Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response: We have proofread the manuscript.

• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write the text on lines 19-22 to avoid this overlap.

Response: We have corrected the text on these lines.

• Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

Response: The ethics statement now occurs before the numbered protocol.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: We have added additional details about the protocol steps in the revised manuscript.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

Response: We have adjusted the numbering and alignment of the protocol section to follow JoVE's instructions for authors.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Response: We highlighted the relevant portions of text.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response: We have rewritten portions of the discussion to ensure that it covers the required topics in paragraph form. The revised discussion also addresses reviewer comments regarding control experiments.

• **References:** Please spell out journal names.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response: All our figures either are new representations of data or comply with the re-use policies of the publisher. We confirmed this with the publisher and we have uploaded the text of the reprint permission as a Word document to the Editorial Manager site.

Comments from Peer-Reviewers:

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript describes a novel way to study different responses to classical fear conditioning in rodents, aiming at utilizing a within-subjects design. The authors designed a modified Pavlovian fear conditioning paradigm that allows for the study of both freezing and flight behaviors in response to the unconditioned stimulus. In addition, this paradigm uses higher-than-average shock intensities, a greater number of pairings between conditioned and unconditioned stimulus, and the added presentation of a white noise stimuli following the tone stimuli. Pavlovian fear conditioning is used to model and study fear and anxiety-related behaviors in rodents, but too often focuses on one response behavior: freezing. This new paradigm is intended to allow the researchers to study a different response behavior: flight or any escape like behavior. The authors conducted a pilot study with the novel paradigm, and briefly discuss the results: Mice exhibit freezing in response to the tone stimulus, but flight in response to the white noise stimulus. Expanding the behaviors that are studied in fear conditioning will allow for a broader understanding of the multitude of behavioral outcomes that result from fear- and anxiety-related disorders in humans. This novel paradigm takes important steps in broadening our understanding of the complex behavioral responses to Pavlovian fear conditioning and this manuscript is extremely helpful in outlining how to properly set-up, conduct

and analyze the data for the new paradigm. There are a few points and questions that should be addressed prior to publication.

Response: We are thankful to the reviewer for their comments.

Major Concerns:
1. A figure depicting what the two different contexts look like would be helpful for those trying to re-create the paradigm (lines 107-109, section 2.1.2).

Response: We have modified Figure 1 to include illustrations of the two different contexts.

2. How did the authors select the specific parameters that they did for fear conditioning (i.e. how long the average ITI should be, how long the session is in total, shock intensity, tone frequency)?

Response: During the development of this paradigm, we tested the effects of several experimental variables to determine which conditions would elicit the most robust flight behavior. For example, we compared behavioral responses following conditioning with a pure tone or white noise and found that conditioning with white noise led to higher flight scores (Fadok et al. 2017). The duration of the ITI was determined by a combination of empirical evidence and literature demonstrating that longer, pseudorandom ITIs are better for Pavlovian learning (Lavond and Steinmetz, 1952; Barnet et al., 1995; Lattal, 1999). The total session length is a product of having a baseline period that allows assessment of contextual learning, the time it takes for the trials and ITIs, and a one-minute post-conditioning period. Finally, we have empirically determined that conditioning with lower shock intensities results in lower flight scores (unpublished data).

3. It would be helpful if the authors summarized some of the findings and control experiments done for previous manuscripts (e.g. reversal of compound stimulus order, extinction results), to help new readers appreciate the scope of what is already understood about this novel paradigm.

Response: We have added a paragraph to the discussion that summarizes the results of previously published experiments using this paradigm. These results include the single-cue conditioning experiments (see response to Point 2, above) and no-shock control experiments published in Fadok et al. 2017. We also reference the findings of Hersman et al. 2020 who explored the role of stimulus salience in the intensity of the conditioned flight response. That paper contains experiments testing the reversal of compound stimulus order, but it should be noted that Dong et al. 2019 obtained opposite results with a reversed SCS.

Finally, our recent data show induction of robust freezing and flight responses to the tone and white noise, respectively, in both male and female mice (Borkar et al. 2020). We did not find significant sex differences for the flight response, although generalized/contextual freezing was higher in female mice (Borkar et al., 2020).

4. More discussion of the extinction results would be useful as well; it is interesting that the presentation of the white noise in the neutral context elicits more freezing than the tone does. Any thoughts on the reason for this switch in white noise responding?

Response: We agree that the behavioral changes observed in the extinction and context transfer experiments are very interesting. During extinction training within the conditioning context, we observe that flight responses during the white noise are rapidly abolished and are subsequently replaced by freezing (Fadok et al. 2017). We conclude from these experiments that the imminence of the threat is vital for the flight response. If mice are conditioned in this paradigm and then tested in a neutral context, freezing values are significantly higher during the white noise compared to the tone (Fadok et al. 2017). We conclude from these data that this is a demonstration of the importance of context in modulating defensive responding. Only in the conditioning context is the perceived threat imminence strong enough to elicit flight responses.

Unfortunately, the author instructions for JoVE do not allow us to add an extensive discussion of these issues to the manuscript as all discussion must be centered on the protocol itself.

Minor Concerns:
1. Line 274, there might need to be a space between "lastmost"

Response: This has been corrected.

2. Line 301, there is an extra "s" in "selection"

Response: This has been corrected.

3. Line 417, there is a missing hyphen between "pre" and "SC"

Response: This has been corrected throughout the manuscript.

Reviewer #2:
This technical report details procedures for performing serial compound stimulus conditioning, an important new procedure useful for studying threat imminence perception and defensive behavior action selection. The report is thorough and comes from the person who developed the protocol, making it a valuable addition to the field. Based on our experience running these experiments, I have just a few minor additions that might be helpful for those who plan to set up SCS conditioning for the first time:

Response: We are thankful to the reviewer for their suggestions, which were useful for improving the quality of the paper.

1. One thing that might be emphasize more is the importance of placing one camera at the top of the chamber to acquire speed data. This is implied in "2. Preparation" section, but could be explicitly stated in "3. Video tracking" section, since over-head cameras are not standard equipment in all commercial fear conditioning packages (e.g. this is not part of Med Associates package, and so would need to be added).

Response: This point has been added to the relevant section.

2. In "5. Quantification of behavior" section, it states that freezing behavior can be manually annotated by frame-by-frame analysis, but this can also be done automatically by thresholding frame-by-frame pixel changes… something commonly done using a side view camera recording with plain background. Med Associates offers a software package for this (VideoFreeze), though other custom programs could also be used.

Response: We have clarified our analysis method and we have expanded this section to better demonstrate how we quantify freezing behavior. We use an automatic freezing detector that is part of the Cineplex software package (Plexon). This software uses contour tracing to identify the subject against the background and then provides a frame-by-frame analysis of pixel changes. An observer blinded to the experiments performs the initial thresholding and then the algorithm automatically detects freezing events. We have added a note addressing the fact that other systems, or manual scoring, can also be used.

Reviewer #3:
Manuscript Summary:
This concisely written manuscripts introduces a behavioral paradigm, which allows to study the switch between passive (freezing) and active (flight) fear responses. It is based on classical conditioning to a sine wave tone and white noise, with subsequent re-exposure to the acoustic stimuli in a neutral or the original conditioning context. This experimental procedures holds the promise of becoming a standard routine in threat research. I have a few minor points which should be addressed during the revision process:

Response: We appreciate the reviewer's efforts to improve the manuscript.

Minor Concerns:
(1) Title: I cannot see the "selection" aspect in the behavioral response. To me, the switch between passive and active fear is rather reflexive and just a matter of threat intensity (accumulation of contextual fear + the stronger auditory-cued fear in case of white noise). In the same line of reasoning - I consider it slightly overstated that the protocol employed results in "conditioned flight" (otherwise, this response should have been prevalent also in the neutral test context). Therefore, I would reconsider this term (and change the title, e.g., "to study the switching between passive and active fear"). In general, it would be helpful to present data of control experiments, which show that conditioning with sine wave tones ~ foot shock alone (i.e., without white noise) do not result in increased flight behavior, if the animals are exposed to white noise in the conditioning chamber after conditioning.

Response: We agree with the reviewer that our paradigm elicits transitions between different, innately programmed, defensive behaviors as a product of threat intensity. This paradigm is useful for understanding how brain circuits operate to determine selection of defensive behavioral output; however, we agree that selection as used in the title is not necessary. We have removed this word from the title. We have also removed "conditioned flight" from the title. In general, we use this term in the same way that many in the field use the term "conditioned freezing"—an adaptive defensive response that occurs during the CS only after conditioning. We have removed this term from the title because the paradigm elicits both flight and freezing within subjects.

The results of control experiments and the published results of other experiments using this paradigm (Dong et al. 2019; Fadok et al. 2017; Hersman et al. 2020), are discussed in response to Reviewer 1 and are now part of the discussion section of the protocol. Unfortunately, we do not have results from the control experiments that the reviewer mentions. One could hypothesize that a nonassociative process could induce a behavioral response to white noise in such an experiment (i.e. sensitization induced by the shock), as has been described previously in traditional fear conditioning (e.g. Kamprath and Wotjak 2004).

(2) Point 1.1. (p. 2): The statement about "power analysis" is not very meaningful without specifications of the effect size, the beta error and a priori knowledge about the variance of the behavior.

Response: We have removed this statement.

(3) The correct nomenclature would be "C57BL/6J".

Response: This oversight has been corrected.

(4) Other than stated at p. 7, l. 308/309, it is not evident why this model should follow the "predatory imminence theory in terms of threat intensity, proximity and context". This requires further explanation. To me, the switch from passive to active fear primarily relates to the strength of activation of the fear matrix (as simulated by increasing electrical or chemical stimulation of the PAG and related brain structures).

Response: We have removed this statement from the discussion. We developed this paradigm using the principles of the predatory imminence theory as a model, but a detailed discussion of why we believe the results follow this theory is not germane to the implementation of this protocol in another laboratory and is therefore beyond the scope of a JoVE protocol.

(5) Figure 1: Too schematic - the protocol described at p. 3/ section 3.2. mentions repeated pips, which are not evident from the sketch (which indicates a continuous presentation of the sine wave tone and white noise). Please revise!

Response: We have corrected Figure 1 to illustrate the use of pips in this paradigm.

(6) Figure 2: Please add individual data to the bar graphs (to get an impression about the distribution of the data).

Response: We have added individual data to the bar graphs in the revised manuscript.

(7) Legend to Fig. 2: Here and in the text, there is an apparent discrepancy in the nomenclature of the experimental days compared to the figure (e.g., "d-1" vs. "Day 1). I would suggest to stick to the nomenclature chosen in the figure (in particular, since "d-1" could be read as "day minus 1"). There is some confusion when referring to the experimental days in the legend to Fig. 2A and 2B ("d-1" and "d-2" have to be replaced by "Day 2" and "Day 3", respectively). Please mention the experimental day for the data shown in Fig. 2F and G.

Response: We have corrected this throughout the manuscript.

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