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Context-dependent modulation of natural approach behaviour in mice

Nicole M. Procacci, Kelsey M. Allen, Gael E. Robb, Rebecca Ijekah, Hudson Lynam and Jennifer L. Hoy

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Review timeline

Original submission:	27 May 2020
Revised submission:	5 August 2020
Final acceptance:	7 August 2020

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSPB-2020-1189.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Major revision is needed (please make suggestions in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Acceptable

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Marginal

Is the length of the paper justified?
No

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
Yes

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
In this study the authors investigate the relationship between visual stimulus features (size and motion speed) and spontaneous mouse behavior. The work is motivated by earlier studies in which some of the authors were involved showing that mice rely on vision during prey capture. By using parameterized visual stimuli modeled after prey items such as crickets, they found that even naive mice preferably approach visual objects of a specific size and speed. Further, the authors show that prey capture experience affects some of the behavioral measures used in this study such as approach frequency.

The data obtained in this study are very valuable to the community studying neural circuits of vision in mice, and might give new insights into how ethologically relevant stimuli can be studied in the lab. However, I have a number of concerns about the paper in its current form, mainly related to lacking/inconsistent information and data analysis/interpretation.

Major comments:

1. While the paper is generally well written, important information is spread across the paper, inconsistent, or missing (or at least I could not find it). For example, to assess the quality of the data it would be important to provide more details on mouse tracking, including quantification of reliability (e.g., minimum likelihood for tracked body parts used in the study and percentage of frames with likelihood above cutoff). Moreover, details on how the data were recorded are largely missing, e.g., what was the resolution of the camera and the distance from the experimental environment? Was the camera calibrated? How was mouse speed computed in real-world units?

Another example is line 88: "The ellipse was centered on one of three possible locations along the azimuth of the target screen and the bottom edge was maintained at 1 cm from the floor in elevation. Maintaining this aspect ratio, we randomly varied the size of the stimulus along the horizontal axis from 0.5 to 8 cm, and quantified behaviors elicited within 60 s of the start of stimulus presentation." What were the the possible locations on the target screen? What does "aspect ratio" relate to? The ellipse? If yes, what is the ratio? Moreover, the methods section states (line 448) "We varied the major axis of the stimulus from 0.5 to 10 cm.". As I understand the ellipse's major axis coincides with the horizontal axis. Could the authors please clarify this?

There are more examples:
- line 220: "spatial bias": what does this mean? Bias for a specific location in the environment or head angle relative to parts of the environment? The paragraph is mostly about head angle and bias in spatial position (where the animal is) and head angle (where its head is pointing to) are two potentially independent descriptions.

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Potential approaches could be to repeat the same analysis for a baseline condition (and compute screen approaches regardless of any stimulus) or use randomization of stimulus conditions across trials. The authors state in the Methods section that they used the last 60 s segment from the first 180 seconds of each experiment as baseline condition (lines 459-460). What was the motivation for choosing this baseline period rather than the 60 s inter-stimulus-intervals (line 462).

3. The mouse visual system is probably one of the best studied systems in neuroscience. While the authors mention some potential future directions in the Discussion, there is little mention of how the results relate to what is already known.

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lines 39-40: "... more salient cue that indicates possible thread"
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Review form: Reviewer 2

Recommendation
Accept with minor revision (please list in comments)

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> **Is it adequate?**
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This study paves the way for a subsequent investigation of the neural substrates that underlie the stimulus selectivity for the various behaviors, as well as the changes in these substrates that are associated with predatory experience.

SPECIFIC COMMENTS

Mouse gender: Would be useful to specify the gender of the experimental animals (or numbers of females and males). Is there any reason to expect gender-related differences in the behaviors examined?

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Review form: Reviewer 3 (Aman Saleem)

Recommendation
Accept with minor revision (please list in comments)

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Excellent

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Decision letter (RSPB-2020-1189.R0)

10-Jul-2020

Dear Dr Hoy:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Editors have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

To submit your revision please log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions", click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" - in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (https://royalsociety.org/journals/ethics-policies/). You should pay particular attention to the following:

Research ethics:
If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:
If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article. Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

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Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the

accompanying article so that the supplementary material can be attributed a unique DOI. Please try to submit all supplementary material as a single file.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,
Dr John Hutchinson, Editor
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Associate Editor: Doug Altshuler

This is a very interesting manuscript that makes use of virtual reality to investigate voluntary approach behaviour in mice. Through a series of elegant experiments the independent effects of object size, object speed, and prior experience are considered. The results are clear and should be of interest to a broad readership. The three referees generally agree on the merits of the work. All three also list missing elements that currently prevent full evaluation of the experiments. I would be keen to see if the authors can submit a revised manuscript that is responsive to these comments.

Reviewer(s)' Comments to Author:

Referee: 1

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Author's Response to Decision Letter for (RSPB-2020-1189.R0)

See Appendix A.

Decision letter (RSPB-2020-1189.R1)

07-Aug-2020

Dear Dr HOY

I am pleased to inform you that your manuscript entitled "Context-dependent modulation of natural approach behavior in mice" has been accepted for publication in Proceedings B. Congratulations!!

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

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Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,
Dr John Hutchinson
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor:
Board Member
Comments to Author:
Associate Editor: Doug Altshuler

The authors have submitted a revised manuscript that is highly responsive to the helpful suggestions of the reviewers. These changes have made a strong manuscript even better, and I expect this will be an important contribution to the understanding of visual guidance behavior in rodents.

Appendix A

University of Nevada, Reno

31st July2020

Re: Revised Manuscript submission and response to reviewers.

Dear Dr. Hutchinson,

We are humbled by the overall interest in our work and find all of the reviewer's comments clear and constructive. We have now implemented all suggested revisions which we agree significantly improve the utility, clarity and readability of the manuscript, and are likely to enhance the ability of others to replicate and/or expand on this work.

Overall, we have: 1) included more experimental details in the methods and supplemental methods to make our approach easier to discern and replicate, 2) corrected poor writing and better defined our terms while improving the consistency of use of those terms, 3) added all suggested additional analyses that increase the interpretability of the study and enhance its impact, 4) added additional graphical models (Supplemental Data 3) and movie of the mouse's probable view during a representative trial where an approach and several freezes can be observed that summarize "take-home" observations 5) provided additional quantification of visual stimulus appearance dynamics, 6) corrected and clarified figures and presentation of main data, and 7) heavily edited throughout, especially the introduction and discussion, to make more concise and better synthesize our work with how it relates to what is known about mouse vision, specifically citing the extensive work that has quantified direction and size selectivity throughout the mouse visual system as well as spatial topography and the likely role of the superior colliculus in mediating the observed behavioral responses.

We point out the specific revisions in line with the reviewer's specific comments below in blue text.

Please note that the line numbers cited correspond to the "tracked-changes" document attached here with the changes "hidden" in order to create continuous line numbering.

Again, we are very grateful for the careful reading and clear, considered suggestions of all the reviewers. We do hope that this timely work is now in a suitable form for publication at Proceedings of the Royal Society B.

Sincerely,

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Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
In this study the authors investigate the relationship between visual stimulus features (size and motion speed) and spontaneous mouse behavior. The work is motivated by earlier studies in which some of the authors were involved showing that mice rely on vision during prey capture. By using parameterized visual stimuli modeled after prey items such as crickets, they found that even naive mice preferably approach visual objects of a specific size and speed. Further, the authors show that prey capture experience affects some of the behavioral measures used in this study such as approach frequency.

The data obtained in this study are very valuable to the community studying neural circuits of vision in mice, and might give new insights into how ethologically relevant stimuli can be studied in the lab. However, I have a number of concerns about the paper in its current form, mainly related to lacking/inconsistent information and data analysis/interpretation.

Major comments:

1. While the paper is generally well written, important information is spread across the paper, inconsistent, or missing (or at least I could not find it).

We have now included the critically missing information pointed out by this reviewer regarding our approach in clearer and more detailed methods and supplemental methods.

For example, to assess the quality of the data it would be important to provide more details on mouse tracking, including quantification of reliability (e.g., minimum likelihood for tracked body parts used in the study and percentage of frames with likelihood above cutoff).

This is now specified in the methods, lines: 417-420.

“Tracked points with a “likelihood” value of < 0.99 were rejected and dropped as inaccurate. The average percentage of dropped frames for the tracked points was 1.55, 0.05, 0. 03, 1.89 and 1.51 percent for the nose, ears, tail base and stimulus, respectively. Dropped frames were omitted from analysis and not interpolated.”

Moreover, details on how the data were recorded are largely missing, e.g., what was the resolution of the camera and the distance from the experimental environment? Was the camera calibrated? How was mouse speed computed in real-world units?

This information is now included in the Supplemental methods.

“Behavior was video recorded at 30 Hz frame rate and 1920 x 1080 resolution from a camera fixed at 90 cm above the floor of the arena. The objective spatial scale of each recording session was calibrated based on the measured length of the base of arena walls prior to the day’s recording session. The final 60 s of the habituation period prior to stimulus presentation was used to assess spontaneous mouse orienting behaviors relative to either of the screened walls in the absence of a stimulus (**Supplemental Data 1**). After habituation, a randomized order of stimulus speeds or sizes were presented to the mouse for 60 s each stimulus and with an inter-stimulus-interval of 60 s.”

Another example is line 88: "The ellipse was centered on one of three possible locations along the azimuth of the target screen and the bottom edge was maintained at 1 cm from the floor in elevation. Maintaining this aspect ratio, we randomly varied the size of the stimulus along the horizontal axis from 0.5 to 8 cm, and quantified behaviors elicited within 60 s of the start of stimulus presentation." What were the possible locations on the target screen?

We have included this information in the results, lines: 80-82

“The ellipse was centered on one of three possible locations along the azimuth of the target screen (center or midway between center and left adjacent or right adjacent wall).”

What does "aspect ratio" relate to? The ellipse? If yes, what is the ratio?

This has now been clarified in the results, lines: 76-78.

“We parametrically varied the size (**Fig. 1**) or speed (**Fig. 2**) of stimuli, black ellipses with a 2:1 aspect ratio of the major (horizonal) to minor axis…”

Moreover, the methods section states (line 448) "We varied the major axis of the stimulus from 0.5 to 10 cm.". As I understand the ellipse's major axis coincides with the horizontal axis. Could the authors please clarify this?

As above, this has been clarified on lines 76-78 and we are grateful that this critical omission was caught by the reviewer.

There are more examples:
- line 220: "spatial bias": what does this mean? Bias for a specific location in the environment or head angle relative to parts of the environment? The paragraph is mostly about head angle and bias in spatial position (where the animal is) and head angle (where its head is pointing to) are two potentially independent descriptions.

We apologize for confusing these distinct aspects of the mouse’s behavior. To correct this, we clarified that we focus our analysis and discussion on what we are now better naming “stimulus angle” (the angle between the mouse’s head and stimulus) as suggested by reviewer number 3. This makes it clearer that we are interested in reporting the relative, ”egocentric”, behavioral measures in order to gain insight into likely visual processing that is critical for these behaviors.

This should alleviate confusion of what specific aspect of the mouse’s behavior relative to the stimulus we are interested in and keep our naming conventions more consistent relative to other fields such as those studying foraging and spatial navigation, lines 87-89.

“We calculated the mouse’s approach frequency, range (distance between stimulus and mouse head at approach start), locomotion speed, and stimulus angle, angle between the mouse’s head and stimulus. “

Our reference to “spatial bias” was referring to our observation of subtle, but consistent biases in approach and freeze starts that depended on whether the stimuli were in the left visual field or right visual field regardless of where the mouse was in the arena or which wall the stimulus was shown on. While we do not greatly expand on this observation and are careful in our discussion of this finding, we think that noting it will prove useful to others in the field (Figure 4F and Supplemental Data 3). Regardless, based on this reviewer’s point, we have removed reference to “spatial bias” and simply present the raw data and more generally point out the change in visual stimulus angle preference exhibited between naïve and prey capture experienced mice.

In general, it would be really useful (1) to provide the information relevant for understanding the experiments in the results section, (2) to introduce/define all terms, and (3) to use consistent terminology throughout the paper.

We have attended to this issue by carefully re-reading all sections in the manuscript with this in mind, corrections are highlighted with the track changes function. We thank this reviewer for their careful reading and helpful detection of missing critical information. We hope that we have fully addressed this concern while keeping the wording concise in the latest version.

2. Data analysis/interpretation: during spontaneous locomotion in an open field, mice show a wide range of behaviors. A major challenge with naturalistic paradigms like the one used in this study is to understand how experimental manipulations (here: visual stimuli) are reflected in an animal's behavior. Throughout the paper, the authors compare freeze vs approach during 60 s trials in which stimuli were shown (e.g., Fig. 2A). However, in order to support the findings in the paper (in particular those in naive mice), the authors need to confirm that this is not just a consequence of spontaneous mouse behavior.

Potential approaches could be to repeat the same analysis for a baseline condition (and compute screen approaches regardless of any stimulus) or use randomization of stimulus conditions across trials. The authors state in the Methods section that they used the last 60 s segment from the first 180 seconds of each experiment as baseline condition (lines 459-460). What was the motivation for choosing this baseline period rather than the 60 s inter-stimulus-intervals (line 462).

This reviewer raises an excellent point and we regret that it was unclear how we measured spontaneous behavioral rates and that we did not address this issue as thoroughly as this reviewer suggests.

First, we calculate freezing frequency and approaches towards screens in the last 60 seconds of a 3 minute habitation trial that occurs before any visual stimulus is shown, this is now termed the “No Stim” condition in Supplemental Data 1. This analysis is now properly shown in Supplemental Data 1 and demonstrates that mice rarely freeze and never approach screens (data not shown) where stimuli may be presented to within 2 cm when there are no stimuli shown on them. We chose this time period as mice had no reason to expect the appearance of anything on the wall and it gave them time to acclimate to the arena.

Second, all stimuli are indeed shown in a randomized order and randomly presented on one of two opposite facing screens, that is, each mouse is exposed to a different sequence of sizes of stimuli or speeds of the 2 x 1 cm stimulus that appear in unpredictable locations. Thus, the frequency and type of behavioral responses are correlated with the specific “relative” stimulus sizes, speeds of motion or location within the visual field of a given mouse and not: stimulus order, novelty or specific physical position in the arena. This was made clearer in the revision in the results, lines102-103

“Freezing, a period of immobility lasting at least 500ms, was not significantly increased relative to habituation epochs with no stimuli shown (**Supplemental Data 1A**) “

And Supplemental methods:

“The final 60 s of the habituation period prior to stimulus presentation was used to assess spontaneous mouse orienting behaviors relative to either of the screened walls in the absence of a stimulus

(**Supplemental Data 1**). After habituation, a randomized order of stimulus speeds or sizes were presented to the mouse for 60 s each stimulus and with an inter-stimulus-interval of 60 s.”

We thank the reviewer for making their suggestion clear by articulating their ideas to effectively demonstrate this important point.

3. The mouse visual system is probably one of the best studied systems in neuroscience. While the authors mention some potential future directions in the Discussion, there is little mention of how the results relate to what is already known.

This is a good point and so we have modified the discussion significantly to include a more specific discussion of how the prominent visual features of direction of motion, visual field location (lower versus upper visual field and central/binocular versus peripheral/monocular) and object size relate to our behavioral observations as well as the likelihood that superior colliculus circuitry plays a role in mediating the observed behaviors.

We have modified the discussion substantially and would be grateful to the reviewers or editors for a careful rereading and providing any additional suggestions for citations or relevant literature we may have missed deemed critically relevant to our observations.

Related to this, the authors should consider extending the analysis of the head angle relative to the stimulus. In particular, it would be very useful to not only show the distribution of head angles relative to the stimulus at approach start (Fig. 4F) but also before and after, including the relative motion between stimulus and the mouse's head. This would help to relative approach dynamics to what is known about different parts of the mouse's visual field.

We have significantly augmented our study to include the interesting analyses suggested by this reviewer. These data are presented in Supplemental Data 3 and an additional Supplemental video (Video 7) that depict the probable mouse view before and during an approach or several freezes in order to summarize our observations more intuitively.

In Supplemental Data 3 we show in detail: 1) peri-event changes in visual stimulus angle from 500 ms before behavior through the duration of the behavior for both approaches (Supplemental Data 3A) and freezes (Supplemental Data 3D), 2) the estimated angular size of stimuli (degrees of the visual arc) and position within the visual field preceding and during the quantified behaviors (Supplemental Data 3B & 3E), and 3) a graphical summary of mouse preferences for stimulus size, motion and visual field location preceding and during quantified behaviors (Supplemental Data 3C & F). Of note, these are just representations of preferences, but we are careful to note that they do not indicate that the particular stimuli depicted did not exclusively evoke the indicated behavior.

We also added a calculation of the average angular velocity of the visual stimulus from approach starts and show that this is significantly different between naïve mice and prey capture experienced mice, lines 260-264

“Additionally, we observed that mice adjusted their stimulus angle more rapidly at approach initiation (**Supplemental Data 3A**, angular velocity of 107 +/- 17 deg/s vs. 156 +/- 10 deg/s, naïve vs. prey capture experienced, respectively, $p < 0.05$, Mann Whitney U) and there was a significant difference in the distribution of stimulus angles preceding approach for prey capture experienced mice (**Fig. 4F** and **Supplemental Data 3A**).”

Together, these new data additionally highlight the relative likelihood of the stimulus occupying the monocular versus the binocular visual zone before and during approaches versus freezes, and provide a more intuitive idea of the features of visual stimuli coupled to distinct moments during behavior and whether they change or not as a function of prey capture experience. This summary makes it clearer that we see selective changes in visual stimulus perception preceding and during approaches as a function of prey capture experience and that this experience alters relatively little about the stimulus features that precede freezes. A discussion of this analysis and its implications for how mice differentially process visual stimulus information during behavior are now included in the discussion section of the manuscript.

Minor comments:
lines 39-40: "... more salient cue that indicates possible thread"
While freezing might be associated with thread, it might also be relevant to perception and action preparation (see also Roelofs (2017) cited in the paper). Thus one potential role of the "freeze" behavior reported in the paper could be to improve visual perception of the moving stimulus which might be impaired during self-motion (see also above comment on relative motion between head and stimulus). The authors investigate the possibility of thread detection in their final analysis but it might be good to mention this earlier to avoid confusion.

To avoid the confusion this reviewer points out, we now discuss the possibility that freezes do not represent fearful responses explicitly in the introduction, lines: 57-62:

"These freezing responses precede about 50% of accurate and successful approaches towards novel moving objects, are triggered by distinct visual features relative to approach starts, and do not predict an increase in fleeing or avoidance behavior. Therefore, the frequent freezing observed in the context explored here may relate better to its ability to improve motion perception and/or action preparation in response to potentially appetitive objects [19]"
line 67: typo "... interpreted AS potential thread."

Corrected
Fig. 1C: add time unit (as in Fig. 4G,H)

Corrected Fig.1C

Fig. 3A: why different histogram bins for freeze and approach?
We thank the reviewer for catching this, we have now set bin width to be consistent for this measure in Figure 3A and Supplemental Data 5.

line 268: additional "speeds"

We have removed the superfluous "speeds".

Fig. 4F: y-axis label reads p(x). Should probably be p(head angle)?
This label has been corrected to "p(stimulus angle)", Figure 4F.

line 451: "a 2X1 cm" -> "a 2 x 1 cm"
Corrected

Referee: 2

Comments to the Author(s)
GENERAL COMMENTS

This study investigates the effectiveness of a visual object - a dark ellipse on a white background, presented on a computer monitor screen - in eliciting 'freeze' and 'approach' responses in mice. A series of object sizes and speeds are trialed to determine the stimulus conditions that best elicit freezing behavior, approach behavior, and interception. The same experiments are then repeated on mice that have had the opportunity to capture live, moving prey (crickets). The study finds, not surprisingly, that prior experience with detecting, approaching and capturing prey alters (or 'tunes') the behavior in relation to the optimum stimulus parameters for evoking freezing, approach and interception, as well as the topography of the visual fields that are associated with these behaviors.

This study paves the way for a subsequent investigation of the neural substrates that underlie the stimulus selectivity for the various behaviors, as well as the changes in these substrates that are associated with predatory experience.

SPECIFIC COMMENTS

Mouse gender: Would be useful to specify the gender of the experimental animals (or numbers of females and males). Is there any reason to expect gender-related differences in the behaviors examined?

We apologize for omitting our explicit analysis of this factor and stating the sexes in the methods. We used both sexes (and do as a default) and now specify this in the methods, line 388

We also included our statistical tests showing no significant difference in the frequency of approaches or freezes between male and female mice to moving stimuli in Supplemental Data 2B.

The aspect ratio of the ellipses (ratio of major and minor axes) is never specified anywhere, as far as I can tell. I am guessing (from the wording in the text) that the aspect ratio was kept constant at 2:1, while the size was varied. It would be important to specify this clearly in 'Methods'.

We regret this significant omission. The aspect ratio of the stimulus is indeed 2:1 in all conditions and this is now specified in the results more clearly, lines: 76-78 and 83-84.

"We parametrically varied the size (**Fig. 1**) or speed (**Fig. 2**) of stimuli, black ellipses with a 2:1 aspect ratio of the major (horizonal) to minor axis"

"Maintaining the 2:1 ratio, we varied the size of the stimulus along the horizontal axis from 0.25 to 8 cm…"

Stimulus speed range: Lines 141-142: It is stated here and in "methods' that the stimulus speeds ranged from 2cm/s to 50cm/s, but the graphs in Fig. 3 B,C and D seem to display a larger range:

We apologize for this confusion and have now added additional information to the figure legend for panel 3B to clarify what is shown. We hope that this change conveys that objective stimulus speed is represented as a category in these plots and not a continuous measurement. We clarify that we separated the x positions of the freeze and approach data on these plots and added a small jitter in order to facilitate the visualization of the raw data distribution. In addition, we directly plotted angular size and speed data (relative speed data) in Figure 3D in order to better show the relationship between behavioral response frequency and relative size and speeds features as suggested by the other reviewers.

Please do not hesitate to let us know if these alterations do not fully satisfy this reviewer or if the concern may need further clarification on our part.

"(B) Mean trial-averaged range at all three speeds (each speed condition is represented as a separate category on the x-axis, approach and freeze data are also separated along the x-axis and jittered in the x dimension to improve visualization of each distribution) where approaches started (green circles) or freezing occurred (grey circles) "

Line 104: It is ambiguous when you say "2cm diameter stimulus". This is an ellipse, not a circle. I think you are referring to the length of the (horizontal) major axis.

Yes, we are referring here to the horizontal major axis and we regret the imprecise language and have now better clarified the shape and dimensions of our stimulus when they are mentioned in the results section to make this clearer for readers. Throughout the manuscript we are more careful in our description of the geometry of the stimulus.

Lines 113: I think you mean to say "…a preferred angular size of stimulus.."

Yes, we have now corrected this, and other similarly unclear statements.

Lines 144-145: You say "Overall, from stimulus onset, mice froze more frequently and earlier". Compared to what? Compared to the condition when the object was stationary? Clarify.

We are thankful for this reviewer alerting us to this ambiguity. We are referring to the relative number of freezes overall versus the overall number of approaches to moving stimuli that is apparent in the ethograms, Figure 2A & 2B.

In Figure 2B, we see that the time to first observe a given behavioral response, ~18 sec for freezes to stimuli moving 2cm/sec versus ~7 sec for freezes to stimuli moving 50cm/sec, is earlier for freezes relative to approaches when the stimulus moves more quickly, We have now clarified this on lines: 140-142

"From stimulus onset, mice froze more frequently and immediately relative to when they began approaches to the same stimuli (**Fig. 2A & B and Supplemental Data 1)**."

Lines 149-151: It is stated that "..the proportion of approaches preceded by freezing steadily increased as stimulus speed increased, 31%, 63%vs. 80% at stimulus speeds of 2 cm/s, 15 cm/s and 50 cm/s, respectively. Is this evident from the graphs shown in Fig 2, or is it an independent observation obtained from analyzing the data?

We clarify now in the text that this was derived from quantifying the data observed in the ethograms in Figure 2A, but is not represented elsewhere as its own plot, lines 145-147:

“and the proportion of approaches preceded by freezing steadily increased as stimulus speed increased, 31%, 63% vs. 80% at each speed increment, respectively (see ethogram in **Fig. 2A**).”

Lines 178-179: This observation is not evident from inspection of Figs. 2 and 3. There is no 2D plot showing freezing frequency as a function of stimulus size and speed. Might be useful to summarize the overall behavior by including 2D plots – one for freezing and one for approach - showing how each response depends upon the two stimulus variables: object size, and object speed. Also for the results obtained post-predation.

This is an excellent idea and indeed a better way to make it clear which angular sizes and speeds of stimuli mice preferred. We agree that this makes the key relationships between angular stimulus size and speed (relative stimulus features) and the mouse’s behavior much clearer. We have replaced Fig. 3D & E with the suggested plots (Fig. 3D and Supplemental Data Fig. 5D), same data, different representation. The frequency is still indicated as plotting the raw data points, but the density of data points and their transparency on this plot we hope makes the stated point more clear.

Lines 211 and 213: I think you are referring to the upper and lower right-hand panels of Fig. 3A in each case, not Fig. 3C.

We thank the reviewer for pointing this out to us, we believe that you can see stimulus angle biases preceding approach in the raw distribution in Fig. 3A as well as the averaged data in Fig. 3C. We have therefore updated these references to refer to both panels as well as revised Supplemental Data 3, lines: 199-201

“The stimulus angle at approach start when approaches were most frequently observed was most often less than 60 deg (**Fig. 3A & C**, green circles and **Supplemental Data 3A - C**)”

Line 448: You specify the range of sizes of the major axis of the ellipse (0.5 to 10cm), but there is not specification of the sizes of the minor axes or the aspect ratio.
This has been corrected, along with an error on the size ranges tested, on lines 83-84:

“Maintaining the 2:1 ratio, we varied the size of the stimulus along the horizontal axis from 0.25 to 8 cm”

Lines 481-484: It seems to me that you were calculating the angular stimulus sizes and speeds for all object sizes - not just for the 2cm size that this sentence seems to suggest.
We thank the reviewer for indicating the lack of clarity in this description. It is now corrected as suggested by the reviewer by indicating that 2 cm is an example of a possible measurement in the supplemental methods:

“Angular stimulus size (a) was estimated as $a = 2\arctan(L/2D)$, where L is the length of the major axis of the stimulus in cm (e.g. 2 cm) and D is the distance between the point centered between the mouse’s ear and the stimulus center. “

The Introduction and the Discussion are rather wordy and repetitive. I think they could each be shortened by about 25% without seriously compromising the content.
We appreciate this concern and have heavily edited the introduction and discussion to be more concise and informative. We reduced the introduction by about 15% and the discussion by 7% by word count. We hope that this is satisfying to the reviewer.

TYPOS AND STYLE:

All typos have been addressed as suggested by this reviewer and we thank them for the careful reading and bringing these issues to our attention.

Referee: 3

Comments to the Author(s)
In this study, Context-dependent modulation of natural approach behavior in mice, Procacci and colleagues report some very interesting features of mouse approach behaviour. Using a virtual prey, an oval visual stimulus on a monitor, they quantify the visual features that modulate the likelihood and vigour with which mice approach a virtual prey. First, using static stimuli they find the best size that elicits approach. Next, using the best stimulus size they investigate the modulation of approach and freeze behaviors as a function of stimulus speed. Finally, they investigate the influence of prior exposure to prey (crickets) on the approach behaviour and find that experience increases the approach behaviour, particularly for stimulus speeds that are similar to the natural prey.

This report does an excellent, systematic characterisation of the features that trigger approach and freeze behaviors in mice. Below are the few questions and suggestions related to the analysis and presentation of the data:

1. Were there any freeze responses to the stationary dots presented in the first part of the report? Given the number of freezes observed at all speeds, it seems likely, and it would be good to include the analysis of freezing behaviour for the stationary stimuli as well.

This is an excellent point! While there were some freezes when we presented stationary stimuli or even no stimuli, remarkably, our mice rarely froze in the presence of stationary stimuli and nearly every response we observed correlated to the stimulus were approaches. We show this now in Supplemental Data 1A.

It is indeed an interesting case where “object” motion appears to the major feature driving freezing responses in our study. This is consistent with the idea that freezing in this particular context may subserve better motion extrapolation and/or help mice transition between behaviors (e.g. moving in one direction and exploring the arena, to changing direction in response to a suddenly appearing stimulus. See also “Procacci et al.,_Video 7 Mouse View_Freeze_approach_ESM,” a video rendering the probable visual experience surrounding orienting behaviors relative to the stimulus.

2. How does the stimulus repeat when moving? At mid and high speeds (15-50cm/s) the stimulus would cover the complete extent of the screen (60cm) within a few seconds. What happens after that? Does the stimulus reappear again in the old spot, does it move to the other screen? The results and methods do not specify these details.

These details are now specified in the methods, lines 402-404:

“Once stimuli traversed the screen, they reappeared from where they exited after 1 second and traversed in the opposite direction until the full 60 second trial was complete.”

We have now also included the stimulus appearance times over the 60 second trial as grey shading added to the ethograms shown in **Figure 2A and 4A**. The stimulus does indeed reappear with a 1 second delay from the location of the screen it left.

3. Related to above, the ethograms of Figure 2, show some small discreet segments of behaviour for freeze/approach. Do the time spans of these segments relate to the time the stimulus is on the screen in any way?

This is an important point. We analyzed behavioral responses and their lag relative to the onset of each “stimulus sweep” (see graphs below). However, we do not find a consistent lag between either type of response and the onset of stimulus appearance on the screen at each of the three speeds. The starts of behaviors are highly distributed relative to the onset of each “sweep” of the stimulus and do not follow with a consistent lag (see graphs below).

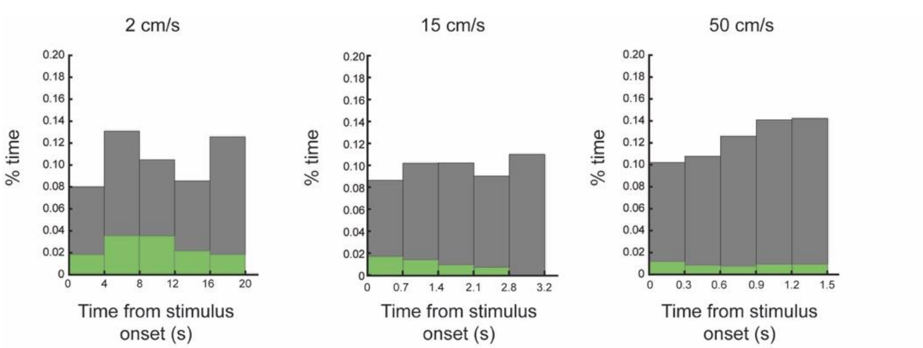
This is consistent with the idea that mice respond to stimuli as a function of when the stimulus is in a particular place within the visual field or at a particular range as they are freely roaming. Furthermore, mice rarely freeze in the absence of stimuli and do not approach the region of the screens during habituation where stimuli subsequently appear during the stimulus presentation trials (Supplemental Data 1). We also removed subjects that produced no behavioral responses in a given trial (e.g. remained in a corner grooming the full 60 seconds). The number of subjects removed from each type of trial was reported and was also not significantly different between stimulus speeds and was reported in the results section, lines 152-155:

“Despite this shift, mice found each stimulus similarly behaviorally salient, as the percentage of trials where at least one approach or freezing event was observed was not significantly different as a function of stimulus speed (91%, 78% vs. 87%, each speed increment respectively, N = 23, Fisher’s exact test, $p > 0.05$ for all pairwise comparisons, **Fig. 2A**).”

Taken together, these observations all support that mice are responding to stimulus motion, size and relative position within the visual field. This point is now made much more clearly in the additional analysis provided in Supplemental Data 3. Behavioral responses are clearly well-aligned to where visual stimuli appear in the visual field of each subject and our videos and arena quadrant occupancy analysis show that most mice roam widely through the environment for all conditions, reported in the results section. Lines: 205-209:

”These biases are unlikely to be explained by preferences for occupying specific allocentric locations within the testing environment as stimuli were randomly presented on either screen and we observed no spatial occupancy bias within the arena in the absence of stimuli (16.7 ± 6.2%, 18.3 ± 4.9%, 18.3 ± 5.5%, 13.3 ± 4.4% percent time in each quadrant, 1-4, respectively during the baseline habituation period, N = 23, pairwise Mann Whitney U, $p > 0.05$).”

Please do not hesitate to let us know if this reviewer would additionally like a separate figure of the histograms shown here included in the final publication.



4. Mean #Approaches is described per mouse. It would be good to have a supplementary analysis in terms of #Approaches per trial (i.e. number of times the dot traverses the screen)

Again, this is a great point. We had initially done the analysis as shown above and it showed no consistent lag between the start of each stimulus sweep and behavioral onset (see below). Rather than include this re-presentation of the data as a separate supplement, we instead indicate on our ethogram in Figure 2 of the main text the moments when the stimulus appears on the screen to give a better sense of the relationship between stimulus presentation onset and each behavioral response start time. We hope this satisfies this reviewers concern and makes our approach and observations clearer for all readers.

5. Fig 2F might be nice to see as a scatterplot between number of approaches versus number of freezes, as it is more straightforward to interpret compared to an index.

This is an excellent suggestion and we now include this representation of the data as Supplemental Figure 2A in order to satisfy different preferences for ways to visualize and summarize this key data per individual subject. Indeed, we too often prefer scatter plots to visualize key relationships on our data upon first pass. However, we ultimately decided on the index in the main figure to make it slightly easier to view the relative performance at each speed separately on one plot. We hope this is satisfying to this reviewer.

6. I found it a bit counter intuitive to call the angle between the stimulus and direction the animal is pointing towards as 'Head angle', as head angle more commonly refers to allocentric frame or head direction (in hippocampal literature). However, stimuli are usually defined in egocentric reference frame and a suggestion could be 'stimulus angle' for this.

This is an excellent suggestion and changing all references from “head angle” to “stimulus angle”, will work to clarify the confusion of the other reviewers regarding this term as well. We therefore renamed each instance of “head angle” as “stimulus angle” and ensure that we defined this term clearly and early on, lines 87-89:

“We calculated the mouse’s approach frequency, range (distance between stimulus and mouse head at approach start), locomotion speed, and stimulus angle, angle between the mouse’s head and stimulus.”

7. Fig 3D-E. Rather than looking at the stimulus size and speed as a function of stimulus speed, it would be interesting to look at the combination of the two. If these are plotted against each other, is there a specific line that separates the likelihood of freeze/approach. If there is, it would be really interesting. Especially, if the line of separation is shifted by experience!

The angular stimulus size and speed data have now been replotted in Fig. 3D, 4E and Supplemental Data 5D, as suggested.

We agree that directly visualizing this relationship in the angular features of stimuli more clearly relayed that the mice behave as a function of the combination of these specific visual stimulus features. Further, as this reviewer correctly predicted, it makes the point much clearer that the “line” along which stimuli fall when mice approach is different between naïve (lower line) and prey capture-experienced mice (middle line), with prey capture-experienced mice preferring to approach larger and faster stimuli than naïve mice. One can also now better visualize that freezing frequency steadily increases at each “line” in this space as well in all plots.

8. Fig 4F: is the sign of head angle relevant. Probably better to look at the distribution of absolute head angle.

In general, the sign of the stimulus angle is not strongly predictive of behavioral response and the main results point to the strongest differences in behavioral responses related to central (presumably binocular) versus peripheral (presumably monocular) visual field differences.

However, we see small but consistent effects in the likelihood that animals respond with different behaviors to stimuli on one side of the visual field relative to the other (right field of view versus left field of view) and we would like to represent some of the data in this way for future work interested in understanding possible left versus right asymmetries in sensory-motor orienting responses, particularly as they may be enhanced at other stages of development or by different states.

Otherwise, we do agree that the general difference between central versus peripheral stimulus detection is the most prominent effect seen in this study and might be better appreciated by quantification of absolute measures and we did that in several cases, importantly in Supplemental Data 3 where we show visual angle change trajectories (panel A).

9. Fig 1C took a while to interpret. One suggestion is to show light grey arrows indicating the previous positions/directions that the animal was in.

We have attempted to clarify Figure 1C as suggested by this reviewer by adding light grey arrows to indicate previous trajectory and stimulus angles during an approach. We hope that this satisfies this reviewer's concern.

10. It might be nice to have an additional figure akin to a graphical abstract: summarising the stimuli and conditions that best elicit approach/freeze (like the illustration in Fig 3A)

This was an excellent suggestion and lead to inclusion of the analysis and summary provided in Supplemental Data 3. Furthermore, it lead us to consider how to render stimulus visualization from the mouse's "perspective". These efforts are now summarized in Supplemental Data 3B, C, E & F and Procacci et al.,_Video 7 Mouse View_Freeze_approach_ESM.

11. "Computer generated" - maybe "artificially generated" is better given that now a days such visual stimuli can be generated by a range of devices including raspberry Pi, iPads, etc., which aren't necessarily termed 'computers'.

Agreed, we have altered this language according to this reviewer's suggestion and altered all references to "computer generated". Lines: 70, 75 & 113 (Figure 1 legend), 240.

12. Line 386-388: A fast moving dot has been shown to trigger escape behaviors in addition to freeze behaviors. Was any flight behaviour observed in this study? If not, probably worth discussing it in relation to the presence/absence of a nest within the arena.

This is a great point and though we previously noted the absence of fleeing behavior which first alerted us to the fact that mice were not necessarily threatened by our stimuli, we had failed to include mention of this in the previous version of the paper and discuss our interpretation explicitly.

We now clarify that in the absence of a shelter provided, we expected fleeing to corners or edges of arena and increased thigmotaxis in addition to freezing if mice felt threatened as this is what mice do when experimenters approach to remove them from the arena, lines 273-277:

"Finally, we sought to determine whether freezing responses were likely to reflect threat detection in the absence of a shelter. We therefore quantified the probability that mice responded to our stimuli with additional appetitive behavior such as pursuit (following stimulus after approach), versus active avoidance behavior such as increased thigmotaxis"

We confirm that we did indeed look for this behavior and did not observe it, which can be confirmed by looking at the videos deposited in the dryad database that accompany this study (doi:10.5061/dryad.mw6m905v3), lines 282-286:

"Mice also did not exhibit active avoidance behaviors such as fleeing to corners as scored by three independent observers (see all videos, doi:10.5061/dryad.mw6m905v3), nor increased thigmotaxis after stimulus onset, relative to baseline conditions without stimuli (79 ± 5.6 % thigmotaxis in baseline condition without stimuli, **Supplemental Data 6** and Hoy et al., 2019 [20,26])."

We also clarify our interpretation of what freezing behavior may relate to in this study if not "avoidance" or "fear", discussion, lines 332-342:

"freezing responses did not change significantly after prey capture experience and were consistently robust to small, fast moving stimuli in the lower visual field (**Supplemental Data 3D – F, 4 and 5**). Our data are most parsimonious with the idea that freezing in this context is enabling accurate perception of external motion [19,29] and augmenting the perception of objects [30] as opposed to reflecting a response to threat (**Fig. 4G & H and Supplemental Data 6**). Indeed, freezing was specifically modulated by increasing stimulus motion (**Supplemental Data 1**), preceded approximately 50% of approaches for naive mice (**Video 1 & 4**) and mice often followed the trajectory of moving stimuli with saccadic head movements as stimuli moved towards their peripheral visual field (**Video 4, 6 & 7**). However, future studies that apply dimensionality reduction methods to infer distinct stimulus-driven behavioral states will better address this issue [31-33]."