Pheromones Hot Paper

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The Sex Attractant Pheromone of Male Brown Rats: Identification and Field Experiment

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Abstract: *Trapping brown rats is challenging because they avoid newly placed traps in their habitat. Herein, we report the identification of the sex pheromone produced by male brown rats and its effect on trap captures of wild female brown rats. Collecting urine- and feces-soiled bedding material of laboratory-kept rats and comparing the soiled-bedding odorants of juvenile and adult males, as well as of adult males and females, we found nine compounds that were specific to, or most prevalent in, the odor profiles of sexually mature adult males. When we added a synthetic blend of six of these compounds (2-heptanone, 4-heptanone, 3-ethyl-2-heptanone, 2-octanone, 2-nonanone, 4-nonanone) to one of two paired food-baited trap boxes, these boxes attracted significantly more laboratory-strain female rats in laboratory experiments, and captured ten times more wild female rats in a field experiment than the corresponding control boxes. Our data show that the pheromone facilitates captures of wild female brown rats.*

Brown rats (*Rattus norvegicus*) are significant global pests.[1,2] They inflict harm by vectoring disease-causing pathogens,[3–5] soiling food,[6] spreading allergens,[7,8] diminishing yields of agricultural crops,[3,9] endangering island seabird colonies,[10] and as an invasive species harming indigenous fauna.[11,12] These many adverse effects caused by brown rats in urban centers[13] and in agricultural or ecosystem settings[14] have prompted ongoing efforts to trap or poison rats, in turn exerting selective pressure on rats to evolve counter-adaptations. Neophobia (the fear of new objects) is one such well documented counter-adaptation that helps rats avoid being trapped.[15] Neophobic rats do not readily accept or enter new objects such as bait boxes in their habitat. Yet, tamper-proof trap boxes are mandated in rodent management as they minimize the risk of accidentally poisoning pets and humans, and the capture of non-target animals.[16] The lag time for neophobic rodents to become accustomed to the presence of trap boxes in their habitat, and to enter them and get trapped, greatly reduces the expediency of rat control.[17]

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Pest-management experts engaged in rat control have often observed that those traps that have captured a rat are more likely than new traps to yield another capture,[18] possibly because traps with prior captures carry some sort of rat odor. There is also emerging experimental evidence that urine odorants from conspecific rodents alleviate bait or trap shyness, as demonstrated for desert gerbils (*Meriones hurrianae*),[19] Indian gerbils (*Tateri indica*),[20] Gambian giant pouched rats (*Cricetomys gambianus*),[21] and roof rats (*Rattus rattus*).[22] We have recently shown[23] that trap boxes were most effective in trapping wild brown rats when they were baited not only with a food mix[24] and with synthetic rat pup sound but also with urine- and feces-soiled bedding material of laboratory-kept brown rats. Combined, all of these observations imply that specific scent cues or pheromone signals could be identified and formulated to enhance trap captures of rodents.

Some 131 compounds have been identified in the urine and/or preputial glands of brown rats (Supporting Information, Table S1)[25] and inferred or speculated to play a role in sexual or social interactions. Only nine compounds were subjected to some kind of behavioral test.[25] Most of these tests entailed the insertion of "odor-painted" glass rods into a cage housing a single rat and then recording the rat's sniffing or licking responses.[25] Still, it remained unknown whether male or female brown rats actually produce a sex attractant pheromone that mediates long-range chemotactic attraction, and if such a pheromone was produced, whether it would have a positive, negative, or neutral effect on trap captures of wild rats in the field.

Our search for a pheromone took various considerations into account. We predicted that the components of a sex attractant pheromone would need to be sufficiently volatile to attract potential mates over some distance. For that reason, we opted not to extract odorants or proteins from urine, but instead to capture the odorants in the volatile headspace emanating from urine. We further predicted that pheromone components would be specific to the urine odor of the producing sex, and would appear only as juveniles become sexually mature adults. Based on these predictions, we focused our pheromone search on volatile components specific to, or most prevalent in, sexually mature rats.

To obtain the urine headspace volatiles of brown rats as they matured from juveniles to adults, we used four-week old rats and housed them in the Animal Research Centre of Simon Fraser University. We kept four groups of five females each and four groups of five males each in separate cages lined with corn cob bedding. Rats in randomly assigned treatment groups, but not in (naive) control groups, had intermittent opportunity to see and smell rats in opposite-sex

groups without making physical contact. We replaced urine- and feces-soiled bedding with new bedding every week, captured odorants from soiled and from clean bedding (positive control) on Porapak Q absorbent,[26] desorbed the odorants with pentane and ether, and analyzed aliquots of the Porapak Q extracts by GC-MS.

The analyses revealed complex odor blends emanating from the soiled bedding of male and female rats (Figure 1). Whereas many of the 39 identified odorants were common to both males and females, there were sex-specific differences. Nine compounds (3-ethyl-2-pentanone, 2-heptanone, 4-heptanone, 3-ethyl-2-heptanone, 2-octanone, 2-nonanone, 4-nonanone, 2,3,5-trimethylpyrazine, tetramethylpyrazine) were either specific to, or most prevalent in, male bedding (Figure 1 A), suggesting that some may have a pheromonal function and attract females. Similarly, seven compounds (2-methylbutyric acid, 3-methylbutyric acid, heptanal, hexanoic acid, 2-phenylacetaldehyde, nonanal, and decanal) were specific to, or more abundant in, female bedding (Figure 1 B).

The hypothesis that the male-specific compounds have pheromonal activity was supported by the fact that five of the ketones (3-ethyl-2-pentanone, 2-heptanone, 4-heptanone, 3-ethyl-2-heptanone, 4-nonanone) steadily increased in abundance as males sexually matured during weeks 5–11 (Figure S1), with three ketones (3-ethyl-2-pentanone, 4-heptanone, 4-nonanone) appearing for the first time as males progressed from week 5 to week 6 (Figure S1). As the two pyrazines did not increase in abundance as the males sexually matured (Figure 2), we did not consider them to be candidate pheromone components.

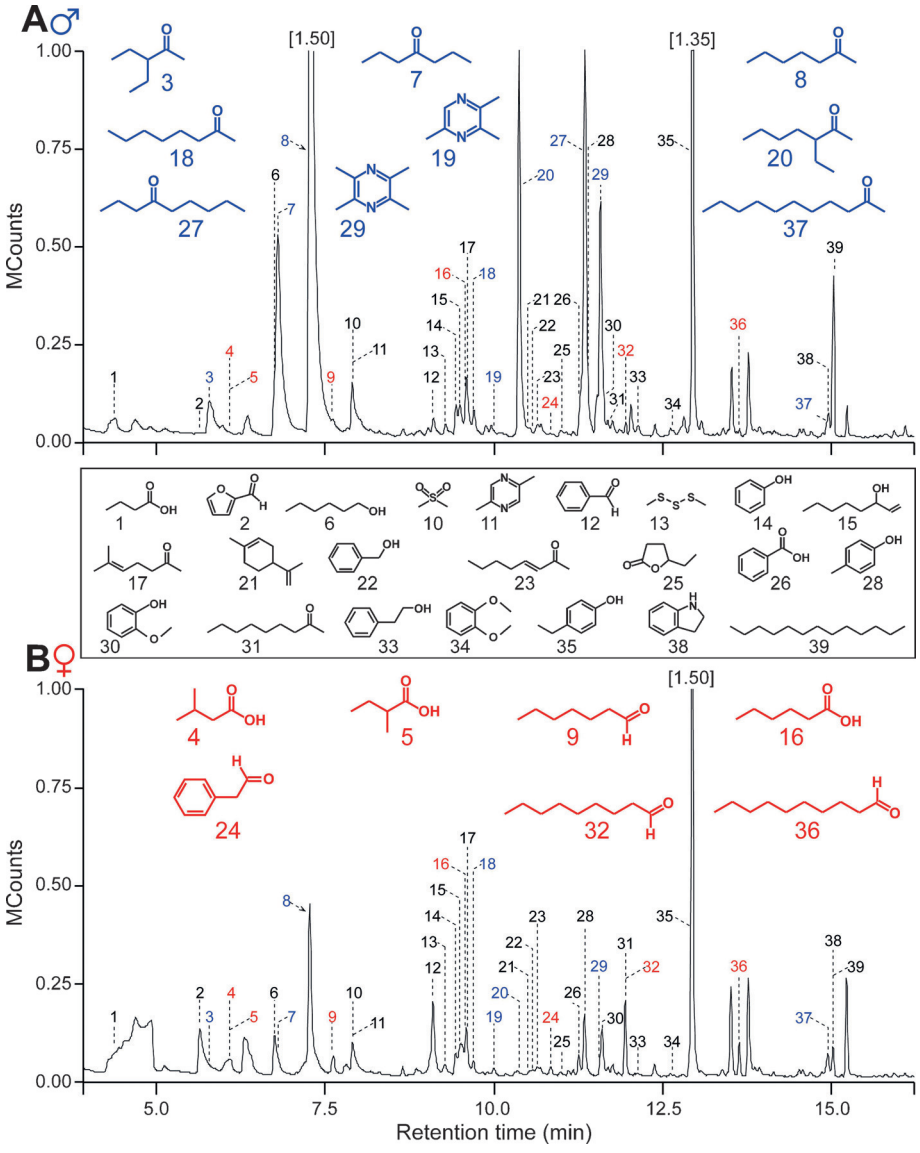

Figure 1. Representative ($n = 8$) total ion chromatograms (2 µl aliquots each) of volatile components emanating from urine- and feces-soiled bedding material of adult male (A) and female (B) brown rats. Compounds highlighted in blue (**3**, **7**, **8**, **18**, **19**, **20**, **27**, **29**, **37**) or red (**4**, **9**, **12**, **16**, **22**, **24**, **32**, **36**) were specific to, or more prevalent in, the volatile profiles of male and female rats, respectively. Compounds presented in the shaded area between the chromatograms were common to both sexes. Numbers in parentheses indicate MCounts of the respective chemicals; a peak of 1 MCount corresponds to approximately 10 ng.

To test the hypothesis that these male-specific ketones constitute an attractive pheromone, we formulated a synthetic blend (1 mg total; 2-heptanone, 4-heptanone, 3-ethyl-2-heptanone, 2-octanone, 2-nonanone, 4-nonanone) in mineral oil (10 g) for laboratory and field experiments. 3-Ethyl-2-pentanone was absent from the blend because we identified it only after the onset of our behavioral experiments.

For laboratory testing of the candidate pheromone blend (CPB), we used a large steel arena with two metal boxes in opposite quadrants (Figure S2 a).[23] We baited both boxes with a food lure,[24] and the randomly assigned treatment box with the CPB. For each experimental replicate, we removed a rat from its home cage, placed it into a gated "transportation container" and positioned the container along the wall of the arena equidistant to both traps, allowing the rat to leave the container on its own accord and to explore the arena and the boxes. For each responding rat, we recorded the box it entered first and the time it spent in arena quadrants associated with a box. Following each replicate, we thoroughly cleaned the arena and traps.

The CPB proved very effective in these laboratory experiments (Figure 3 A). Both males and females spent significantly more time in quadrants associated with the male CPB than in opposite control quadrants (males: df = 18,

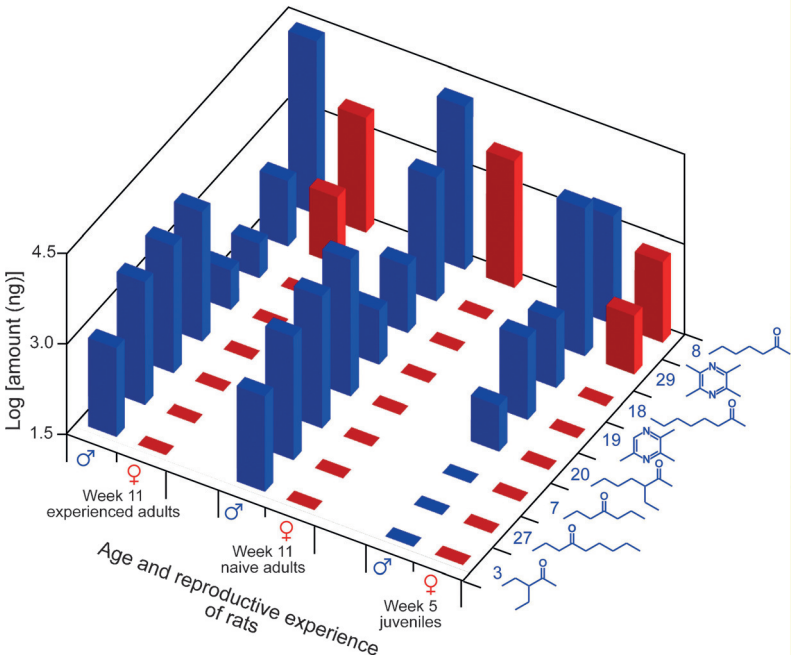

Figure 2. Graphical representation of selected headspace volatile compounds from soiled bedding of male and female brown rats. Components **3**, **27**, **7**, **20**, **19**, **18**, and **29** were specific to, or more prevalent in, the odor profiles of males. Note that these compounds increased in abundance as males progressed from juveniles to sexually mature adults, and that sexual experience (exposure or not to females) had no modifying effects.

$t = 2.57$, $P = 0.019$; females: df = 19, $t = 13.23$, $P < 0.001$; Figure 3A). Moreover, both male and female rats chose to first enter the box baited with the male CPB significantly more often than the corresponding control box (males: $\chi 2 = 5.26$, $P = 0.022$; females: $\chi 2 = 6.05$, $P = 0.014$; Figure 3A).

Despite the positive behavioral bioassay data obtained in these laboratory experiments with laboratory-strain brown rats, we wanted to obtain definitive evidence in a field experiment that the synthetic male CPB indeed attracts wild rats. We ran this field experiment on commercial premises, placing paired trap boxes ($n = 64$) along the interior and exterior walls of buildings with 50 cm spacing between paired traps and at least 5 m spacing between pairs (Figure S2b). Each trap box contained an armed and food-baited snap trap. The CPB (formulated in mineral oil) was randomly assigned to one trap in each pair and the mineral oil control to the other. Once per week, we checked the traps and replaced all test stimuli (food lure, CPB, mineral oil). Whenever a rat had been captured or the snap trap sprung, we replaced both the trap box and snap trap with new ones to make sure that the odor of the captured animals would not affect future captures.

In this field experiment, snap traps in trap boxes baited with the CPB captured 32 female brown rats, whereas control trap boxes without the CPB captured only three females ($\chi 2 = 22.4$, $P < 0.001$; Figure 3B). This tenfold difference in trap captures clearly indicates that the male CPB was highly attractive to wild female brown rats. Conversely, in contrast to the laboratory results (Figure 3A), the same CPB strongly repelled wild brown rat males (Figure 3B). Of the 21 males captured in this experiment, we found only four in traps baited with the CPB and 17 in control traps without the CPB ($\chi 2 = 6.86$, $P = 0.009$).

Our laboratory and field data, in combination, strongly support the conclusion that the CPB contains essential components of the sex attractant pheromone produced by male brown rats. We selected these components based on their specificity or prevalence in the odor profiles of males and their ever increasing abundance in sexually maturing males (Figure S1). It is conceivable that there is some redundancy and plasticity in the pheromone blend in that one or more components could possibly be deleted or replaced by other components, such as 3-ethyl-2-pentanone, without significantly affecting the blend's efficacy. This possibility would have to be investigated in field experiments that would test the effect of partial blends on rat captures. However, given that all blend components are inexpensive, reducing the blend to a minimum number of components seems not to be urgent.

Our data also demonstrate that results of laboratory behavioral experiments with rats may have limited predictive value for the outcome of equivalent field experiments. There are at least two reasons for the contrasting results with male rats from laboratory and field experiments (Figure 3). First, the male rats in the laboratory experiments originated from a long line of laboratory-bred animals that over time may have become more social and tolerant compared to their wild territorial counterparts. Second, laboratory male rats were caged in groups of five. Following their isolation for experimental testing, they may have simply responded to the pheromone in attempts to re-establish contact with their cage mates.

Three of the sex pheromone components of male brown rats that we report here were previously detected in urine extracts of male brown rats (numbers 51, 52, 98 in Table S1)[25] but their effects on rat behavior were either not tested, or tested only in laboratory bioassays that assessed sniffing or licking by rats in response to odor-painted glass rods inserted into their cage.[25] The lack particularly of experimental field data with wild rats may be the reason why compounds such as 2-heptanone and 4-heptanone were previously referred to as "potential male pheromones",[25] as were other compounds such as 9-hydroxy-2-nonanone,[25] which we did not detect and did not consider as potential pheromone components in our study.

Our field data demonstrating that the male sex pheromone attracts females and repels males (Figure 3) are consistent with life history traits of brown rats. It is the territorial polygamous male that continually marks his territory,[27] thereby deterring potential male intruders while retaining females within his deme[28] and attracting wandering females to it.

The female-specific odorants (Figure 1, Figure S3) afforded only a few captures of male brown rats in the field

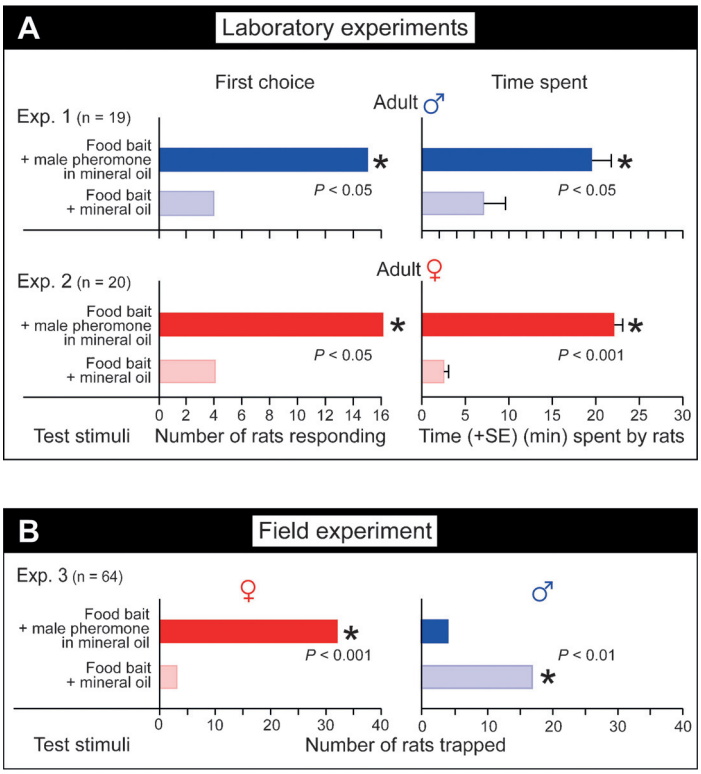

Figure 3. Effect of the synthetic sex attractant pheromone blend (1 mg in total; 2-heptanone, 4-heptanone, 3-ethyl-2-heptanone, 2-octanone, 2-nonanone, and 4-nonanone in a blend ratio of 100:10:10:1:1:10) on the behavioral responses of brown rats in laboratory experiments and on trap captures in a field experiment. In laboratory experiments, we recorded the trap each rat entered first ("first choice") and the time it spent ("time spent") in each of the two arena quadrants with a treatment or control trap. An asterisk (*) denotes a significant preference ($P < 0.05$) for a test stimulus, and *n* indicates the number of single rats tested in the laboratory experiments or the number of replicates run in the field experiment.

(Figure S4, Exp. 6), and thus likely do not represent the complete sex attractant pheromone of female brown rats. As females scent-mark mostly when they are in estrus,[29] estrus-related odorants may need to be present to strongly attract males. Whereas female-specific odorants appear less attractive to females than the male sex attractant pheromone (Figure 3, Exp. 3; Figure S4, Exp. 6), these odorants still increased captures of females in the field (Figure S4, Exp. 6). Conceivably, wandering females responding to female odorants may seek the relative safety of a breeding deme, as indicated by the scent of females.

In conclusion, we have reported the identification of a multiple-component sex attractant pheromone produced by male brown rats. The synthetic pheromone greatly enhances trap captures of female brown rats, apparently alleviating their neophobic trap-avoidance behavior. Operational implementation of our findings could greatly improve the efficacy of rat control tactics in urban and agricultural settings.

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