO, USA; 2,3,5-trithiahexane: DeLong Chemicals America, New Haven, CT, USA; benzaldehyde: Fisher Scientific, Hampton, NH, USA; 2-*sec*-butyl-4,5-dihydrothiazole

and 3,4-dehydro-*exo*-brevicomin: synthesized in Gries-lab applying published procedures (Musso *et al.*, 2017 and references cited herein); 3-octanone: oxidized from 3-octanol (Sigma-Aldrich) in Gries-lab.

Nest box occupancy field experiment 1

Replicates of the field experiment were initiated during 25–29 March 2019. Each replicate (n = 46) consisted of paired nest boxes mounted to trees (Fig. 1a) in six florally rich locations across the Lower Mainland of British Columbia (BC), Canada. Most replicates were set up in botanical gardens and parks which generally have a high nest site density of bumble bees (Osborne *et al.*, 2008; Lye *et al.*, 2011). Boxes were mounted via packaging straps (STRAP~PAC ®, PAC Strapping Products Inc., Exton, PA, USA), with 0.4-m spacing between entrance holes within pairs and > 2 m between pairs. Each box (20 × 18 × 14 cm; Hobbs *et al*., 1960) was built from 2-cm thick spruce plywood (Home Depot, Burnaby, BC, CA) and fitted with unbleached cotton (30 g) as nesting material. A 2-cm hole in the front panel of the box served as an entrance, and a plastic sheet (Bennett 6-mm Heavy Weight Plastic Drop Sheet Roll, Home Depot, Burnaby, BC, CA) cut to size (30 cm^2) and stapled to the top of the box provided protection from rain. Each box received a cheese cloth bag (VWR International, Randor, PA, USA) which contained 200 g of corncob bedding which was kept clean (control box) or was previously soiled by mice (treatment box). Soiled bedding contained the metabolic waste produced by one male and one female mouse over the course of three days (see above).

Each week, nest box pairs were monitored for 5 min to assess bumble bee occupancy. Nine weeks after initiating experimental replicates, one worker from each occupied box was collected while exiting the box and identified to species in the laboratory. At the end of the season, during 8–16 July, all boxes were dismounted and opened for final recordings of occupancy, colony size, number of brood cells, and evidence for parasitism.

7.3.3. (H2) Lures of synthetic rodent odor can be used to guide queen bumble bees to nest sites

Design of nest box occupancy field experiment 2

The design of field experiment 2 largely followed the design of experiment 1 (see above), with some modifications. During 22–26 March 2021, paired nest boxes (Fig. 1 a) were mounted on trees in eight florally rich locations across the Lower Mainland of BC. By random assignment, the treatment box in each pair (n = 97) was baited with a synthetic mouse odor lure (see below) and the control box was left unbaited. A 44-mm hole in the bottom panel of each box accommodated an inverted plastic vial (89 × 42.9 mm; Uline, Lacey, WA, USA) with a flip-top lid to facilitate lure replacement, and with a perforated bottom to enable odor release into the box (Fig. 1 c). To ensure the sustained release of synthetic mouse odor, every other week for a period of eight weeks the lid was opened from the outside without disturbing potential bee occupants in the box, and the old lure was replaced with a new one.

Each week, the boxes were monitored for signs of bumble bee occupancy, such as queen or worker bees entering or exiting a box though the entrance hole. Seven weeks after mounting nest boxes, each box was opened and one worker from every occupied box was removed for definitive species identification. At the end of the field season, between 10–16 August, all boxes were dismounted and opened for final recordings of occupancy, colony size, number of brood cells, and evidence for parasitism.

Constituents of the synthetic mouse odor lure

Select components of the 14-component synthetic mouse odor blend were assigned to one of five separate lures: (1) known sex attractant pheromone components of male mice (Novotny *et al*., 1985); (2) candidate sex attractant pheromone components of male mice (Varner *et al*., unpublished); (3) sex attractant pheromone components of female mice (Varner *et al.*, 2019); (4) non-pheromonal urine/feces odorants of mice which consistently elicited antennal responses from bumble bee queens and workers; and (5) ammonium and CO_2. The amounts and ratios of lure odorants were formulated and carefully adjusted until they generated a headspace odorant blend *equivalent* to that emanating from previously soiled bedding (see above) of one male or female mouse

over the course of 24 h. The release rate of ammonium approximated that from urine/feces deposits of one male and one female house mouse over 24 h (Washington & Payton, 2016).

The lure of known male sex attractant pheromone components consisted of brevicomin (1 mg) and thiazole (1 mg) formulated in mineral oil (10 mL) and released from a 20-mL glass scintillation vial (Fig. 1 c). The lure of candidate male sex attractant pheromone components consisted of 3-methyl-2-pentanone (0.3 mg), 1-hexanol (0.3 mg), and 2,3,5-trithiahexane (50 mg) formulated in mineral oil (100 mL) of which 700 µL were pipetted into a 0.5-dram glass vial. The lure of known female sex attractant pheromone components consisted of butyric acid (14 mg), 2-methylbutyric acid (1.4 mg), and 4-heptanone (0.1 mg) formulated in mineral oil (1 mL) of which 200 µL were pipetted into a 400-µL polyethylene microcentrifuge tube (Evergreen Scientific, Rancho Dominguez, CA 90220, USA) with a pierced (1.5 mm) lid. The lure of non-pheromonal mice odorants mice consisted of 2-heptanone (300 mg), 3-octanone (20 mg), benzaldehyde (40 mg) and acetophenone (10 mg) formulated in 100 mL of mineral oil, of which 140 µL were pipetted into a 400-µL polyethylene microcentrifuge tube with a pierced (1.5 mm) lid. Ammonium and CO_2 originated from ammonium bicarbonate (25 g) which was dissolved in water (1 L), of which 2 mL were placed in a 4-mL vial. Release rates of ammonium were measured with a MultiRAE PGM-6228 (Pine Environmental, Burnaby, BC, CA). The control nest box in each pair was fitted with the same glassware containing the same volume of mineral oil and water.

7.3.4. Statistical analyses

Data of bumble bee nest box occupancy in synthetic mouse odor-scented (treatment) and unscented (control) boxes were analyzed by a χ^2 goodness of fit test [R version 3.5.0 (R Core Team, 2019)] with Yate's correction.

7.4. Results

7.4.1. (H1) Queen bumble bees antennally sense, and behaviorally respond to, natural rodent odor

GC-EAD analyses of natural house mouse odor

In GC-EAD analyses, 10 headspace odorants of soiled mouse bedding consistently elicited responses from the antennae of queen *Bombus vosnesenskii* (Fig. 2) and queen *B. impatiens*, and the antennae of worker *B. vosnesenskii*, *B. mixtus*, and *B. flavifrons*. GC-MS analyses of these antennally-active odorants indicated, and GC-MS analyses of authentic standards confirmed, that they were 1-hexanol (**1**), 4-heptanone (**2**), 2-heptanone (**3**), benzaldehyde (**4**), 1-octen-3-ol (**5**), 3-octanone (**6**), 3,4-dehydro-*exo*-brevicomin (**7**), acetophenone (**8**), 2-*sec*-butyl-4,5-dihydrothiazole (**9**), and 2,3,5-trithiahexane (**10**). Five of these compounds (**1**, **2**, **7**, **9**, **10**) are sex attractant pheromone components of male or female house mice (Novotny *et al*., 1985; Varner *et al*., 2019, unpublished). All these odorants were absent in the extract of clean bedding, except for acetophenone which was present at a 7-fold lower amount.

Nest box occupancy field experiment 1

Four out of the 46 tree-mounted nest box pairs were tampered with by curious garden visitors and were excluded from data analyses. In 23 of the remaining 42 nest box pairs, one box in each pair was colonized. In these pairs, bumble bees established colonies in 17 mouse-scented (treatment) boxes and in six unscented (control) boxes, resulting in a significant difference in nest box occupancy ($\chi^2 = 4.35$, $P = 0.037$, Fig. 3). In one additional nest box pair, both the treatment and the control box were colonized by bumble bees (*B. sitkensis; B. flavifrons*). This ’saturated’ nest box pair was excluded from statistical analyses because it could not be ascertained whether the treatment or the control box was colonized first.

As many as 18 (43%) of all mouse-scented nest boxes were occupied, whereas only seven (17%) of all unscented boxes were occupied, reflecting a significant difference in occupancy rate ($\chi^2 = 4.0$, $P = 0.045$).

The propensity of bumble bees to colonize mouse-scented rather than unscented nest boxes was evident for three out of the four species represented in our study by at

least three colonies. Eight of nine *B. mixtus,* three of four *B. sitkensis,* and three of three *B. melanopygus* established colonies in mouse-scented boxes. The single colony of *B. flavifrons* was in an unscented control box (Fig. 3). The 8-fold preference of *B. mixtus* for mouse-scented boxes was statistically significant (χ^2= 7.11, *P* = 0.008), but sample sizes for the remaining species were too small to warrant statistical analyses.

As it became evident in July, all colonies (n = 25) in both treatment and control boxes were parasitized by larvae of the bumble bee wax moth, *Aphomia sociella,* that had destroyed wax cells for storage of nectar and pollen and for housing of bumble bee larvae. Because of this parasitism, we could not count the number of brood cells and measure colony size.

7.4.2. (H2) Lures of synthetic rodent odor can be used to guide queen bumble bees to nest sites

Nest box occupancy field experiment 2

Sixteen out of 97 nest box pairs were tampered with by park and garden visitors and thus needed to be excluded from analyses. In 19 of the remaining 81 nest box pairs, one of the two boxes was colonized. In these pairs, bumble bees established colonies in 13 baited boxes and in six unbaited control boxes (χ^2 = 2.58, *P* = 0.108, Fig. 4). Among the four species colonizing boxes, *B. mixtus* favored baited to unbaited boxes (7:1; χ^2 = 4.5, *P* = 0.034, Fig. 4), whereas the remaining three species did not show a preference. *Bombus sitkensis* established colonies in each of three baited and three unbaited boxes, *B. melanopygus* established colonies in each of two baited and two unbaited boxes, and *B. flavifrons* established one colony in a baited box. The sample size of these three species was too small to warrant statistical data analyses.

As it became evident in early June, all occupied nest boxes (n = 19) were parasitized by larvae of the bumble bee wax moth, *Aphomia sociella,* much earlier in the season than anticipated. Because of this parasitism, we could neither count the number of brood cells nor measure colony size.

7.5. Discussion

The reproductive success of a Spring queen bumble bee emerging from overwintering hinges on her ability to locate a suitable nest site, such as a dry and well-insulated cavity (Donovan & Wier, 1978). Abandoned rodent burrows seem to meet essential nest site requirements because many queens establish their colonies in burrows of rodents or shrews such as the wood mouse, *Apodemus sylvaticus,* common shrew, *Sorex araneus*, bank vole, *Clethrionomys glareolus*, and field vole, *Microtus arvalis* (Sladen, 1912; Fussell & Corbet, 1992; Lye *et al.*, 2012). How queens locate these burrows is not well understood but there are at least two complementary observations suggesting that rodent odorants emanating from burrows guide queens.

Nest site-searching queens exhibit a characteristic zig-zag flight pattern with an overall forward motion (Kells & Goulson, 2003) which is reminiscent of odor-tracking behavior in many animal taxa (Svensson *et al.*, 2014). This flight pattern suggests that queens exploit rodent odorants to locate the often visually obscured entrance holes of rodent burrows. Direct evidence that rodent odorants guide Spring queens to rodent burrows was presented in an experimental study showing that burrows had higher bumble bee occupancy when they were recently occupied by mice (Fye & Medler, 1954) but these results could not be repeated (Hobbs *et al.*, 1960).

To test the hypothesis that Spring queens indeed antennally sense, and behaviorally respond to rodent odorants, we conducted both laboratory and field studies. As odorants from wild mice are difficult to acquire for testing in electrophysiological and behavioral experiments, we relied on laboratory-strain mice on the assumption that their odorants closely resemble those of their wild counterparts. For an odorant source, we used bedding material soiled by laboratory-kept house mice, drawing on our experience that soiled bedding emanates a plethora of mouse urine and feces odorants, including sex pheromone components (Varner *et al.*, 2019; unpublished). Analyzing headspace odorants of soiled bedding by GC-EAD revealed as many as 10 odorants that elicited responses from bumble bee queen and worker antennae (Fig. 2). Remarkably, four of these odorants (1-hexanol, 3,4-dehydro-*exo*-brevicomin, 2-*sec*-butyl-4,5-dihydrothiazole, 2,3,5-trithiahexane) are sex pheromone components of male mice (Novotny *et al.*, 1985; Varner *et al.*, unpublished) and one odorant (4-heptanone) is a sex pheromone component of female mice (Varner *et al.*, 2019). That bumble bee antennae

accommodate olfactory receptors tuned to rather unique rodent pheromone components implies a functional role of these odorants in the context of locating rodent burrows as nest sites. After all, sensory receptors are costly to maintain (Niven & Laughlin, 2008) and the physical space taken up by rodent pheromone receptors could otherwise be occupied by receptors facilitating floral foraging (Leonard *et al*., 2010) or social communication between nest mates (Gamboa *et al.*, 1987). Experimental evidence that the queens' ability to sense rodent odorants improves their success in finding rodent burrows could be demonstrated conclusively only in field experiments. In the first experiment, we substituted rodent burrows with nest boxes, and presented natural rodent scent in the form of bedding soiled by laboratory-kept mice. In each of 46 replicates, we offered Spring queens a choice between a 'mouse-scented' box and an unscented control box, monitoring nest site selection and colony establishment throughout the season. The 3-fold higher occupancy rate of 'mouse-scented' nest boxes (Fig. 3) provides convincing evidence that rodent odorants serve as a 'road map' to rodent burrow-seeking queens. Preferential selection of mouse-scented boxes by queen *B. mixtus*, *B. melanopygus* and *B. sitkensis* (Fig. 3) further indicates that rodent odorants are a universal nest site location cue. All but one of the species present in our field sites more readily located, or preferentially chose, the mouse-scented boxes. That the single *B. flavifrons* colony was established in an unscented control box, could have happened by chance, or could possibly be attributed to this box being in a superior micro-climate, which is an important nest site selection factor (Goulson, 2009).

Our findings that mouse-scented nest boxes had significantly higher bumble bee occupancy (43%) than unscented control boxes (17%) offer new opportunities for bumble bee conservation. Thus far, supplemental nest boxes, due to their low occupancy rates, have been deemed impractical for bumble bee conservation (Svensson & Lundberg, 1977; Richards, 1978; Fussell & Corbet, 1992; Lye *et al.*, 2011), and only few studies have focused on nest site selection behavior using nest-searching queens as a proxy (Kells & Goulson, 2003; Lye *et al.*, 2009; O'Connor *et al*., 2017). Our experimental data conclusively show that rodent odorants inform Spring queens about potential nest sites and that these odorants enhance occupancy rates of nest boxes. Soiled house mouse bedding, however, is an impractical solution as a bait for bumble bee nest boxes and must be replaced with a synthetic lure for large-scale bumble bee conservation. To this end, we prepared a synthetic lure that – conservatively – included

all currently known or putative sex attractant pheromone components of house mice, all non-pheromonal constituents in soiled house mouse bedding that bumble bee antennae sensed (Fig. 2), and even ammonium as a universal gas emanating from mammalian urine that blood-seeking horse flies and mosquitoes exploit during host foraging (Kristensen & Sommer, 2000; Venkatesh & Sen, 2017). We took this 'all-inclusive' approach for lure preparation to maximize the likelihood of lure attractiveness.

Our data provide proof of concept that synthetic lures can be developed for guiding nest site-seeking queen bumble bees to nest boxes. That the synthetic lure was not quite as effective as natural rodent odor may be due to either components still missing from the lure or suboptimal release dynamics of lure constituents. For example, major urinary proteins in urine deposits of mice bind to sex attractant pheromone components and facilitate their slow release (Robertson *et al.*, 1993), thus prolonging the effectiveness of pheromonal urine markings (Armstrong *et al.*, 2005). To help ensure that a synthetic nest box lure is adopted widely for bumble bee conservation, the lure composition must be simplified and effective systems for disseminating lure constituents must be designed. Determining the key lure constituents is a first key step towards lure development. The sex attractant pheromone components of mice may be these key constituents because sex pheromones, in general, are 'designed' to stand out, and persist, in chemically noisy settings, thereby enhancing the likelihood of detection by intended receivers (Peake, 2005). This conspicuousness, however, makes pheromones also susceptible to interception, or eavesdropping, by other community members (Peake, 2005), such as queen bumble bees seeking nest sites. Alternatively, generic urine cues such as ammonium may drive queen attraction. In any case, a rigorous systematic approach, such as testing the effect of partial (incomplete) lures with specific lure constituents omitted, is needed to determine the lure constituents that attract queen bumble bees. Once these constituents have been determined, their formulation and sustained release can be studied towards lure development.

In conclusion, we have shown that Spring queen bumble bees antennally sense, and behaviorally respond to, urine and feces odorants from laboratory-kept house mice, and that queens exploit these odorants to find nest sites. Moreover, we have shown that *Bombus mixtus* queens are effectively guided to nest boxes by a synthetic lure of house mouse odorants, and that boxes baited with synthetic lures have higher occupancy rates

than unbaited boxes. With these findings, it seems that future bumble bee conservation programs will be able to provide both floral resources and readily detectable nest sites.

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7.7. References

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7.8. Figures

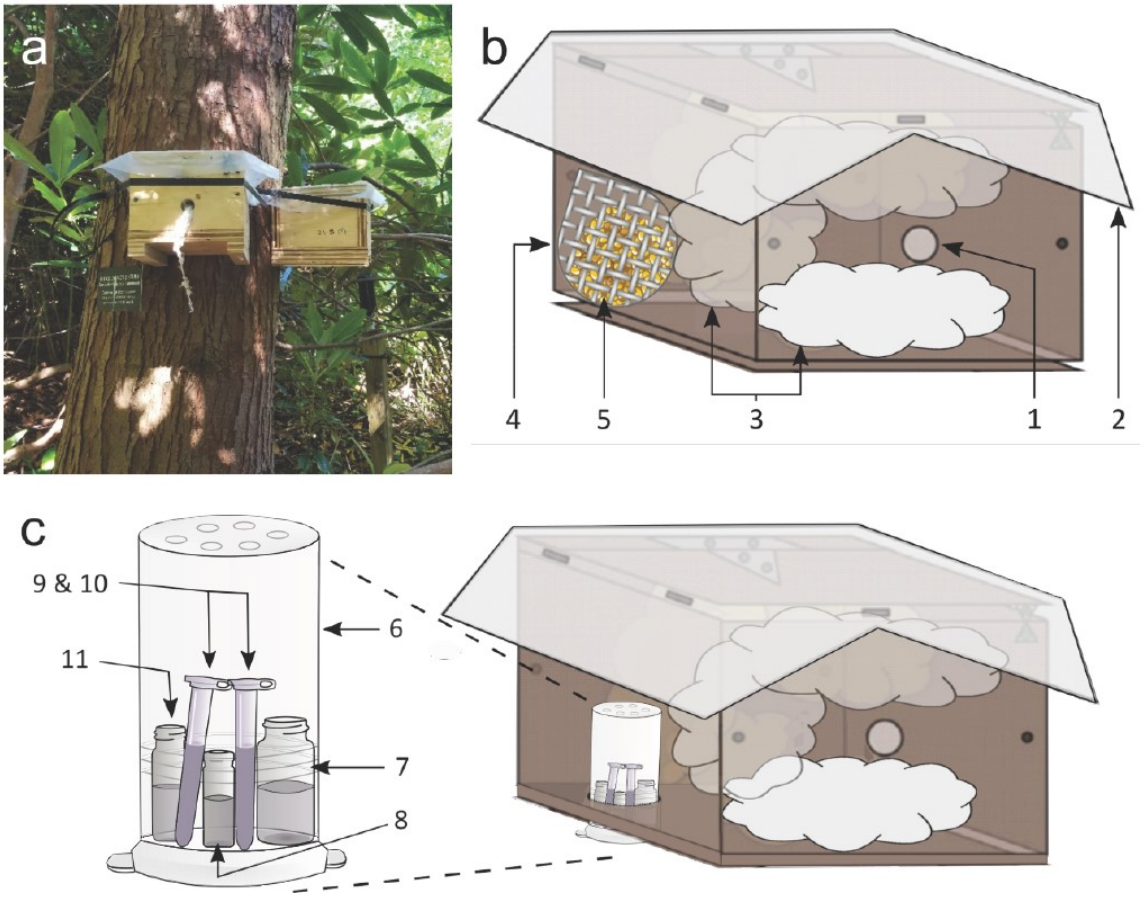

Figure 7.1. **Photograph and drawings illustrating (a) the paired nest box design used in two field experiments, and (b, c) a single nest box and its contents (image modified from Wildlife Preservation Canada 2018) used in experiment 1 (b) and in experiment 2 (c). Boxes were mounted to trees via packaging straps, with 0.4-m spacing within pairs and > 2 m between pairs. Each box (20 × 18 × 14 cm; Hobbs *et al*. 1960) featured a 2-cm hole in the front panel to serve as an entrance (1), and a plastic sheet (2) stapled to the roof to provide protection from rain. All boxes were fitted with unbleached cotton (30 g, 3) as nesting material. Boxes in experiment 1 received – enclosed in a cheesecloth bag (4) – 200 g of corncob bedding (5) which was kept clean (control box) or was previously soiled by mice (treatment box). Soiled bedding contained the metabolic waste produced by one laboratory-strain male and female house mouse over the course of three days. Boxes in experiment 2 had a 2-cm hole in the bottom panel to accommodate an inverted plastic vial with a flip top lid (89 × 42.9 mm) (6) to facilitate lure replacement and a perforated bottom to enable odor release into the box. The vial contained separate lures for known sex attractant pheromone components of male mice (7), candidate sex attractant pheromone components of male mice (8), sex attractant pheromone components of female mice (9), non-pheromonal urine/feces odorants of mice invariably eliciting antennal responses from bumble bee queens and workers (10) (see figure 2), and ammonium and CO_2 (11).**

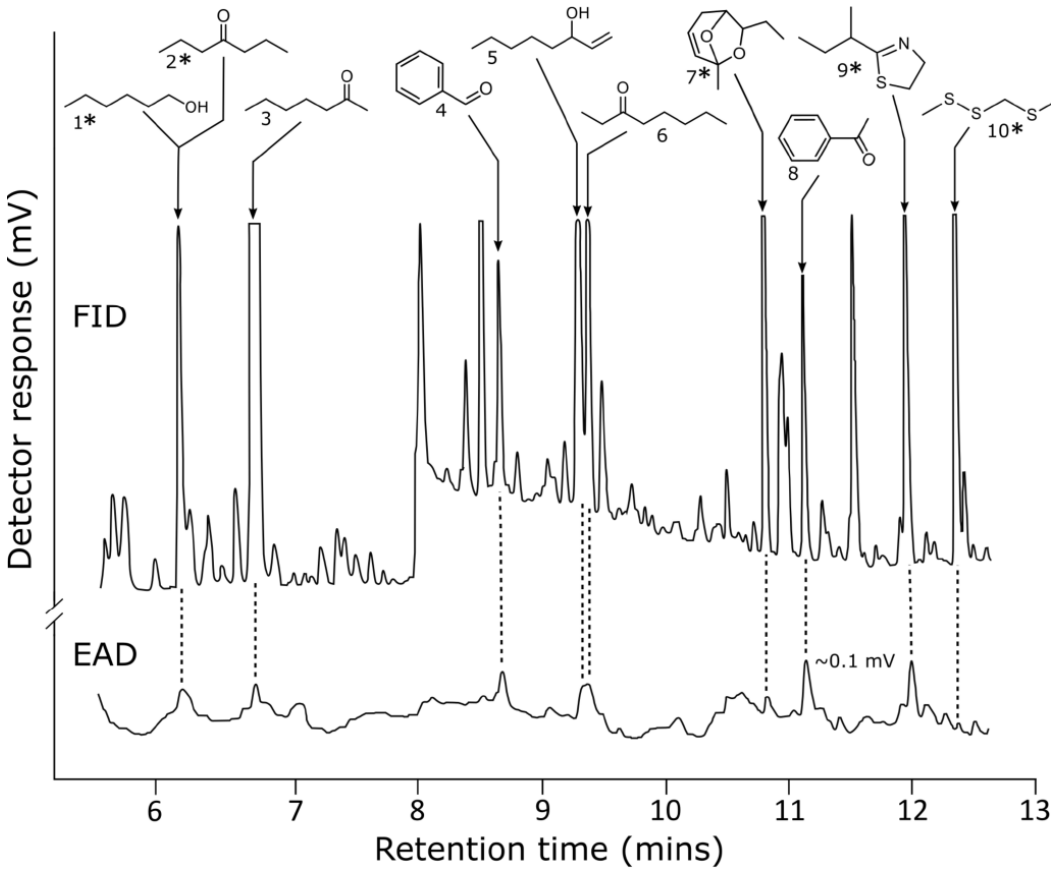

Figure 7.2. **Representative recording of the responses of a gas chromatographic flame ionization detector (FID) and an electroantennographic detector (EAD: antenna of a queen bumble bee *Bombus vosnesenskii*) to aliquots of Porapak Q headspace odorant extract of bedding material soiled by laboratory-kept male and female house mice. Compounds that elicited antennal responses were identified as 1-hexanol (1), 4-heptanone (2), 2-heptanone (3), benzaldehyde (4), 1-octen-3-ol (5), 3-octanone (6), 3,4-dehydro-*exo*-brevicomin (7), acetophenone (8), 2-*sec*-butyl-4,5-dihydrothiazole (9), and 2,3,5-trithiahexane (10); an asterisk (*) denotes an odorant reported as a sex pheromone component of male or female house mice, *Mus musculus*.**

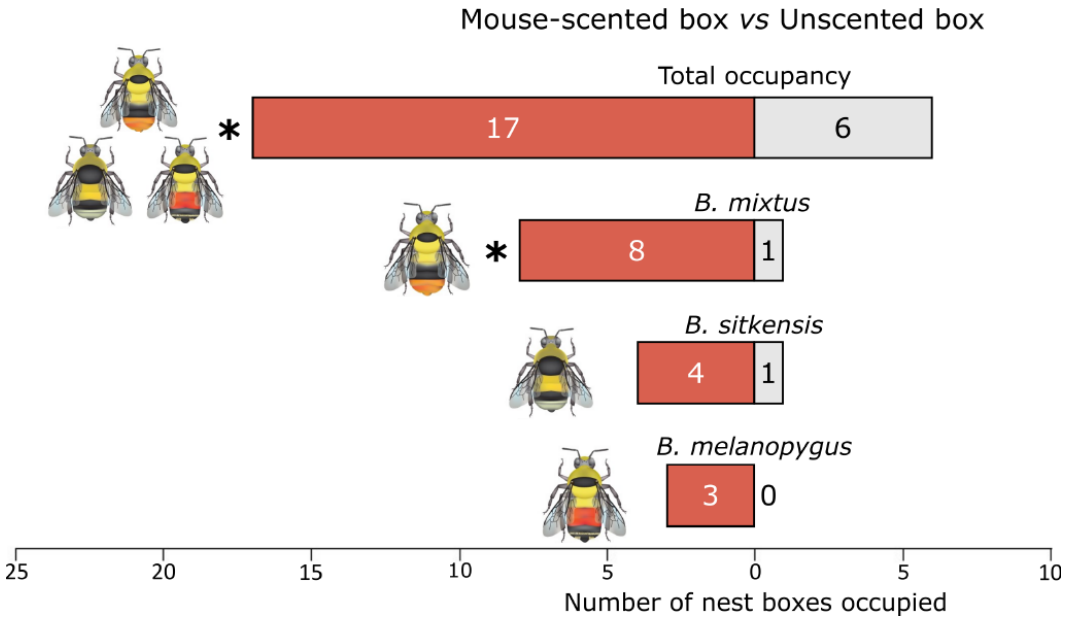

Figure 7.3. **Number of colonies established by *Bombus mixtus*, *B. sitkensis*, and *B. melanopygus* in paired wooden nest boxes (see Fig. 1 a), that were baited (mouse-scented treatment box), or not (unscented control box), with bedding soiled by laboratory-kept male and female house mice, *Mus musculus*. An asterisk (*) denotes that significantly more colonies were established in mouse-scented boxes (χ^2 test with Yate's correction for continuity, $P < 0.05$); total occupancy refers to colonies from all identified species plus additional colonies that could not be identified to species; occupancy data for *B. sitkensis* and *B. melanopygus* were too low to warrant statistical analyses. Bumble bee patterns from Williams et al. (2014) with artwork by Evans et al. (n.d.).**

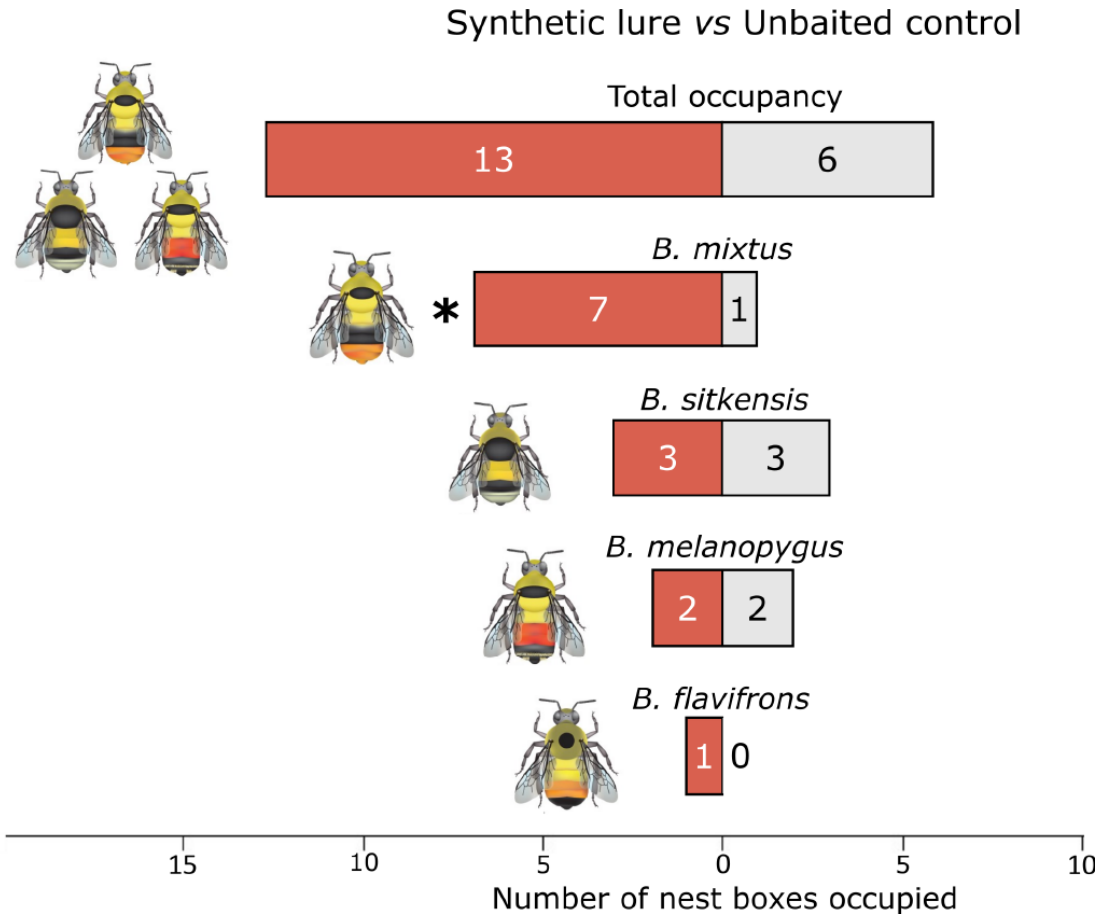

Figure 7.4. **Number of colonies established by *Bombus mixtus*, *B. sitkensis*, and *B. melanopygus* in paired wooden nest boxes (see Fig. 1 a). In each pair, one box was baited with a synthetic lure of house mouse, *Mus musculus*, odor (Fig. 1 c) and the other left unbaited. An asterisk (*) denotes that significantly more colonies were established in mouse-scented boxes (χ^2 test with Yate's correction for continuity, $P < 0.05$); occupancy data for *B. sitkensis*, *B. melanopygus*, and *B. flavifrons* were too low to warrant statistical analyses.**