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TITLE:

Assessment of Mouse Judgment Bias through an Olfactory Digging Task

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SUMMARY:

This article provides a detailed description of a novel mouse judgment bias protocol. Evidence of this olfactory digging task's sensitivity to affective state is also demonstrated and its utility across diverse research fields is discussed.

ABSTRACT:

Judgment biases (JB) are differences in the way that individuals in positive and negative affective/emotional states interpret ambiguous information. This phenomenon has long been observed in humans, with individuals in positive states responding to ambiguity 'optimistically' and those in negative states instead showing 'pessimism'. Researchers aiming to assess animal affect have taken advantage of these differential responses, developing tasks to assess judgment bias as an indicator of affective state. These tasks are becoming increasingly popular across diverse species and fields of research. However, for laboratory mice, the most widely used vertebrates in research and a species heavily relied upon to model affective disorders, only one JB task has been successfully validated as sensitive to changes in affective state. Here, we provide a detailed description of this novel murine JB task, and evidence of its sensitivity to mouse affect. Though refinements are still necessary, assessment of mouse JB opens the door for answering both practical questions regarding mouse welfare, and fundamental questions about the impact of affective state in translational research.

INTRODUCTION:

Measuring affectively modulated judgment bias (henceforth JB) has proven to be a useful tool for studying the emotional states of animals. This innovative approach borrows from human psychology since humans experiencing positive or negative affective states (emotions and longer-term moods) reliably demonstrate differences in the way they process information[1–3]. For example, humans experiencing anxiety or depression might interpret neutral facial expressions as negative, or neutral sentences as threatening[4,5]. It is likely that these biases have an adaptive value and are therefore conserved across species[6,7]. Researchers aiming to assess animal affect have cleverly taken advantage of this phenomenon, operationalizing optimism as the increased expectation of reward in response to neutral or ambiguous cues, and pessimism as the increased expectation of punishment or reward absence[8,9]. Thus, in an experimental setting, optimistic and pessimistic responses to ambiguous stimuli can be interpreted as indicators of positive and negative affect, respectively[10,11].

Compared to other indicators of animal affect, JB tasks have the potential to be particularly valuable tools since they are capable of detecting both the valence and intensity of affective states[10,11]. The ability of JB tasks to detect positive states (e.g., Rygula et al.[12]) is especially useful since most indicators of animal affect are limited to the detection of negative states[13]. During JB tasks, animals are typically trained to respond to a positive discriminative cue predicting reward (e.g., high-frequency tone) and a negative discriminative cue predicting punishment (e.g., low-frequency tone), before being presented with an ambiguous cue (e.g., intermediate tone)[8]. If in response to ambiguous cues an animal 'optimistically' performs the trained response for the positive cue (as if expecting reward), this indicates a positive judgment bias. Alternatively, if animals demonstrate the negative trained response to avoid punishment, this is indicative of 'pessimism' or negative judgment bias.

Since the development of the first successful JB task for animals by Harding and colleagues[8], several JB tasks have been developed for a wide range of species across diverse research fields[7]. But despite their increasing popularity, animal JB tasks are often labor-intensive. Moreover, perhaps because they are methodologically different from the human tasks that inspired them, they sometimes produce null or counterintuitive results[14] and commonly yield only small treatment effect sizes[15]. As a result, JB tasks can be challenging to develop and implement. In fact, for laboratory mice, the most widely used vertebrates in research[16,17] and a species heavily relied upon to model affective disorders[18], only one JB task has been successfully validated as sensitive to changes in affective state[19] despite many attempts over the past decade (see supplementary material for a summary by Resasco et al.[19]). This article describes the recently validated murine JB task, detailing its biologically relevant design, and highlighting the ways that this humane task can be applied to test important hypotheses relevant to mouse affect. Overall, the protocol can be implemented to investigate the affective effects of any variable of interest on JB in mice. This would include categorical treatment variables as described here (drug or disease effects, environmental conditions, genetic background, etc.), or relationships with continuous variables (physiological changes, home cage behaviors, etc.).

PROTOCOL:

Experiments were approved by the University of Guelph's Animal Care Committee (AUP #3700), conducted in compliance with Canadian Council on Animal Care guidelines, and reported in accordance with ARRIVE (Animal Research: Reporting of *In Vivo* Experiments)[20] requirements.

1. Experiment preparation

1.1. Experimental design (see **Table 1**)

NOTE: This behavioral test is a scent-based Go/Go digging task, in which mice have to dig for high- or low-value rewards. It uses a rectangular arena (**Figure 1**) with two arms, in which one arm is scented while the other one is unscented. As outlined below, mice are trained to discriminate between positive and negative odor cues before being presented with an ambiguous odor mixture.

1.1.1. Pseudorandomly assign cages to mint or vanilla positive discriminative stimulus (DS+) groups as follows.

NOTE: This protocol has only been validated for mice assigned to vanilla DS+ odor mixture (see Representative Results and Resasco et al.[19] for details). However, it is strongly recommended to conduct pilot tests to confirm that the DS+, DS-, and ambiguous mixtures meet requirements for valid JB assessment (steps 4.6.3 and 5.3). Thus, the methods outlined here include the testing of both a vanilla and a mint DS+, to provide an example of a group that successfully meets requirements for assessment of JB (the vanilla DS+ mice) and a group that fails to do so (the mint DS+ mice). Prior pilot tests would identify this type of problem in advance.

1.1.2. Mint DS+ mice: for these mice, during arena setup for training (see step 1.4 below), mark scent dispensers and pots containing a high-value reward with the mint odor cue, and those containing no reward with the vanilla odor cue.

1.1.3. Vanilla DS+ mice: for these mice, during arena setup for training (see step 1.4 below), mark scent dispensers and pots containing a high-value reward with the vanilla odor cue, and those containing no reward with the mint odor cue.

NOTE: This task consists of reinforced training trials and unreinforced test trials. During unreinforced test trials, no rewards are accessible to the mice (see step 1.4.3 below) and the ambiguous odor cue for both mint and vanilla DS+ mice is the same 1:1 mint/vanilla mixture.

1.1.4. Pseudorandomly assign cages to left or right scented arm groups (counterbalancing across DS+ odor groups) as follows.

1.1.5. Left: for these mice, during arena setup for training and testing (see step 1.4 below), mark the left arm with the appropriate DS+ or DS- odor cue and ensure the right arm is always unscented (marked only with distilled water).

1.1.6. Right: for these mice, during arena setup for training and testing (see step 1.4 below), mark the right arm with the appropriate DS+ or DS- odor cue and ensure the left arm is always unscented (marked only with distilled water).

1.1.7. Randomly assign each animal a "blind code" as follows.

NOTE: Blinding allows the researcher handling the mice and scoring their behavior to remain unaware of the animal ID or treatment, avoiding undesirable subjective bias. This is a mandatory step to comply with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines and other reference documents[20].

1.1.8. Add a column for blind code in a spreadsheet containing each animal ID and the corresponding treatment they have been assigned to.

1.1.9. Randomly assign each animal a unique code (e.g., a letter and number combination "A2") that is unrelated to cage number or treatment.

NOTE: All randomization should be conducted using a random number generator (e.g., Random.org). During this randomization step, counter-balance across treatment, strain, etc., where applicable. Let this be done by a research assistant who will remain "unblind" to treatment throughout experiments. This individual must not collect data during testing, to avoid biased assessments.

1.1.10. During all data collection, provide the "blind" researcher who is live- and video-scoring latency to dig and digging duration only with the animal's blind code to keep the researcher blind to treatment.

NOTE: Spreadsheets used during data collection will only include the animal's blind code and the corresponding latency to dig and digging duration for each trial. At the end of the experiments, the corresponding animal ID and treatment information can be added to the spreadsheet, thus "unblinding" researchers for data analyses.

1.2. Material preparation

1.2.1. High-value food rewards: break dried sweetened banana chips into approximately 0.5 cm x 0.5 cm pieces by hand.

1.2.2. Low-value food rewards: using a cutting board and knife, cut rodent chow (from animals' regular diet) into approximately 0.125 cm^3 pieces.

NOTE: Pilot tests to identify high- and low-value rewards should be conducted prior to starting the task.

1.2.3. Mint and vanilla essences: Using a 1 mL syringe or micropipette, add mint and vanilla

extract into labeled centrifuge tubes. Dilute extracts 1:4 with distilled water and mix by inverting several times. Make these daily and invert repeatedly before use to ensure that the mixtures are fresh and consistent.

1.2.4. Ambiguous odor mixture: after mint and vanilla essences have been diluted, add equal volumes to a centrifuge tube (creating a 1:1 mixture of the diluted essences). Make these on the day of testing responses to ambiguous odor mixture, and invert repeatedly before use to ensure that the mixture is fresh and consistent.

NOTE: Pilot tests to identify appropriate dilutions and intermediate odor mixtures are strongly encouraged to ensure the utility of ambiguous probes, since intensity may vary between manufacturers, batches, etc. If multiple ambiguous cues are offered, randomly assign mice to the near positive, midpoint, near negative test cues. See Discussion for further details.

1.2.5. Cotton pads: cut each circular cotton pad into six equal pieces (allowing them to fit within the tissue cassettes used as scent dispensers).

NOTE: The number of high- and low-value food rewards, cotton pads, and volumes of odor mixtures will be dependent on the number of subjects being tested. Please refer to **Table 1** for the number of trials per subject in each phase and **Table 2** for the materials required in each trial type.

1.2.6. Identify the experimental area: Conduct training and testing on a bench in the colony room or elsewhere. Conduct them under red light during the dark phase when mice are active.

1.3. Pre-training (1 week prior to experiments) for digging in the home cage

1.3.1. For each cage being tested, pour a small amount of corncob bedding into two digging pots (just enough to cover the bottom of the pot) to help treats remain in the center of the pot when being buried.

1.3.2. In one pot, place pieces of high-value reward on top of the corncob layer so that each mouse in the cage can have one piece (e.g., three pieces of banana chips for a cage of three mice). In the other pot, place pieces of low-value reward on top of the corncob layer so that each mouse in the cage can have one piece (e.g., three pieces of chow for a cage of three mice).

1.3.3. Slowly pour corncob bedding over the treats in each pot, covering them and filling the pots to a height of 3 cm.

1.3.4. Simultaneously place one high-value and one low-value pot in each cage for 10 min. After 10 min, remove the pots from all cages.

1.3.5. Discard any corncob and treats remaining in the pot. Wipe all pots thoroughly with 70% ethanol to prevent animal and cage odors from influencing future trials.

1.3.6. Repeat steps 1.3.1–1.3.4 once per day for 5 consecutive days.

NOTE: The goal of this phase is to allow all cage mates the opportunity to dig and eat a treat prior to the onset of formal training. This also facilitates habituation to food rewards.

1.4. Arena set up for training and testing

1.4.1. Place the arena on a workbench under red light. Wipe all components of the arena, the digging pots, and scent dispensers thoroughly with 70% ethanol to remove dust and any odors from previous trials.

1.4.2. Prepare the digging pots as follows.

1.4.3. Place the appropriate food rewards into the "inaccessible compartment" (see **Figure 1**).

NOTE: Rewards included in this compartment are dependent on which treats (if any) are buried in a given trial (see **Table 2**). Thus, each pot will always contain one piece of banana chip and one piece of chow across the two compartments.

1.4.3.1. DS+ odor pots: during reinforced trials, place one piece of chow in the inaccessible compartment and bury one piece of banana chip in the accessible area of the pot.

1.4.3.2. DS- odor pots: during reinforced trials, place one piece of chow and one piece of banana chip in the inaccessible compartment. No food rewards will be available in the accessible area of the pot.

1.4.3.3. Unscented pots: during reinforced trials, place one piece of banana chip in the inaccessible compartment and bury one piece of chow in the accessible area of the pot.

1.4.3.4. During all unreinforced test trials (positive, negative, and ambiguous), place one piece of chow and one piece of banana chip in the inaccessible compartment. No food rewards will be available in the accessible area of the pot.

NOTE: This step is to prevent the scent of the buried treats from revealing which pot is rewarded. As such, the barrier between the two compartments is perforated to facilitate odor transmission.

1.4.4. Pour a small amount of corncob bedding into each pot to keep food rewards centered when being buried. Place one piece of the appropriate food reward (see **Table 2**) on top of the corncob layer and slowly pour corncob bedding over the treats, covering them and filling the pots up to a height of 3 cm.

1.4.5. Using a 1 mL syringe or micropipette, draw up 0.1 mL of the appropriate odor mixture

(i.e., mint, vanilla, or ambiguous mixtures) or distilled water (see **Table 2**) and inject it directly on top of the corncob in a circular motion.

1.4.6. Prepare scent dispensers

1.4.6.1 Place one cotton pad piece in the base of the tissue cassette. Using a 1 mL syringe or micropipette, draw up 0.1 mL of the appropriate odor mixture (i.e., mint, vanilla, or ambiguous mixtures) or distilled water (see **Table 2**) and inject it onto the cotton piece. Cover the tissue cassette with its lid to enclose the scented cotton and create a scent dispenser.

1.4.7. Place the digging pots at the ends of the arms and scent dispensers at the beginning of each arm. Insert the removable "door" immediately before the cassette slots to block entry to the arena arms and create the start compartment (see **Figure 1**).

NOTE: Digging pots, scent dispensers, and syringes must be clearly labeled and only used for one scent throughout experiments to avoid unintentional mixing of odor cues (i.e., use a different set of materials for mint, vanilla, unscented, and ambiguous mixtures throughout).

2. Digging training: 5 days, two positive trials per day (**Table 2**).

2.1. Fast mice for 1 h prior to training in their home cage by removing food from the hopper.

2.2. Set up the arena for a reinforced positive trial by following the arena setup instructions above (step 1.4).

2.3. On Day 1 of digging training, place the food rewards on top of the 3 cm corncob bedding instead of burying them.

2.4. Progressively bury the rewards deeper under the corncob over the following 4 days, until they are located at the bottom of the 3 cm bedding by Day 5 (i.e., Day 1: on top of corn-cob, Day 2: buried by a very thin layer of corn-cob, Day 3: buried half-way to the bottom of the pot, Day 4: buried three-fourth of the way to the bottom of the pot, and Day 5: buried at the bottom of the pot).

2.5. Move mice from their home cage to an empty transport cage. Place a cue card with the animal's blind code on top of the transport cage so that the researcher conducting experiments remains blind to animal ID and treatment. Carry mice to the experimental area.

NOTE: Steps 2.2 and 2.5 should be completed by a research assistant familiar with the mice. Subsequent steps during trials will be conducted by a researcher blind to animal ID (and treatment, if applicable). Always handle mice using cup handling (open hand) or tunnel handling (with a paper cup or plastic tunnel) to avoid the aversive effects of traditional tail handling[21].

2.6. Move mice from the transport cage to the start compartment of the arena. Remove the start "door", immediately lower the plexiglass lid over the arena, and start the 5-min trial timer.

NOTE: If multiple mice from the same cage are being tested, this should be done simultaneously. However, the number of animals being tested at the same time will depend on the number of blind observers available; ideally, a researcher will observe and handle one animal at a time, but one individual can observe and handle two animals simultaneously, if necessary.

2.7. Live-score latency to dig and latency to eat the reward in both arms.

2.7.1. Record latency to dig as the time at which the first occurrence of digging is observed. Digging is described as a mouse actively pushing or manipulating corncob bedding with the forepaws and/or muzzle.

2.7.2. Record latency to eat as the time at which the first occurrence of eating is observed. Eating is described as a mouse consuming a reward while holding it in the forepaws and sitting on haunches.

2.8. When the trial ends, lift the plexiglass lid, and move the mouse back to its transport cage.

2.9. Discard all corncob bedding and treats left in the pots. Open tissue cassettes and discard cotton pieces. Wipe all components of the arena and the digging pots and scent the dispensers thoroughly with 70% ethanol.

2.10. Set up the arena for a second positive trial (step 1.4). Repeat steps 2.6–2.9 for a reinforced positive trial.

2.11. Return the transport cage to the research assistant so mice can be placed back into their home cage.

2.12. Repeat steps 2.1–2.11 for 5 consecutive days.

3. Discrimination training: 10 days, four trials per day.

3.1. Set up the arena for a positive or negative trial (see **Table 2**) following instructions in step 1.4.

3.2. Conduct one positive trial followed by one negative trial on days 1 to 5. On days 6–10, pseudorandomize the order of trials so that each mouse undergoes two positive and two negative trials per day.

3.3. Follow instructions in step 2.1 at the beginning of each training day, and then follow steps 2.2–2.10. Repeat until mice have undergone four trials in total.

3.4. Return the transport cage to the research assistant so mice can be placed back into their home cage.

3.5. Repeat steps 3.1–3.4 once per day for 10 days in total (i.e., two consecutive 5-day work weeks, separated by a 2-day weekend).

4. Testing

NOTE: Testing duration is 3–5 days (depending on the time taken for each mouse to meet learning criteria), five trials per day for the sessions in which positive and negative test trials are conducted, and three trials per day when the ambiguous test is conducted.

4.1. Fast mice for 1 h prior to training/testing in their home cage by removing food from the hopper.

4.2. Perform one positive trial and one negative trial in a randomized order (see **Table 1**) by following arena setup instructions in step 1.4 and reinforced trial instructions in steps 2.2–2.10.

4.3. Perform one video-recorded unreinforced test trial.

NOTE: The unreinforced trials are identical to reinforced trials, except for the place in which the rewards are placed. Therefore, in the unreinforced trial, one piece of each high- and low-value reward are placed in the inaccessible compartment both for the scented and unscented pots.

4.3.1. Conduct one positive or negative test trial for each mouse daily until learning criteria are met (maximum 4 days; learning criteria is described in step 4.7.3). Ensure to conduct positive and negative test trials in alternating order across days (e.g., Day 1: positive test, Day 2: negative test, Day 3: positive test, etc.).

4.3.2. Follow arena setup instructions in step 1.4.

4.3.3. Move mice from the transport cage to the start compartment of the arena. Set up a video camera on a tripod so that both pots at the ends of arms are in view, and start recording. Ensure to record the cue card with the animal's blind code and the trial type (Positive Test or Negative Test) during video scoring.

4.3.4. Remove the start "door", immediately lower the plexiglass lid over the arena, and start the 2 min trial timer.

4.3.5. When the trial ends, stop recording, move the camera to the side, lift the plexiglass lid, move the mouse back to their transport cage.

4.3.6. Discard all corncob bedding and treats left in the pots. Open tissue cassettes and discard

cotton pieces. Wipe all components of the arena, the digging pots, and scent dispensers thoroughly with 70% ethanol.

4.4. Perform one positive trial and one negative trial in a randomized order (see **Table 1**) by following arena setup instructions in step 1.4 and reinforced trial instructions in steps 2.2–2.10.

4.5. Return the transport cage to the research assistant so mice can be placed back into their home cage.

4.6. Once all mice have completed their five daily trials, transfer videos from camera memory card to a computer for video scoring.

4.7. Score positive and negative test trial videos on the day of testing to assess whether mice have met learning criteria. Ensure same-day video scoring since animals who meet learning criteria will undergo ambiguous tests the following day.

4.7.1. Using event recording software or a stopwatch, let the researcher who is blind to treatment record each mouse's latency to dig and digging duration in each pot during the first minute of positive and negative test trials.

4.7.2. Record latency to dig as the time at which the first occurrence of digging is observed. Digging is described as a mouse actively pushing or manipulating corncob bedding with the forepaws and/or muzzle. Record digging duration as the total time a mouse spends digging.

4.7.3. Compare digging duration in the scented arm between positive (DS+) and negative (DS-) trials to determine whether animals can discriminate the task. Consider that the learning criterion is met if digging duration in the DS+ scented arm is at least double that for the DS- scented arm during the first minute of testing (with a minimum DS+ digging duration of 3 s).

4.8. Repeat steps 4.1–4.7 daily until mice have met the learning criterion.

4.8.1. Exclude individuals that have not met criterion by day 4 from ambiguous trials (and thus, judgment bias assessment).

4.9. For mice that have met learning criteria, test responses to the ambiguous odor mixture.

4.9.1. Fast mice for 1 h prior to training/testing in their home cage by removing food from the hopper.

4.9.2. Perform one positive and one negative trial in a randomized order by following arena setup instructions in steps 1.4 and reinforced trial instructions in steps 2.2–2.10.

4.9.3. Perform one video-recorded test trial as described in step 4.3 using the ambiguous odor mixture (see **Table 2**).

4.10. Score Ambiguous trial videos to assess judgment bias.

4.10.1. Using event recording software or a stopwatch, let a researcher who is blind to treatment record each mouse's latency to dig (see step 4.7.2) in each pot during the first 1 min and 2 min of test trials.

5. Data analysis

NOTE: Exact analyses required will depend on the details of the experimental design. A general overview is outlined here, but researchers are strongly encouraged to refer to Gygax[22] when planning analyses for animal JB experiments, and to Gaskill and Garner[23] when selecting sample size (since required analyses are often too complex for *a priori* power analyses).

5.1. "Unblind" the researcher by adding the corresponding animal ID and treatment information (e.g., enriched or conventional housing; drug or placebo etc.) for each blind code to the spreadsheet. Ensure that the resulting spreadsheet includes three rows for each animal that met the learning criteria (i.e., for the positive, negative, and ambiguous test trials) indicating latencies to dig in the scented arm.

5.2. Run a repeated measures generalized linear mixed model, using preferred statistical software. Here, the outcome variable will be latency to dig. Ensure that the model includes Treatment (or continuous variable of interest), Trial Type, and the Treatment x Trial Type interaction as fixed effects. Include Mouse ID (nested within treatment) as a random effect. Additional terms included in the model will depend on the experimental design applied.

NOTE: If mice are group-housed (as is appropriate for this social species[24]) and cage mates are tested, then cage nested within treatment (if present) must be included in the model as a random effect (and Mouse ID must subsequently be nested within the cage).

5.3. Plot the least-square means of latency in each trial type to confirm that the ambiguous cue presented was interpreted as intermediate (i.e., the latency least-square means estimate for the ambiguous trial should fall at a midpoint between the positive and negative latencies). Assess mouse JB only if this requirement is met.

5.4. Assess the simple effect of treatment (or continuous variable of interest) on latency to dig in the ambiguous trial by investigating the Treatment x Trial Type interaction to determine whether mice display affect-modulated JB.

[Figure 1 here]

[Table 1 here]

[Table 2 here]

REPRESENTATIVE RESULTS:

Results presented here reflect relevant findings from Experiment 1 of Resasco et al.[19]. Subjects in this experiment were 18 female C57BL/6NCrl ('C57') and 18 Balb/cAnNCrl ('Balb') mice. Animals arrived at the facility at 3–4 weeks of age and were randomly assigned to environmentally enriched or conventional housing treatments (EH or CH, respectively) in mixed strain quartets[25]. Each cage contained one C57 and one Balb, in addition to two DBA/2NCrl mice being used in another experiment. Here, the use of female mice allowed group housing of this social species with environmental enrichment, while avoiding the elevated aggression and resource guarding that can occur in male mice[26] (although note that the task has also been applied in non-enriched male nude mice[19]). CH mice were kept in open-top, transparent polyethylene cages (27L x 16W x 12H cm; n = 9) with corn cob bedding, two types of nesting material (crinkled paper strips and cotton nestlets; **Figure 2A**), and a paper cup 'shelter'. EH cages were opaque plastic, measuring 60L x 60W x 30H cm^3 with one red plexiglass window for observations (n = 9; **Figure 2B**,**C**). These conditions are known to improve welfare: containing diverse enrichments (as described previously by Nip et al.[27]) that mice are motivated to access[28], and which reduce behaviors indicative of poor welfare (e.g., stereotypic behavior, aggression, and depression-like inactivity[27–29]. Each EH cage also included an attached 'annex' cage (identical to CH cages but containing only bedding), which mice could freely access *via* the tunnel (**Figure 2C**). Annex cages allowed for ease of catching and handling EH mice trained to enter this attachment for food rewards when a cup full of sweet oat cereal was shaken. Once a mouse entered the annex cage, the access tunnel could be blocked and mice could be easily removed from cages using cup or tunnel handling[21]. This approach thus avoided stressful 'chasing' through complex enriched environments[30]. Food and water were available *ad libitum* and the colony room was maintained at 21 ± 1 °C and 35%–55% relative humidity, on a reverse 12:12 h light cycle (lights off at 06:00 and on at 18:00). Mice were differentially housed for 5 weeks prior to commencement of digging training in the apparatus (see timeline in **Figure 2D**).

Mice underwent training and testing in the JB task as outlined in the Protocol above. Latency to dig in the scented arm during the first 1 and 2 min of positive, negative, and ambiguous test trials was used to test for housing effects on JB. Here, data were analyzed using Generalized Linear Mixed Models, applying transformations where necessary to meet assumptions of normality and homogeneity. See Resasco et al.[19] for a detailed description of analyses (e.g., model selection). Briefly, the repeated measures models always included Trial Type, Housing, Strain, Trial Type x Housing, DS+ Odor, Trial Type x Strain, Trial Type x DS+ Odor, Trial Type x Housing x DS+ Odor, Cage (a random effect nested in Housing and DS+ Odor), and Mouse ID (a random effect nested in Cage, Housing, DS+ Odor and Strain). The simple effects of Housing on latency were determined from the Trial Type x Housing when calculating the Least Squares Means[31]. Note, two-tailed p-values are reported throughout to demonstrate investigation of treatment effects, but the original validation work by Resasco et al.[19] used one-tailed p-values where appropriate[32] since one specific response was required to validate the task (see Resasco et al.[19] for validation discussion).

Before judgment bias can be assessed in any animal task, it is crucial that two technical criteria be met: first, animals must successfully discriminate between positive and negative cues (i.e., meet learning criteria). For animals meeting this criterion, it must then be demonstrated that the ambiguous cue is interpreted as intermediate. If either of these is not met, then inferences cannot be made about judgment bias and corresponding affective states. In this experiment, all but four C57 mice met learning criteria and one C57 was removed before testing for barbering a cagemate (n = 31). In both the first 1 and 2 min of testing, Trial Type x DS+ Odor was significant (1 min: $F_{2,62}$ = 5.67, p = 0.006; 2 min: $F_{2,62}$ = 5.74, p = 0.005), revealing that Mint DS+ mice unexpectedly interpreted the ambiguous odor mixtures as positive (as if 100% mint), while Vanilla DS+ mice treated the same ambiguous odor mixtures as intermediate (**Figure 3A,B**). This finding indicated that only Vanilla DS+ mice met the technical requirement of treating the scent mixture as intermediate between the DS+ and DS-, and thus Mint DS+ mice were excluded from subsequent JB analyses.

For Vanilla DS+ mice, simple effects of housing were calculated from the Trial Type x Housing term[31]. Within this group, Housing influenced digging latencies with CH animals being slower than EH to dig in ambiguous trials, but not in positive or negative trials. This was true after 1 min (ambiguous*: t* = 2.27, d.f. = 92.94, p = 0.014, *Cohen's d* = 1.148; positive: t = 0.22, d.f. = 92.94, p = 0.414, *Cohen's d* = 0.110; negative: t = 0.80, d.f. = 92.94, p = 0.214, *Cohen's d* = 0.404; see **Figure 4A**) and after the full 2 min (ambiguous: t = 2.14, d.f. = 91.89, p = 0.018, *Cohen's d* = 1.083; positive: t = 0.39, d.f. = 91.89, p = 0.348, *Cohen's d* = 0.198; negative: t = 0.61, d.f. = 91.89, p = 0.273, *Cohen's d* = 0.308; see **Figure 4B**), even though Trial Type x Housing x DS+ Odor (1 min: $F_{3,65.37}$ = 0.36, p = 0.7835; 2 min: $F_{3,65.37}$ = 0.49, p = 0.688) and Trial Type x Housing (1 min: $F_{2,62}$ = 1.66, p = 0.198; 2 min: $F_{2,62}$ = 1.41, p = 0.252) did not account for significant variation. These pessimistic interpretations of ambiguous cues by CH mice reflect negative judgement biases indicative of negative affect.

[Figure 2 here]

[Figure 3 here]

[Figure 4 here]

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of experimental apparatus. The JB apparatus includes a rectangular arena with two arms. Each arm contains a scent dispenser located at the start and a digging pot placed at the end. Reprinted from reference [19] with permission from Elsevier.

Figure 2: Housing treatments and experiment timeline. (**A**) CH laboratory cage. (**B**) Overhead view of EH. (**C**) Front view of EH with attached 'annex' cage to facilitate mouse catching/handling. (**D**) Experimental timeline and summary of positive, negative, and ambiguous training and test trials. DS(+): positive discriminative stimulus, DS(-): negative discriminative stimulus, AMB: ambiguous mixture (50% vanilla-50% mint), B: banana chip, C:

Rodent diet ('chow'), X: no food rewards. Reprinted from reference [19] with permission from Elsevier.

Figure 3: Determining whether mice meet requirements for JB assessment. Digging latency least-square means (±standard error) during positive, negative, and ambiguous test trials. (**A**) 1 min digging latency in mice receiving mint (M, n = 15) or vanilla (V, n = 16) as the positive discriminative stimulus (DS+) (data Box Cox transformed). (**B**) 2 min digging latency in the same subjects (data logarithmically transformed). During both time periods, Vanilla DS+ mice met the technical requirement of interpreting ambiguous cues as intermediate. Mint DS+ mice failed to do so and were consequentially eliminated from subsequent JB analyses. Reprinted from reference [19] with permission from Elsevier.

Figure 4: Impact of housing treatment on affect-modulated JB. Digging latency least-square means (±standard error) during positive, negative and ambiguous test trials. (**A**) 1 min digging latency in Vanilla DS+ mice from conventional (CH, n = 7) or enriched (EH, n = 9) housing (data Box Cox transformed). (**B**) 2 min digging latency in the same subjects (data logarithmically transformed). During both time periods, CH animals demonstrated significantly longer latencies to dig during ambiguous trials than EH conspecifics, indicating negative JB. Reprinted from reference [19] with permission from Elsevier.

Table 1: **Summary of experimental design and schedule for training and testing.** Number and order of trials per day for Digging Training, Discrimination Training, and Testing phases in addition to experimental design details. Reprinted from reference [19] with permission from Elsevier.

Table 2. Summary of trial details. Odor cues and rewards presented in each trial type during Digging Training, Discrimination Training, and Testing phases. DS(+): positive discriminative stimulus, DS(-): negative discriminative stimulus, Pos: positive, Neg: negative. Reprinted from reference [19] with permission from Elsevier. See Supplementary Table S2 in the original article for the expanded table.

DISCUSSION:

The scent-based digging protocol and results outlined here demonstrate the ability of this novel JB task to detect changes in mouse affective state. The task thus presents a valuable tool for diverse fields of research. Similar to any JB task, to assess animal affect it is critical that animals first learn to discriminate between cues (step 4.7.3) and that the ambiguous stimulus is interpreted as intermediate (step 5.3). Though simple, meeting these requirements can be challenging, particularly in laboratory mice for which over 15 past attempts to develop and implement a mouse JB task have failed[19]. Here, multiple components played an essential role in meeting these technical criteria and contributed to the success and utility of the task.

First, the ethological design of the task promoted successful discrimination learning since both the discriminative cues and required responses were biologically relevant: mice have impressive olfactory abilities, making them capable of rapid learning and considerable memory

spans when presented with odor stimuli[33], and they are naturally driven to perform digging for general exploration, foraging, and burrow construction[24,34]. Further, counterbalancing DS+ odors revealed differences in the ways that Vanilla DS+ and Mint DS+ mice interpreted the ambiguous mixture, confirming that the ambiguous cue was interpreted as intermediate for Vanilla DS+ mice, but not for Mint DS+ mice. It is therefore recommended to use only Vanilla as the DS+ for any future work utilizing the extract brands and mouse strains used here. Importantly, though counterbalancing yielded successful outcomes in the present experiment, we urge researchers to conduct pilot tests to identify appropriate ambiguous mixtures if implementing changes, since counterbalancing can sometimes add considerable noise to the data, increasing the risk of masking treatment effects[35].

Even when these essential criteria are met, JB is not always easy to detect, perhaps owing to the small treatment effect sizes that these experiments commonly yield[15]. Thus, to ensure task sensitivity, a unique Go/Go design is used since this approach has been shown to be more sensitive to changes in animal affect than Go/No-Go designs in other species[15]. However, the use of an unscented arm containing a low-value reward in all trials differentiates this task from previous failed attempts to validate a Go/Go JB task for mice[36–38]. Here, during positive trials mice choose between a high-value reward in the DS+ arm and a low-value reward in the unscented arm; and in negative trials, they choose between no reward in the DS-arm and a low-value reward in the unscented arm. Although this requires mice to learn a more complex task (i.e., discriminating between cues predicting different values of reward, rather than simply reward presence or absence), it appears that having a consistent option, where a low-value reward was always present, may have made training and testing less stressful for mice and enhanced the learning in mice (with 86% of mice meeting learning criteria). While mice are often assumed to be challenging to train or unable to learn difficult tasks[18], results here suggest that their abilities should not be underestimated, and that designing low stress, ethological tasks may be a more effective approach for detecting changes in affect than simpler tasks or those with harsher consequences (e.g., involving punishment like air puffs or white light instead of simply reward absence[39–41]).

Finally, to further reduce stressors that might otherwise interfere with treatment effects and introduce unwanted variability, humane handling methods were implemented[21]. Here, mice were only handled *via* cup or tunnel methods throughout their lives (including to and from transport cages and the JB apparatus) to avoid the aversive effects of traditional tail handling[21]. In addition to this, EH animals were trained to voluntarily enter an annex cage for handling, thus avoiding stressful 'chasing' through complex environments. Together, this approach led to the detection of pessimistic judgment biases in CH mice through longer latencies in response to ambiguous cues. Future researchers should similarly consider whether aspects of their treatments of interest, housing, or husbandry practices have the potential to mask treatment effects or induce floor effects (i.e., where all animals show marked pessimism, negating the ability of the task to detect more subtle treatment differences) so that these can be prevented or mitigated.

Further replication and refinement of this promising task are now needed. To date, this JB task has only been applied to animals experiencing long-term, low-arousal negative affect (as a result of restrictive housing or chronic disease[19]). It is therefore important that future work aims to test the sensitivity of this task to acute stressors and high-arousal negative affective states. Furthermore, maximizing the value of this task would also involve replicate studies investigating test-retest reliability of individuals to the same, or multiple ambiguous probes. Re-testing with the same probes would allow researchers to test hypotheses regarding changes in affect over time, while exposing subjects to a spectrum of ambiguous cues (i.e., near positive, intermediate, and near negative) could potentially allow for the identification of different types of negative states (particularly depression- versus anxiety-like conditions)[11,42]. Additional validation experiments should also study the value of shorter protocols, as well as potential differences between strains and sexes (though the original publication does address these issues, successfully employing a shorter protocol to assess affect in male mice[19]). Indeed, this task could potentially be extended to any rodents intrinsically motivated to make burrows[43,44] provided appropriate size modifications are made, and validation is confirmed. Such replication and refinements are important since no other valid JB tasks have been developed for mice to date and since JB tasks are sensitive to both the valence and intensity of affective states (as outlined in the introduction), something that most indicators of animal affect fail to do (e.g., hypothalamic-pituitary-adrenal activity can be altered in response to both positive and negative experiences[7,45]).

Overall, the development of a mouse JB task represents a promising new tool and opens the door for great progress in the assessment of mouse affect. Mice are the most widely used vertebrates in both basic and translational research[17], and this task provides a means to answer essential questions about the welfare of these tens of millions of animals used globally, as well as the links between affect and the diseases or conditions they are used to model. Though the use of this task is not recommended for day-to-day welfare assessment, experimental investigation of housing and husbandry practices could help identify refinements that promote mouse welfare and aid in identifying more subtle signs of animal suffering that can be observed cageside. Given the humane and potentially enriching nature of this task, and the low economic cost of implementing the protocol, this novel JB task has great utility.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

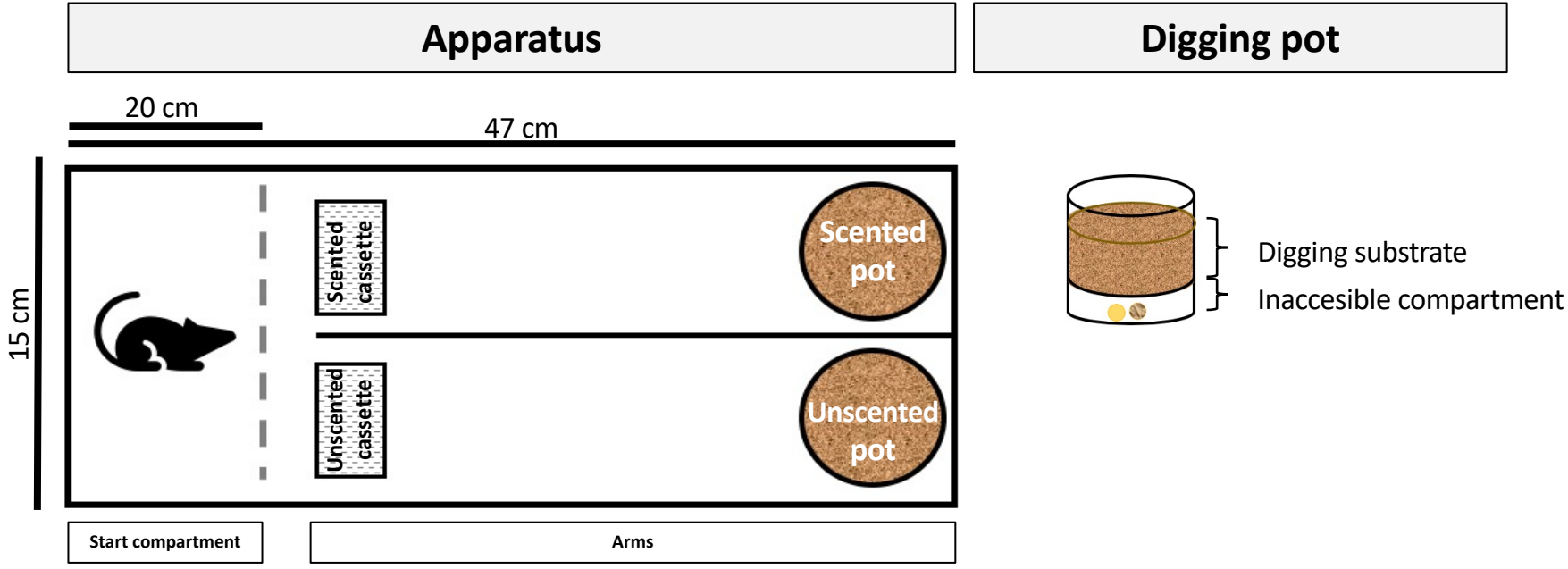
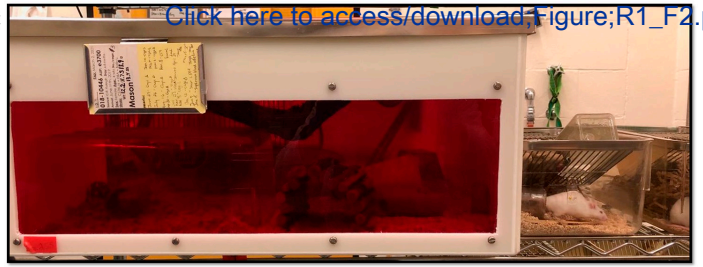


Figure 2



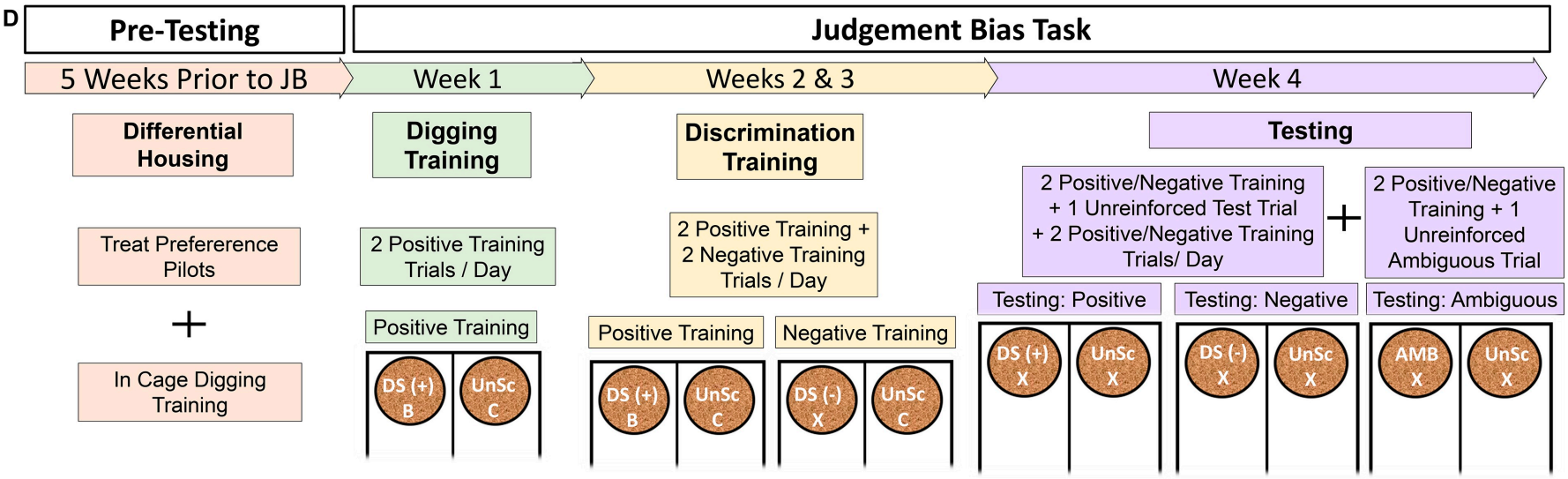


Figure 3

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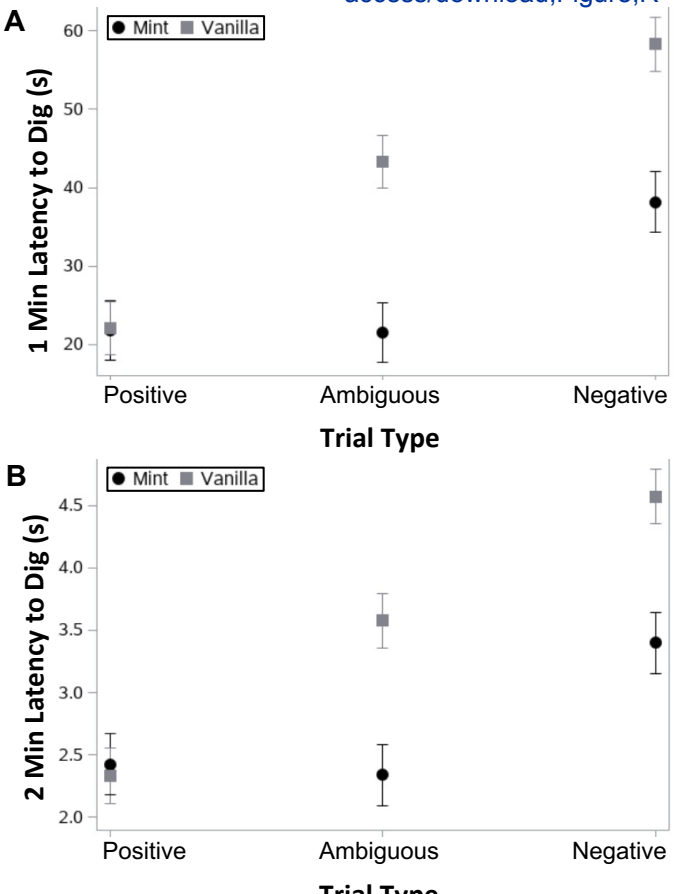

Figure 4

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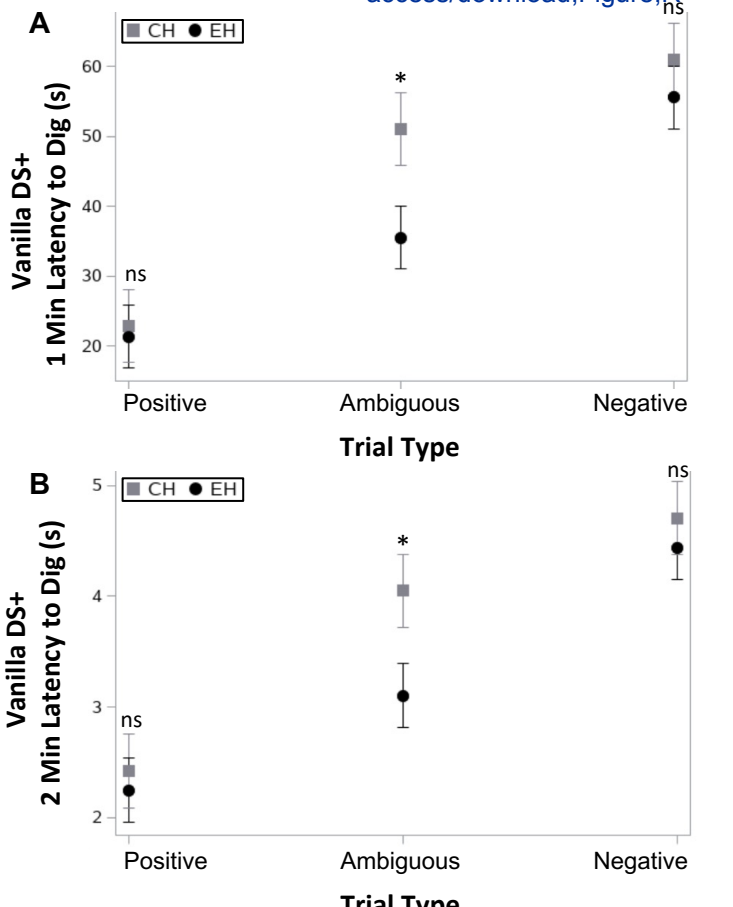

Table 1

Phase:	Experimental Design	
All	High Value Reward	Banana chip
	Low Value Reward	Rodent chow
	DS+	Mint or Vanilla (counterbalanced)
	DS-	Mint or Vanilla (counterbalanced)
Digging Training	Digging Training Schedule	5 days: 2 Pos trials/day
	Digging Trial Duration	5 min
Discrimination Training	Discrimination Training Schedule	10 days: 4 trials/day
	Digging Trial Order	Days 1-5: 4 trials /day Trial 1: Pos Trial 2: Neg Trial 3: Pos Trial 4: Neg Days 6-10: 4 trials /day*
	Discrimination Trial Duration	5 min
Testing	Testing Schedule	3-5 (dependent on time to meet LC) : 5 trials / day
	Testing Phase Order	Trials 1 and 2: Pos or Neg **
		Trial 3: test trial
		Trials 4 and 5: Pos or Neg**
	Test Trial Duration	2 min
* Trials were pseudorandomized so mice always had two Pos and two Neg trials per day		
** Trials were pseudorandomized so mice always had one Pos and one Neg trial before and after the test trial		

Table 2

Trial Details							
Phase	Trial Type	Scented Arm			Unscented Arm		
		Odor Cue	Buried Reward	Inaccessible Reward	Odor Cue	Buried reward	Inaccessible Reward
Digging and Discrimination Training	Pos Training	DS+	Banana	Chow	Water	Chow	Banana
	Neg Training	DS-	No reward	Banana + chow	Water	Chow	Banana
Testing	Pos Test	DS+	No reward	Banana + chow	Water	No reward	Banana + chow
	Neg Test	DS-	No reward	Banana + chow	Water	No reward	Banana + chow
	Ambiguous Test	Mint/ vanilla mixture	No reward	Banana + chow	Water	No reward	Banana + chow
Learning Criterion	Mice must dig twice as long in the DS+ pot (Pos test) than the DS- pot (Neg test), and dig for a minimum of 3 s						

We would like to thank the editor and reviewers for their helpful comments and advice. We have revised our manuscript in response to these, and our point-by-point responses are listed below. All changes made are also indicated in red text in the supplementary Word file submitted.

Editorial comments:

Editorial Changes
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been carefully proofread.

2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

All steps of the protocol are now written in the imperative tense and any information that could not be included in this way has been included as a "Note".

3. Please use SI units as much as possible and abbreviate all units: L, mL, µL, cm, kg, etc. Use h, min, s, for hour, minute, second.

All units are reported in abbreviated SI format.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Allentown Inc, Envigo, Mississauga, Ontario, Canada, CheeriosTM , Harlan® Teklad, Global Diet 14% protein, SAS®9.4, etc.

Commercial language has been removed from the main text.

5. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
Step 1.1.3.1: This statement is confusing. The code allows the researcher to identify the animal and the researcher stays blind to the code ID as outlined in 1.1.3.2.?

We have added more detail throughout the protocol, particularly to steps 1.1.3 regarding blinding, 1.4.3 regarding digging pot preparation, 2.8 and 4.6 regarding live- and video-scoring of digging behavior and 5 regarding data analysis.

Step 1.1.3 noted in the comment now reads:

1.1.3 Randomly assign each animal a "blind code".

NOTE: Blinding allows the researcher handling the mice and scoring their behavior to remain unaware of the animal ID or treatment, avoiding undesirable subjective bias. This is a mandatory step to comply with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and other reference documents[20].

1.1.3.1 In a spreadsheet containing each animal ID and the corresponding treatment they have been assigned to, add a column for blind code.

1.1.3.2 Randomly assign each animal a unique code (e.g. a letter and number combination "A2") that is unrelated to cage number or treatment.

NOTE: All randomization should be conducted using a random number generator (e.g. Random.org). During this randomization step, counterbalance across treatment, strain etc., where applicable.

1.1.3.2.1 Have this done by a research assistant who will remain "unblind" to treatment throughout experiments. This individual must not collect data during testing, to avoid biased assessments.

1.1.3.3 During all data collection, only provide the "blind" researcher who is live- and video scoring latency to dig and digging duration with the animal's blind code, keeping them blind to treatment.

NOTE: Spreadsheets used during data collection will only include the animal's blind code and the corresponding latency to dig and digging duration for each trial. At the end of experiments, the corresponding animal ID and treatment information can be added to the spreadsheet, thus "unblinding" researchers for data analyses.

6. Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

All highlighted steps now include an action written in the imperative tense.

7. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure and Table Legends together at the end of the Representative Results in the manuscript text.

Figures have now been edited to meet these requirements.

The author instructions state to "Indicate via brackets if figure/table placement at another location in the text is preferred [Place Figure 1 here]." This was the approach we had followed for Figure 1, Table 1 and Table 2 (to be placed at the end of the Protocol), but this can be changed if required.

8. As we are a methods journal, please also include in the Discussion the following along with citations:
a) Critical steps within the protocol

L 622-628 now reads: The task thus presents a valuable tool for diverse fields of research. Like any JB task, to assess animal affect it is critical that animals first learn to discriminate between cues (step

4.6.2) and that the ambiguous stimulus is interpreted as intermediate (step 5.3). Though simple, meeting these requirements can be challenging, particularly in laboratory mice for which over 15 past attempts to develop and implement a mouse JB task have failed[19]. Here, multiple components played an essential role in meeting these technical criteria, and contributed to the success and utility of the task.

b) Any limitations of the technique

Limitations of the technique are detailed within L675-693, and specific goals for future work to replicate and refine this task are discussed (e.g. application in different mouse strains and rodent species, assessing repeated testing with multiple or repeated ambiguous probes).

c) The significance with respect to existing methods

We have now expanded on this within the discussion.

L645-660 now reads: Thus, to ensure task sensitivity, we used a unique Go/Go design since this approach has been shown to be more sensitive to changes in animal affect than Go/No-Go designs in other species[15]. However, the use of an unscented arm containing a low value reward in all trials differentiates this task from previous failed attempts to validate a Go/Go JB task for mice[e.g., 36–38]. Here, during positive trials mice choose between a high value reward in the DS+ arm and a low value reward in the unscented arm; and in negative trials they choose between no reward in the DS- arm and a low value reward in the unscented arm. Although this requires mice to learn a more complex task (i.e. discriminating between cues predicting different values of reward, rather than simply reward presence or absence), it appears that having a consistent option, where a low value reward was always present, may have made training and testing less stressful for mice and enhanced learning (with 86% of mice meeting learning criteria). While mice are often assumed to be challenging to train or unable to learn difficult tasks[18], results here suggest that their abilities should not be underestimated, and that designing low stress, ethological tasks may be a more effective approach for detecting changes in affect than simpler tasks or those with harsher consequences (e.g. involving punishment like air puffs or white light instead of simply reward absence[e.g., 39–41]).

L689-693 now reads: Such replication and refinements are important since no other valid JB tasks have been developed for mice to date; and since JB tasks are sensitive to both the valence and intensity of affective states (as outlined in the introduction), something that most indicators of animal affect fail to do (e.g., hypothalamic-pituitary-adrenal activity can be altered in response to both positive and negative experiences[7, 45]).

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The protocol describes a method of evaluation of judgement bias in mice. The procedures are well described and should be easy to follow.

Major Concerns:
None

Minor Concerns:
I miss indication of statistical significance in Figures 3 and 4. Also, I feel, that statistical considerations might be a specific paragraph, including power analysis, etc.

Thank you for pointing this out and for the helpful suggestion. Statistical significance is now indicated in Figure 4. We did not add the statistical significance in Figure 3 because its objective was to confirm (or visually inspect) that one of the technical criteria (step 5.3) was achieved. This is now made clear in the title of the figure too, which reads "Figure 3. Determining whether mice meet requirements for JB assessment."

We have also included details on data analysis within the protocol. Step 5 (L458-487) now reads:

5 Data analysis:

NOTE: Exact analyses required will depend on the details of the experimental design. A general overview is outlined here, but researchers are strongly encouraged to refer to Gygax[22] when planning analyses for animal JB experiments and to Gaskill and Garner [23] when selecting sample size (since required analyses are often too complex for a priori power analyses).

5.1 "Unblind" the researcher by adding the corresponding animal ID and treatment information for each blind code to the spreadsheet. The resulting spreadsheet will include 3 rows for each animal that met learning criteria – one for the positive, negative and ambiguous test trial – indicating their latencies to dig in the scented arm.

5.2 Run a repeated measures generalized linear mixed model using preferred statistical software. Here, the outcome variable will be latency to dig, and the model must include Treatment (or continuous variable of interest), Trial Type and the Treatment*Trial Type interaction as fixed effects. Mouse ID (nested within treatment) must be included as a random effect. Additional terms included in the model will depend on the experimental design applied.

NOTE: If mice are group housed (as is appropriate for this social species[24]) and cage mates are tested, then cage nested within treatment (if present) must be included in the model as a random effect (and Mouse ID must subsequently be nested within cage).

5.3 Plot the least square means of latency in each trial type to confirm that the ambiguous cue presented was interpreted as intermediate (i.e. that the latency least square means estimate for the ambiguous trial falls at a midpoint between the positive and negative latencies). Mouse JB can only be assessed if this requirement is met.

5.4 Assess the simple effect of treatment (or continuous variable of interest) on latency to dig in the ambiguous trial (i.e. investigating the Treatment*Trial Type interaction) to determine whether mice display affect-modulated JB.

I mostly work with rats, so I am curious whether the protocol is transferable to rats.

Though the task would require validation in rats, we believe that this task could be applied in other rodent species that are motivated to burrow.

Thus, Line 796-798 now reads: Indeed, this task could potentially be extended to any rodents intrinsically motivated to make burrows[43, 44] provided appropriate size modifications are made, and validation is confirmed.

Reviewer #2:
The manuscript describes a mouse judgement bias task that has previously been validated by Resasco et al 2021.

I only have a few minor comments:
* In the Experimental preparation section, the authors describe preparation of positive discriminative stimulus (DS+) with both vanilla and mint scents. In the Resasco et al. 2021 paper, Mint DS+ mice interpreted intermediate odor mixtures as positive and not ambiguous. The authors address this issue in the Discussion (Lines 544 - 548). It would be useful for the reader to highlight this issue in the Experiment preparation part, in the same way as the authors urge the reader to conduct pilot tests to identify high and low value rewards prior to starting the task (Line 136).

Thank you for this excellent suggestion! L99-108 now reads:

1.1.1 Pseudorandomly assign cages to mint or vanilla positive discriminative stimulus (DS+) groups:

NOTE: This protocol has only been validated for mice assigned to vanilla DS+ odour mixture (see Representative Results and Resasco et al.[19] for details). However, we strongly encourage authors to conduct pilot tests to confirm that the DS+, DS- and ambiguous mixtures used meet requirements for valid JB assessment (steps 4.6.2 and 5.3). Thus, the methods outlined here include the testing of both a vanilla and a mint DS+, to provide an example of a group that successfully meets requirements for assessment of JB (the vanilla DS+ mice), and a group that fails to do so (the mint DS+ mice).

* In general, many aspects of this paradigm have only been validated in one sex (females) and two strains of mice (C57BL/6NCrl and Balb/cAnNCrl) and the reader should be encouraged to conduct pilot studies if using other strains.

Thank you, we have now acknowledged this.

L684-687 now reads Additional validation experiments should also study the value of shorter protocols, as well as potential differences between stains and sexes (though the original publication does address these issues, successfully employing a shorter protocol to assess affect in male mice[19]).

For example, according to the protocol, the mice are fasted for one hour prior to training (Lines 244) and 334). This may have worked in this experiment, but is often not sufficient to motivate mice to perform in cognitive tasks as the animals will often shift their feeding schedule. It also depends when in the dark

phase the training takes place - mice usually feed at the beginning of the dark phase. These are all minor details that can affect learning the task. Perhaps it is not the scope of this manuscript but maybe the authors can direct the reader to other options of food restriction and deprivation commonly used, which may facilitate discrimination learning resulting in fewer drop outs (Lines 387).
Some exampels here:
Wahlsten. "Chapter 11 - Motivating Mice", In Wahlsten, Douglas, and John C. Crabbe. "Behavioral testing." The mouse in biomedical research. Academic Press, 2007. 513-534.
Graulich, Dana Marie, et al. "Looking on the bright side of bias—Validation of an affective bias test for laboratory mice." Applied Animal Behaviour Science 181 (2016): 173-181.

We understand the reviewer's concern. However, we believe that the short fasting time is a strength of this protocol for multiple reasons. First, fasting can alter a number of physiological and behavioral variables and as a consequence, it is recommended that it be kept to a minimum (Jensen et al 2013). Indeed, hunger itself might affect appraisal in cognitive bias tasks (Verbeek, Ferguson & Lee, 2014). Moreover, if mice fasted for longer periods of time are at risk of losing preference for high vs. low value food rewards (Novak et al. 2016), a critical component of this task (i.e., ensuring animals prefer the DS+ arm over the unscented arm). Finally, the mice used in these experiments were unlikely to have shifted their feeding schedule since order of testing was random/opportunistic across days. All in all, our mice were extremely successful in learning the task, as 31/35 mice met the learning criterion, and replicate experiments in our lab have shown similarly high success rates (over 80%).

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