

Kairomonal communication in mice is concentration-dependent with a proportional discrimination threshold

Vasudevan, Anand; Vyas, Ajai

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RESEARCH ARTICLE

REVISED

Kairomonal communication in mice is concentration-dependent with a proportional discrimination threshold [v2; ref status: indexed, <http://f1000r.es/2h5>]

Anand Vasudevan, Ajai Vyas

School of Biological Sciences, Nanyang Technological University, 637551, Singapore

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Abstract

Odors of predators are often co-opted by prey species to serve as warning signals. Perceptual properties of such kairomonal communication are under studied despite their common use in many mammals. We demonstrate that the kairomonal response in mice to rat odors varies monotonically with the volume of rat odor. Moreover, the ability of mice to differentiate between two strengths of rat odors is dependent on the ratio of the two concentrations. These results show that mice can compare kairomonal strength over a large range of values, and that kairomonal communication follows Weber's law.

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Referee Status:

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	1	2	3
version 2 published 09 Dec 2013	 report	 report	 report
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- Joanne Yew**, National University of Singapore Singapore
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Corresponding author: Ajai Vyas (avyas@ntu.edu.sg)

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REVISED Amendments from Version 1

The main modifications to the text based on comments from the referees are to the materials and methods section of the paper. In response to referee 1, we have clarified the way in which the various urine doses were prepared. In response to referee 3, we have made additions to the methods that clarify the procedure of how we ensured constant volume of the urine stimuli presented to the animals. In addition, we have provided dimensions of the bisects of the arena that was used to define the area from which time spent near the rat urine stimuli was calculated. For referee 2, we have changed the phrase "detection threshold" to "discrimination threshold" in Figure 2 legend and in the "Proportional discrimination threshold" section in the results. We have explained our rationale in the comments section.

See referee reports

Introduction

Foraging animals continually face a conflict between 1) the need to seek opportunities such as food and mating partners; and, 2) the need to avoid exposure to predators. In response to predation pressure, many prey species have co-opted predator odors as kairomones; chemicals emitted by one species, usually for inter-species communication, but intercepted by other species resulting in benefit for the receiver and detriment of the emitter. In this role, predator odors such as urine, fecal material or body odors initiate a rapid avoidance response in prey, thus reducing the probability of successful predation^{1,2}. Such avoidance of predator cues needs to be 'traded-off' against foraging opportunities. In view of this, it can be speculated that a kairomonal responses may not be an absolute all-or-nothing phenomenon. Rather avoidance is expected to be relative to the intensity of the predator cue. Implicit in this speculation is the idea that animals can quantitatively perceive differences in kairomonal strength.

A wide variety of animals can make quantitative estimates of percepts such as time, foraging opportunities, efforts and rewards³⁻⁷. These quantitative estimates are often derived using a comparative representational system that is dependent on the ratios between opposing quantities³⁻⁷. The ability to make quantitative estimates is important because it allows calibration of behavioral responses to incipient environmental opportunities and challenges. In accordance with the comparative nature of such perceptual systems, it can be predicted that greater quantities of kairomones evoke greater response i.e. that the response is dose-dependent. More importantly, sensitivity to changes in the magnitude of a stimulus decreases when stimulus magnitude increases. In other words, the discrimination threshold (i.e. the 'just-noticeable difference' between two stimuli of different intensities) is smaller when both stimuli are weak compared to when both stimuli are strong. This formulation is often termed Weber's law⁸, and is a fundamental property of many percepts.

Kairomonal communication has been widely studied in insects⁹⁻¹². Additionally, the neurobiology and physiology of rodent kairomones has attracted significant scientific interest in the recent past^{1,13}. Yet, the perceptual properties of kairomonal communication in mammals have so far been under studied, including, the dose-responsivity of kairomonal communication¹⁴ and the relationship of discrimination threshold to stimulus magnitude.

House mice (*Mus musculus*) are preyed by rats (*Rattus norvegicus*)¹⁵⁻¹⁷ and accordingly, the mice express innate avoidance to rat odors^{18,19}. In this report, we investigate the dose-responsivity and discrimination threshold of kairomonal communication in mice.

Materials and methods

Animals

The Nanyang Technological University (IACUC number: ARF SBS/NIE-A-0106AZ) institutional animal care and use committee reviewed and approved all procedures. Fifteen male Balb/c mice (7-8 weeks old, housed five per cage (369 x 156 x 132 mm; 1145T, Tecniplast, UK)) were obtained from the vivarium of the National University of Singapore. Eight male Wistar rats (48 days old, housed two per cage (425 x 266 x 185 mm; 1291H, Tecniplast, UK)) were obtained from the same vivarium and used as a kairomonal source. Standard corn cob cage bedding was changed twice a week. Animals were maintained on a 12 hours light-dark cycle, with temperature and relative humidity ranging between 20-25 degree celsius and 70-80%, respectively. Experiments were conducted during the light phase. Food and water was available *ad libitum*. The diet consisted of standard laboratory chow (PicoLab Rodent Diet 20, 5053) with 20% protein content.

Kairomone collection

Rat urine was collected using metabolic cages (Harvard Apparatus). A single pool of rat urine was used for all subsequent experiments. Rat urine contains volatile compounds and major urinary proteins (MUPs). The urine was treated with menadione (M5625 Sigma-Aldrich, Singapore) to competitively displace volatile compounds bound to the MUPs, followed by centrifugation (Millipore, 3000 g for 5 minutes) through a size-exclusion column (>3 kDa). Only the high molecular-weight fraction containing MUPs and devoid of volatiles was used, in accordance with the prior demonstration that rat MUPs serve as kairomones to mice¹³.

Dose-responsivity to kairomones

The response of mice (n = 10) to increasing doses of MUP fraction of rat urine ((henceforth referred to as rat urine) was studied (trial duration = 600s). Avoidance was quantified by comparing time spent by mice in two opposing bisects of an arena (76 x 9 cm; 15 cm high). The bisects were defined by a virtual division of the arena in two equal halves (38 x 9 cm), with exploration in either half being considered as time spent near the stimulus presented in that bisection. The stimuli were presented at the terminal end of the bisects. Data on time spent in each bisection was collected by automated behavioral tracking software (ANY-maze, version 4.3, Stoelting). Opposing arms contained either rat urine or phosphate-buffered saline. The amount of rat urine was systematically varied from 1X to 16X (3.125, 6.25, 12.5, 25 and 50 µl). X was arbitrarily defined as 3.125 µl of rat urine. The different stimuli were made by a twofold serial dilution to ensure that a constant volume was presented (50 µl) while the concentration varied. The stimuli was dotted (5 drops of 10 µl) on filter paper and positioned at the terminal ends of the two bisects. The animals had direct access to the stimuli. The same set of mice was used in successive testing for all doses (starting from lower to higher doses) with 24 hours elapsing between two successive trials).

Discrimination threshold

Both arms of the arena contained rat urine in this condition. The amount of rat urine in one arm was varied in five discrete doses (6.25–25 μ l), equidistant on a Log_2 scale. The stimuli was prepared in the similar way as before ensuring an equal volume was used, with only the concentration being varied. The opposing arm contained volume that was greater by ratio of either 1.2 or 1.3. The percentage of time that mice ($n = 15$, the same mice that were used in the previous experiment) spent in the arm with the greater volume of urine, was quantified. The same set of mice were used in successive testing for all doses (starting from lower to higher doses), with two successive trials (24 hours apart).

Statistics

All statistical tests were conducted using IBM SPSS software (version 20) One-way analysis of variance followed by 'Fisher's least significant difference' (LSD) post-hoc test was used to analyze increasing doses of kairomones on mouse behaviour. A two-way analysis of variance was carried out to determine main effect and/or interaction of kairomone dose and its corresponding ratios.

Results

Dose responsivity of kairomonal communication

One-way analysis of variance (ANOVA) revealed that the amount of time spent near rat urine decreased with an incremental increase in the amount of rat urine (Figure 1; $n = 10$; $F_{(4,45)} = 6.9$; $p = 0.0002$). Animals spent significantly more time near the weakest odor, compared to the strongest (LSD, Fisher's least significant difference, $p = 0.0002$). The stimulus-response curve exhibited a robust fit to sigmoidal curve (Figure 1; $R^2 > 0.99$; $p < 0.01$), showing a monotonic linear response between concentrations 2X to 8X.

In order to rule out carry-over effects during repeated trials, we tested a separate set of five mice repeatedly at a singular dose (4X) over five days. This set of mice exhibited a comparable aversion to rat urine across all trials, showing a lack of habituation, sensitization or conditioning during repeated testing (one-way ANOVA; $F_{(4,20)} = 0.314$, $p > 0.8$).

Proportional discrimination threshold

The discrimination threshold (i.e the 'just-noticeable difference') was studied for five equidistant doses (Log_2 scale) encompassing the linear part of the dose-response curve (Figure 2). One arm of the arena in this case contained the dose depicted in the abscissa. The discrimination around this dose was studied in two successive trials by providing a greater amount of kairomone in the opposing arm, differing by a ratio of either 1.2 or 1.3. A positive discrimination was noted as less time was spent in the arm containing the greater volume of urine.

A two-way ANOVA for dose and ratio revealed a significant main effect of the ratio (Figure 2A; $F_{(1,138)} = 32.1$, $p = 0.00000008$). The main effect of doses themselves did not reach statistical significance ($F_{(4,138)} = 1.347$, $p = 0.256$). Similarly, interaction between doses and ratios was not significant ($F_{(4,138)} = 1.214$, $p = 0.308$). Thus, regardless of the dose studied, discrimination threshold was constantly

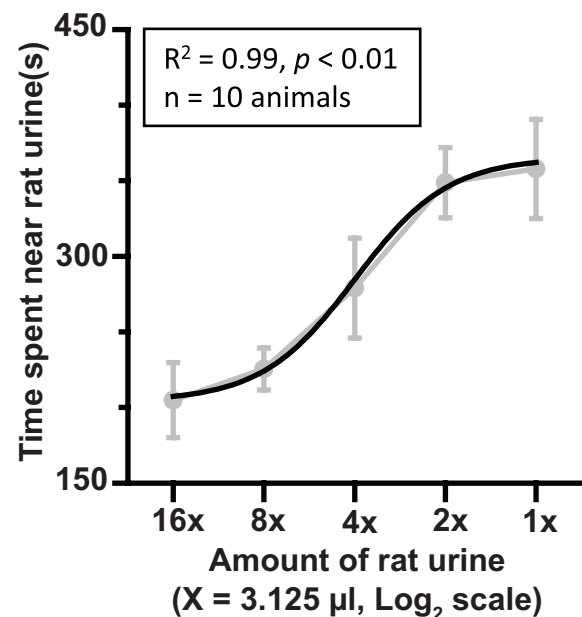


Figure 1. Kairomonal communication in mice is dose-dependent (mean \pm SEM). Aversion of mice to increasing doses of rat urine was quantified by comparing the time spent in two opposing arms of an arena, with one arm containing incremental doses of rat urine and the other containing buffered saline (trial duration = 600 s, $n = 10$ mice). The graph depicts average time spent in rat urine arm. The gray line depicts sigmoidal fit. Abscissa depicts dose of rat urine employed (log_2 scale; x arbitrarily set as 3.125 μ l of rat urine).

proportional to the kairomone strength (Figure 2B) by a ratio ≤ 1.3 but above >1.2 . In other words, the discrimination threshold was smaller for weaker stimuli and bigger for stronger stimuli.

Discussion

Kairomones are compounds emitted by one species and co-opted by another receiving species, resulting in benefit for the receiver and detriment for the emitter. Kairomones can be used by both predators to locate prey and by prey to secure advanced warning of predator presence. Odors used as pheromones in intra-species communication are often the most vulnerable for co-option as kairomones. This is because use in conspecific communication requires robust expression of odors, making them more liable for eavesdropping by other species (reviewed in Kolluru and Zuk²⁰). In agreement with this formulation, rats use urine marks to communicate status and sexual attractiveness^{21–23}, and, proteins secreted with rat urine are sufficient to initiate innate avoidance in mice¹³, a prey species of rats^{15–17}. The salient kairomone in this case has been identified to be a major urinary protein, MUP13¹³. These involatile rodent kairomones provide a unique opportunity to study kairomonal perception by virtue of their stability in the ambient environment and due to their ease of reliably controlling their dosing. This is in contrast with the volatile nature of many kairomones that require onerous delivery methods using olfactometers.

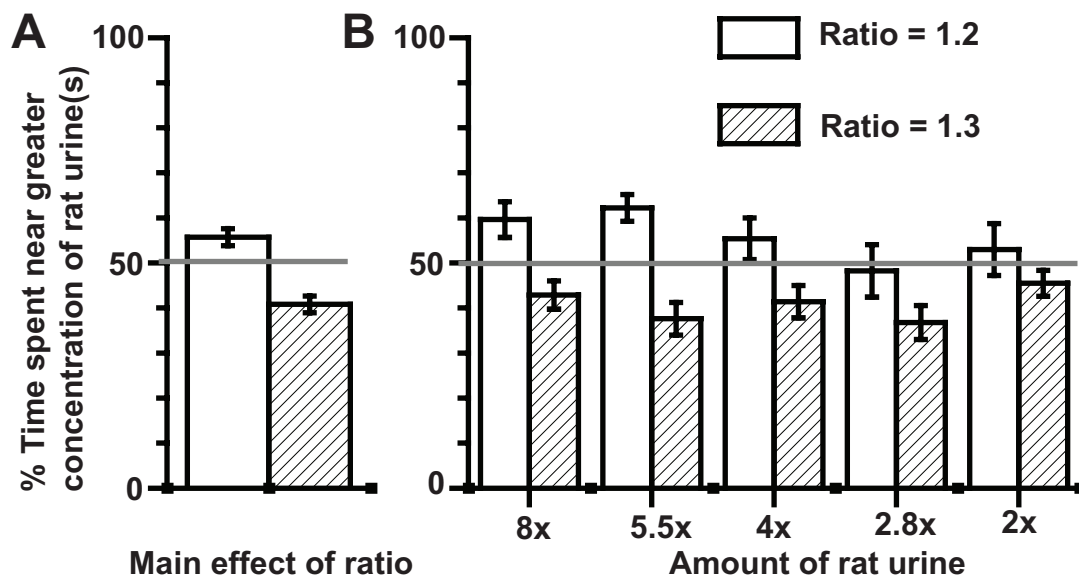


Figure 2. Discrimination threshold in mice is proportional to kairomone strength (mean±SEM). The discrimination threshold at varying doses of rat urine was further examined by setting up an avoidance-avoidance conflict, where mice chose to spend time in arms containing either lower or higher amounts of rat urine. The higher dose was of either a 20% (un-shaded bars) or 30% (shaded bars) greater magnitude (e.g. 120% or 130% of 2X). Abscissa depicts the lower dose used in each of the comparisons (e.g. 2X). Ordinate depicts time spent in arm with the greater amount of rat urine divided by the sum of the time spent in both arms (gray line = 50% chance). N = 15 mice for all comparisons. Log₂ scale; arbitrarily set as 3.125 µl of rat urine.

Several pheromonal responses in insects and mammals show dose responsivity whereby a stronger pheromonal stimulus evokes a greater response (e.g. Coureaud *et al.*, He *et al.* and Perna *et al.*^{24–26}). In contrast, the dose responsivity of kairomonal communication has not been well-studied. In this report, we demonstrate that mice perceive rat kairomones in a dose-dependent manner. We speculate that the relative nature of kairomonal communication permits prey to calibrate foraging responses according to perceived predatory threats. For example, a weak kairomonal stimulus might signal the passage of a long period of time since the urine mark was laid, and thereby evoke lesser avoidance. In contrast, a stronger odor is likely to be fresh and a better indicator of predator presence. Similar dose-responsivity has been previously described during the perception of cat odors by rats¹⁴.

The ability to calibrate avoidance also suggests that mice are able to differentiate between various amounts of kairomones. Animals can indeed discriminate between different amounts of many percepts, including olfactory sensations^{3–7}. In the absence of an absolute numerical system, these discriminations are often dependant on a relative estimation based on comparative perceptions. Weber and Fletcher⁸ formalized one of the hallmarks of such relative estimation by showing that the discrimination threshold is a constant fraction of the stimulus intensity in many perceptual systems ($k = \Delta I/I$; where k is a constant, I is intensity and ΔI is just-noticeable difference). In this report, we demonstrate that kairomonal communication in mice follows Weber's law with Weber's fraction valued at greater than 0.2 but smaller than 0.3. Weber's law has been previously studied in human olfaction for volatile odors, yielding a

comparable sensitivity of 0.28⁷. Similarly, pheromonal communication in Argentine Ants (*Linepithema humile*) has been recently shown to follow Weber's law²⁶. To the best of our knowledge, this is the first demonstration of Weber's law applied to kairomones.

Mouse responsivity and discrimination threshold data to rat kairomones

3 Data Boxes

<http://dx.doi.org/10.6084/m9.figshare.798826>

Author contributions

Anand Vasudevan (AV) and Ajai Vyas (AY) designed the experiments. AV collected the urine samples and carried out the experiments. AV and AY analyzed the data. AY prepared the manuscript. Both were involved in revising the script and approve the final content.

Competing interests

No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Version 2

Referee Report 30 December 2013

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Kazumi Osada

Division of Physiology, Health Sciences University of Hokkaido, Hokkaido, Japan

Although I do not feel the paper is perfectly acceptable, I admit it has been improved by the authors, and am not inclined to reject the paper anymore.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 10 December 2013

doi:[10.5256/f1000research.3209.r2693](https://doi.org/10.5256/f1000research.3209.r2693)



Joanne Yew

Temasek Life Sciences Laboratory, National University of Singapore, Singapore, Singapore

My concerns have been adequately addressed - thank you for doing so.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 10 December 2013

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Fernando Martínez-García, Lluís Fortes-Marco

Faculty of Biology, University of Valencia, Valencia, Spain

After the comments and amendments of the authors, it becomes now clear that they are testing the aversion of mice to different concentrations (rather than different volumes) of the high molecular weight fraction of rat urine, and they are doing so properly.

Even if their results differ from ours, the quality of the paper is high and the results are of interest for the researchers in the area of communication through chemosignals in the rodent model.

We wish the authors good luck in their future research.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 12 November 2013

doi:10.5256/f1000research.2236.r2397



Fernando Martínez-García, Lluís Fortes-Marco

Faculty of Biology, University of Valencia, Valencia, Spain

The manuscript by Vasudevan and Vyas explores the interesting hypothesis that the response of mice to urine-borne kairomones of rats is dose dependent according to the Weber's law. To do so the authors basically analyse the avoidance of mice to different amounts of urine. In fact, the authors purify the MUPs fraction (>3 kD mw), where the kairomone is contained according to Papes *et al.* (2010), and they treat this fraction with high concentrations of menadione to displace odorants contained in the lipophilic pockets of the MUPs. This strategy ensures that the reaction of mice to the stimulus is due to the major urinary proteins and not to urine-borne volatiles.

The results apparently lend support to the authors' hypothesis. There is a highly significant (negative) correlation between the amount of stimulus and the time spent near it by the mice, following a sigmoid dose-response curve.

The authors further test their hypothesis by checking whether mice are able to discriminate different amounts of rat kairomone. To do so they design a simple two-choice test in which the mice can explore two arms of an arena in which two different amounts of MUPs are present, the ratio between the amount in both sides being 1.2 or 1.3. There is a clear, highly significant avoidance of the arm containing the higher amount, as compared to the other arm. This demonstrates that, throughout the linear region of the dose-response curve, mice discriminate two "doses" differing by x1.2 or x1.3.

The main problem with this experimental design is that the authors are checking the response to different amounts (from 3.125 to 50 μ L) of the putative kairomone, rather than to different concentrations of it. If the kairomone were volatile, there would be a direct relationship between amount of sample (volume) and the concentration of the substance in the air: the larger air-liquid interface, the more molecules would be in the air phase. However, a MUP is, due to its high molecular weight, virtually non-volatile. Being a vomeronasal stimulus (according to Papes *et al.*, 2010, *trp2^{-/-}* do not avoid rat urine), for the animal to detect the kairomone it must contact the "drop" of MUP solution and perform vomeronasal pumping.

The important question here is how the MUP solution is presented, and no details are found in Material and Methods. Very likely, a drop of the MUP solution would be deposited in a piece of filter paper or a

similar absorbent material. Depending on the volume of solution, a given area of the paper would become soaked by capillarity.

In a regular piece of absorbent material, a drop of liquid would diffuse giving an approximately circular wet area. Accordingly, one can calculate the theoretical relationship between the volume (V) of MUP solution and the radius (r) of the resulting spot (circular wet area) which would be related to the function: $V = \pi \cdot r^2$. The radius of the spot of urine increases with the volume of the drop. Depending on the size of the area considered in each experiment as “near urine”, this fact, rather than the effect of the “dose”, may explain the results.

In Material and methods, under the heading “Dose-responsivity to kairomones”, the authors explain that “avoidance was quantified by comparing the time spent by mice in two opposing bisects of the area (76x9 cm; 15 cm)”. The term bisect is confusing (it may mean a line sectioning something in two halves, or just sectioning it in two pieces of different size). It is difficult, therefore, to have a clear idea of what the authors are really measuring, for two reasons. First, in spite of what they say (“...comparing the time spent by mice in two opposing bisects”) in their Fig. 1 the authors only represent the time spent “near rat urine”. It seems that this is the measurement analysed statistically (no comparison is made with the side containing PBS).

Second, the arena employed for the tests seems to be a long corridor 76cm long per 9 cm wide, but there is no mention of the actual size of the area that the authors consider “near rat urine”. I assume that they are using an identical area throughout the experiment, maybe a given region at the end of the arm where urine (or MUP solution) is presented. But, if so, as the volume of urine (or MUP solution) increases, the urine spot occupies a larger proportion of the area “near rat urine”. If we assume that contacting the urine allows detecting the kairomone (by vomeronasal pumping) and results in avoidance, the larger the urine spot (which would be the region actually avoided), the shorter the time in the area “near rat urine”. When the spot diameter approaches the size of the area “near rat urine” (e.g. the width of the arena), the time visiting this area reaches an asymptotic line at a minimum. This would explain Figure 1 of the manuscript.

In the same vein, if two spots of different size are located in the two sides of the arena (experiment 2; discrimination threshold) and animals are avoiding contact with the MUP spot, the time in area “near rat urine” on the side having a larger spot (let’s say a 70% of the area “near rat urine”) will be shorter than the time in the side having a smaller spot (which may represent just a 20% of the area “near rat urine”).

As a conclusion, the main problem of the work reported in the manuscript, otherwise well designed and performed, is that the authors are using the term “dose” in a misleading way. The volume of MUP solution is probably not a proper measurement of the “dose” of the stimulus, or the response of the sensory organ (VNO). Vomeronasal neurons respond to the stimulus (MUP) in a concentration-dependent manner (e.g. [Leinders-Zufall et al., 2000](#); [Leinders-Zufall et al., 2004](#)), rather than in a way proportional to the volume of the MUP solution (and the area of the resulting “urine” spot). Therefore, varying the concentration of MUPs in the solution while using a fixed volume would really test the hypothesis that the dose-response to a kairomone follows Weber’s law.

Anyway, the results are difficult to interpret due to the lack of details on procedure. The way in which the stimulus is presented is not properly described, and the size of the area “near rat urine” must be known in order to check if the explanation of the results by the authors is correct or not.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 30 Nov 2013

Ajai Vyas, Nanyang Technological University, Singapore

We have attempted to address the concerns of referee #3 in second version of the manuscript. The referee's comments are based on two strands of criticisms, both of which arise because of our inadequate and unclear description of methods:

- The assumption that the authors varied volume in order to vary amount of the stimulus, rather than concentration.
- The bisect area was undefined in the original methods.

The volume of urine stimuli used was constant; concentrations were varied instead to reach varying amounts. This distinction has now been made clearly in the revised version. Since the volume of the spot was constant, the radius/area of the spot of urine remains constant across the different doses, which addresses the question of variance in the area of the spot of urine affecting our results.

In addition, we have defined the time spent near rat urine by providing dimensions of bisects. In this case, bisects were obtained by equal division of the arena. This point has been clearly mentioned now in the revision. A rather big difference in size of bisect and size of the stimulus spot, along with equal volume of stimuli across doses, avoids substantial interaction between doses and bisect definition.

We believe additional clarifications and elaborations in the material and methods sections adequately respond to all of referee's comments.

In figure 1, we did not show data for PBS because time spent near PBS is directly dependent on time spent near rat urine stimuli. We use two equal bisects to measure time spent near PBS and rat odor (i.e. the arena is virtually divided in two equal compartments). In this design, time spent near PBS is merely subtraction of time near rat odor from total trial duration. Dependence of these two measures then means that statistics for both of them will be redundant.

Competing Interests: I declare that there are no competing interests that might be construed to influence my judgment of the article's or referee response's validity or importance

Referee Report 25 October 2013

doi:10.5256/f1000research.2236.r2187



Kazumi Osada

Division of Physiology, Health Sciences University of Hokkaido, Hokkaido, Japan

This paper showed the quantitative relationship between the amount of crude rat urinary kairomone fraction, and aversive behavior in mice. The figure 1 data is reasonable, but not interesting.

The figure 2 data fails to demonstrate the relationship between the amount of rat urine and the "detection threshold" of the mice. On top of that, this evaluation system is not suitable for detecting the detection threshold of kairomones. This is because if mice spent time evenly in this system, you cannot say that the mice cannot distinguish between these two sources, you can only say that the mice did not distinguish between them.

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 30 Nov 2013

Ajai Vyas, Nanyang Technological University, Singapore

We believe revisions to the script address the main concerns raised by referee #2.

The referee posits that creating an avoidance-avoidance conflict in figure two does not differentiate between possibilities that mice cannot detect rat odor, versus that mice avoid both sources of odor with equal magnitude. Our goal in this figure is not the detection but the discrimination between sets of two odor source. The detection itself is being tested in Figure 1, which does not use avoidance-avoidance conflict.

In other words, we are asking if mice can discriminate an odor (i.e. baseline) from another odor that is incrementally stronger and is presented simultaneously (baseline X Δ). Weber's law predicts that increment in odor strength required for discrimination will be proportional to baseline (i.e. Δ remains constant). Because Weber's law pertains to discrimination and not detection, we tested our hypothesis in Figure 2 using avoidance-avoidance conflict.

We have revised the manuscript to incorporate "discrimination" instead of "detection" in the appropriate results and figure sections.

Competing Interests: I declare that there are no competing interests that might be construed to influence my judgment of the article's or referee response's validity or importance

Referee Report 14 October 2013

doi:10.5256/f1000research.2236.r1883



Joanne Yew

Temasek Life Sciences Laboratory, National University of Singapore, Singapore, Singapore

This article addresses and characterizes rat urine kairomonal effects on mouse avoidance behavior. The study convincingly shows that 1) the effect is dose-dependent and 2) mice display proportional discrimination across several different concentrations.

The title of the study and abstract provide an adequate summary. With respect to the methods, I would like some clarification on whether the different urine doses were made from the same initial sample. If several different urine collections were used, it should be stated how it was verified that the concentration of MUPs in the size exclusion fraction is similar from sample to sample.

Otherwise, the conclusions are justified on the basis of the results and the paper is well-written.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Author Response 30 Nov 2013

Ajai Vyas, Nanyang Technological University, Singapore

The same urine sample was indeed used to create all doses during this experiment. The second version of the manuscript now clearly states this in material and methods section.

Competing Interests: I declare that there are no competing interests that might be construed to influence my judgment of the article's or referee response's validity or importance