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Operant Conditioning Task to Measure Song Preference in Zebra Finches

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W.M. Keck Science Department
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September 19, 2019

To the Editors:

We are pleased to re-submit our manuscript, "An operant conditioning task to measure song preference in zebra finches" for consideration for publication in JoVE.

We thank the reviewers for their comments. We have addressed all the comments and have changed the manuscript substantially. We have 1) included a great deal more description of the procedures in the protocol section. 2) Added more data to the results section. 3) Completely re-written the results section. 3) Added additional figures. 4) Changed some of the discussion. We appreciate the feedback and think the manuscript is improved.

Sincerely,

Nancy Day and Melissa Coleman

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TITLE:

Operant Conditioning Task to Measure Song Preference in Zebra Finches

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KEYWORDS:

songbird, monogamy, pair bond, social, partner preference, dopamine

SUMMARY:

We describe a technique to evaluate song preference in zebra finches. Females are placed in a two-chambered cage and song preference is measured by the number of times she triggers the playback of one song by landing on a perch within one chamber, compared with triggering a different song in the second chamber. Perch landings are counted using infrared sensors.

ABSTRACT:

An operant conditioning paradigm is used to test the song preference of female zebra finches. Finches are placed in a two-chambered cage with a connecting opening and indicate their preference for a song by landing on a perch within each chamber. By interrupting the infrared beam from a photoelectric sensor above each perch, the bird activates the playback of a song through a speaker located on each side of the cage. Freely available software is used to trigger the song playback from each perch. To determine the song preference of each animal, her chamber preference is first identified by triggering no song playback when she lands on each perch. This chamber preference is then compared to her song preference. A minimum activity threshold is set to ensure the preference is real. Using this method, we show that paired females prefer the song of their partner. This method was used to understand the contribution of dopamine to the formation and maintenance of song preference.

NTRODUCTION:

One of the fundamental questions in biology is how animals form affiliative bonds. In particular, what are the neural mechanisms by which these bonds are made and are these basic mechanisms conserved across vertebrate species? The prairie vole has given clues to some of the neurotransmitter systems that are important for pair-bond formation[1–3]. In particular, dopamine acting through receptors in the nucleus accumbens can induce partner preference in both male and female voles[4,5]. It is unclear whether the general principles underlying partner preference formation and maintenance are evolutionarily conserved.

Monogamy is more common in birds than in mammals[6]. Therefore, comparing mechanisms of affiliative behavior in birds to other species is critical to understanding conserved neural foundations[7–9]. In general, across many species of songbirds, male song is thought to serve as an honest indicator of male fitness[10,11]. Male zebra finch songbirds sing to attract mates and influence the formation of monogamous pairs[12,13]. Thus, song can be used to determine partner preference in these birds.

The mechanisms by which females form a preference for their partner's song is unknown. Mesotocin, the avian homologue of oxytocin, appears to play a role in pair-bond formation in finches[14,15]. In addition, dopamine has also been shown to play a role in pair-bond formation[9,16–18]. For example, dopamine levels are higher in the nucleus accumbens of paired versus non-paired finches[9].

To study the role of neurotransmitter systems on pair-bond formation and maintenance, we measured song preference using an operant testing paradigm equipped with infrared sensors to trigger song playbacks[19]. The ratio of song playbacks identified the female's preference for a male's song. Prior to the operant conditioning task, each female was isolated for up to 48 h in an anechoic chamber with her partner for 'paired females' or with an unfamiliar male, for 'unpaired females'. To test the effect of dopamine on song preference, unpaired females were treated twice with a D2R dopamine agonist while isolated with the unfamiliar male. This behavioral paradigm is based on previous studies[19,20] and is amenable to research by undergraduate students.

PROTOCOL:

All experiments were approved by the Institutional Care and Use Committee of the W.M. Keck Science Department, in accordance to NIH guidelines. All animals used were adults (>90 days post hatch).

1. Construction of operant conditioning chamber

1.1. Build a testing cage composed of two identical chambers, 15 x 15 x 17 in each (**Figure 1A**). Make a small door (as an entrance to each chamber) on the side of the cage near the perches by cutting out a small 4.5 x 6 in window that is 2.5 in away from the center of the cage between the chambers. Use a separate piece of the shelving to make a door that covers the window.

NOTE: The cage is made from wire shelving for closets that can be obtained from any home improvement store. The hinge of the door can be made with zip-ties.

1.2. Cut the shelving with wire cutters to make a 4 x 6 in opening between the two chambers to allow the bird to move between chambers.

1.3. In each chamber, place a perch 11 in from the center of the cage and 6 in from the bottom of the cage. Use any perch that is suitable for the type of bird being tested and that will span the width of the cage.

1.4. Place the emitter and receiver of the photoelectric sensor on the cage directly above each end of the perch. Tether the sensors to the wire above the perch using zip-ties.

NOTE: Placing the sensors onto a rigid backing (e.g., a tongue depressor) can help keep the sensors from twisting on the wire cage and becoming misaligned. It is important to test the placement of the sensors prior to each experiment by manually breaking the infrared (IR) beam. If the sensors are not correctly aligned, a bird will not trigger a song, or landing on the perch may be counted as multiple events.

1.5. Place under-cabinet LED lights on the top of the cage to provide enough illumination so that the animals will move freely between the two side chambers.

1.6. Place bird seed and water next to each of the perches.

1.7. Place a speaker at each end of each chamber.

1.8. To reduce sound diffraction and audio and visual distractions, place anechoic foam on the perimeter of the entire cage, with holes left for the cage doors and water bottles.

1.9. Connect the speaker to an audio amplifier and connect the audio amplifier to the sound output on a computer.

2. Connection of sensors

NOTE: There are two sets of IR sensors, each with an emitter and receiver. The emitter has a cable with four wires (brown, blue, black, and white). The receiver has a cable with three wires (brown, blue, and black). The white wire of one emitter is connected directly to input #1 on the digital I/O board. The white wire of the second emitter is connected directly to input #9. The AC power input has two wires (usually red and black).

2.1**.** Install a digital I/O card (**Table of Materials**), its drivers, and the associated program 'Measurement and Automation'.

NOTE: The digital I/O card used here requires a connector block and multifunction ribbon cable to complete installation. Alternatively, a USB I/O device requires no PCI card (or the required connector block and ribbon cable accessories), and can be run on a laptop.

2.2. Connect the two blue wires of each emitter and receiver set together. Attach two small wires to the black wire of the power cable so that the black power wire can connect to the blue wires of each sensor set.

2.3. Connect the black power wire and two blue wires to a common wire. Connect this common wire to either input #2 or # 9 on the connector block.

NOTE: One set of sensors should be connected to input #1 and #2 and the other set of sensors should be connected to input #9 and #10.

2.4. For both sensor sets, connect the brown wire from the emitter and the receiver together. For each sensor set, connect the two brown wires to the red AC power wire.

NOTE: All four brown wires are connected to the red AC power wire. The photoelectric sensors are powered by a 10–30 V input and must be connected to an AC converter (e.g., a 120 V to 12 V converter).

2.5. Within the **Measurement and Automation** program associated with the digital I/O card, determine the Device ID for the I/O card by selecting the **Devices and Interfaces** menu.

2.6. Verify that the I/O detects when the IR beam is broken using the **Test Panels**… option in the program. Note the channel and port IDs for each set of sensors.

NOTE: Green indicators will change color when the status of the beam is altered.

3. Install software and hardware to count perch landings

3.1. Download and install Sound Analysis Pro 2011 (SAP2011) and MySQL from http://soundanalysispro.com/.

NOTE: This is freeware and comes with installation instructions and a user manual.

3.2. Open SAP recorder.exe.

3.3. To configure the sensor input so that SAP can detect perch landings and initiate song playbacks, click the **Operant Devices** tab in the SAP Recorder control window. Check the box **Enable Operant Training (NI Card Installed)**.

3.4. Select the appropriate Device ID (the **Ports** slider can be set to **3**). For each detector, indicate the appropriate port and line from the information collected in step 2.6.

NOTE: When the port and line are set correctly for each detector, the light will change from yellow to red.

3.5. In the **Main** window for the SAP recorder, hit **Train** to activate the sensors for the appropriate channels.

NOTE: A yellow button should appear.

3.6. Record the name of the bird by selecting the channel number of interest (Press **channel 1** or **2** tab on the left side of the screen), and in the **Identification and Mode** window, type in the bird's identification in the **Name** field.

3.7. To ensure that sound is played to the speakers, manipulate the settings in the **Output Selection** tab to select the appropriate device connected to the computer (i.e., speakers) and the channel connected to the speaker.

NOTE: Additional setup information can be found in the Sound Analysis Pro user manual.

4. Isolate pairs of birds and collect male song

4.1. Isolate a male and a female finch in a sound-attenuation chamber for 24−48 h. During this cohabitation, collect the male song, which is referred to as the 'partner's song'.

NOTE: Paired birds are a male and female that have been housed together for at least 2 weeks. Unpaired females are placed with a male for only 24−48 h.

4.1.1. Use a microphone connected to an audio amplifier to capture the partner's song.

4.1.2. Acquire the song using Sound Analysis Pro 2011 and store the song on a hard drive.

4.1.3. To test for the effect of the neurotransmitter systems on female song preference, give the female a 50 µL subcutaneous injection of saline (vehicle control) or quinpirole (1 mg/mL in 0.9% saline) when the pair is first put into the chamber. Repeat the injection after 6–24 h (see **Figure 1B**).

4.2. Create songs for playback in the operant conditioning cage.

4.2.1. Create a .wav file that plays '**silence**' approximately the same length as the song playbacks.

NOTE: This can be done using any sound-editing software (e.g., Audacity).

4.2.2. Cut a representative song (2–3 motifs) from the partner male.

4.2.3. Cut a representative song (2–3 motifs) from an unfamiliar male.

NOTE: Use the same unfamiliar male song for all experiments.

4.2.4. Filter the songs from 300 Hz–10 kHz.

4.2.5. Adjust the volume so that the songs play at ~70 dB (average amplitude over the song duration from the speakers in the behavior chamber).

NOTE: The volume of the song is tested using a sound pressure meter that is placed at the perch.

5. Testing song preference in paired females

5.1. Determine the female's side chamber preference.

5.1.1. Prior to the behavioral testing, place the female in the testing cage to allow her time to adjust to the cage for at least 1 h. Ensure the finch explores both side chambers by using an object to cause the bird to move from one chamber to the other through the opening.

5.1.2. In Sound Analysis Pro, select the '**Playbacks**' tab and then the '**Sounds**' button in the main window.

5.1.3. Select the audio .wav file to play '**Silence**' from chamber 1 and chamber 2.

5.1.4. Go back to '**Main**' and hit '**Reset**' on the top of the boxes on the right.

5.1.5. After the acclimation period, press '**Start**'.

5.1.6. At the end of the session, press '**Stop**'. Write down the number of triggers at each perch, which is displayed in the blue boxes.

NOTE: The data are saved to a MySQL database. Have a minimum number of triggers to count the trial to ensure the female is active enough (e.g., 12 or more perches total).

5.2 Determine song preference.

5.2.1. Repeat the steps in section 5.1, but choose the partner's song to play from the side chamber with the fewest perch triggers (the non-preferred side), and an unfamiliar song from the side chamber with the most perch triggers (the preferred side).

NOTE: The partner's song is played from the side chamber with the fewest triggers during the playback of silence (the non-preferred side).

5.2.2. Press '**reset**' prior to starting the 1 h with song playback.

5.2.3. Calculate the chamber and song preference by dividing the number of triggers on the side chamber playing the partner's song by the total number of triggers.

REPRESENTATIVE RESULTS:

Following the protocol, we found that paired females preferred their partner's song (**Figure 2A**). There was a significant difference between the side chamber preference during silence to that during song playback (t-test corrected for multiple comparisons; $p = 0.004$; $t = 3.35$, $df = 16$). Thus, the female preferentially triggered the song of her partner in comparison to the song of an unfamiliar male.

Females that were paired with an unfamiliar male for 24–48 h and given saline injections did not have a preference for his song over the song of another unfamiliar male. That is, there was no difference between the side chamber preference when landing on a perch that triggered silence compared to triggering song (t-test corrected for multiple comparisons; $p = 0.726$; $t = 0.357$, $df = 16$). Therefore, females housed with a male for less than 48 h did not form a preference for his song.

After showing that we could use the behavioral paradigm to test the song preference of female finches, we used the assay to test the influence of dopamine on formation of song preference. In this case, a naive female was housed with an unfamiliar male for 24–48 h and given two subcutaneous injections of the dopamine 2 receptor agonist quinpirole (**Figure 1B**,**2B**). Overall, females given quinpirole preferred the song of the male she was with for only 24–48 h (t-test corrected for multiple comparisons; $p < 0.001$; $t = 5.25$; $df = 18$). In the example shown, two of the females did not show a preference for her partner's song (**Figure 2A**). In one case the female was older (>5 years). It is not clear why the second female did not form a preference. One possibility is that the female may not have been given a complete dose of the drug.

To ensure there was no difference in total activity of females between treatment groups that could account for the difference in song preference we compared the total number of perch triggers (**Figure 2B**). The total number of perch triggers is the number of times a female landed on both perches during the 1 h testing period for either silence or song playback. The total activity of the birds in any treatment was similar (ANOVA, $p = 0.436$).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of operant conditioning cage and timeline of the experiment. (**A**) The cage has two chambers of equal size with an opening connecting the two side chambers. An opposed pair of photoelectric sensors is placed above each perch that spans the width of the chamber. When a bird lands on a perch she breaks the IR beam from the sensors, which triggers the song playback from the speaker nearest the perch. The dimensions of the cage are shown, in addition to the placement of the perches. (**B**) Timeline of the experiment. This figure was modified from Day et al.[21].

Figure 2: Representative data of female song preference. (**A**) The ratio of perch triggers during operant testing indicates the preference for songs produced by conspecific males. Each female's song preference was compared to her side chamber preference in which no song (silence) was triggered when she landed on a perch. In each case the partner's song was played when she landed on a perch in the non-preferred chamber. Connected points are preferences for each individual. This figure was modified from Day et al.[21]. (**B**) The total number of perches on each side chamber of the cage for females in all conditions.

DISCUSSION:

We describe a method to test the song preference of zebra finches. We used this assay to test the preference of a paired female for her partner's song. In addition, the assay was used to test the effect of dopamine on induction of song preference in naive females. This method is relatively inexpensive and was designed and used by undergraduate students, making it an excellent training tool for students. Several other studies have tested song preference in several song species, making this method useful to many investigators studying auditory preferences in birds[22,23].

This behavioral paradigm is based on an auditory recognition testing system, ARTsy, first described by Gess et al.[19]. These authors used a similar system to test song discrimination in birds. We modified the system to test the preference of a female for one song over another. We adapted the protocol described in Gess et al.[19] to use SAP2011 to play song and collect the data, instead of custom scripts in MATLAB. We found this change for data acquisition easier to use and more accessible to undergraduate students. Operant playbacks can also be triggered using the perches as switches[24].

We found the protocol described here results in data that are easier to analyze than using similar methods. For example, Woolley and Doupe[20] measured the length of time a female spent on each side of a cage during passive playbacks of different song types to identify song preference. Quantifying the time in one chamber compared to another requires filming the birds while they behave, which is not required for the protocol described here. Similarly, others have counted the number of female calls in response to male song as a measure of her preference[25,26]. This requires using manual or semiautomated counting of female calls. The quantification of song preference described here is much simpler as the software automatically keeps track of the number of perch landings.

Using the protocol described here we found that paired females consistently chose to listen to the song of their partner. The task does not test the female's partner preference, only her song preference. Future experiments should examine a partner preference as well as song preference[13,16,27]. This protocol can be used to test the influence of other neurotransmitter systems on formation of song preference to compare the conserved evolutionary mechanisms underlying social behavior network shared by many vertebrates[7,28]. For example, future experiments can target directly specific areas in the brain while animals are learning the task to gain access to the neural circuits underlying social behaviors.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

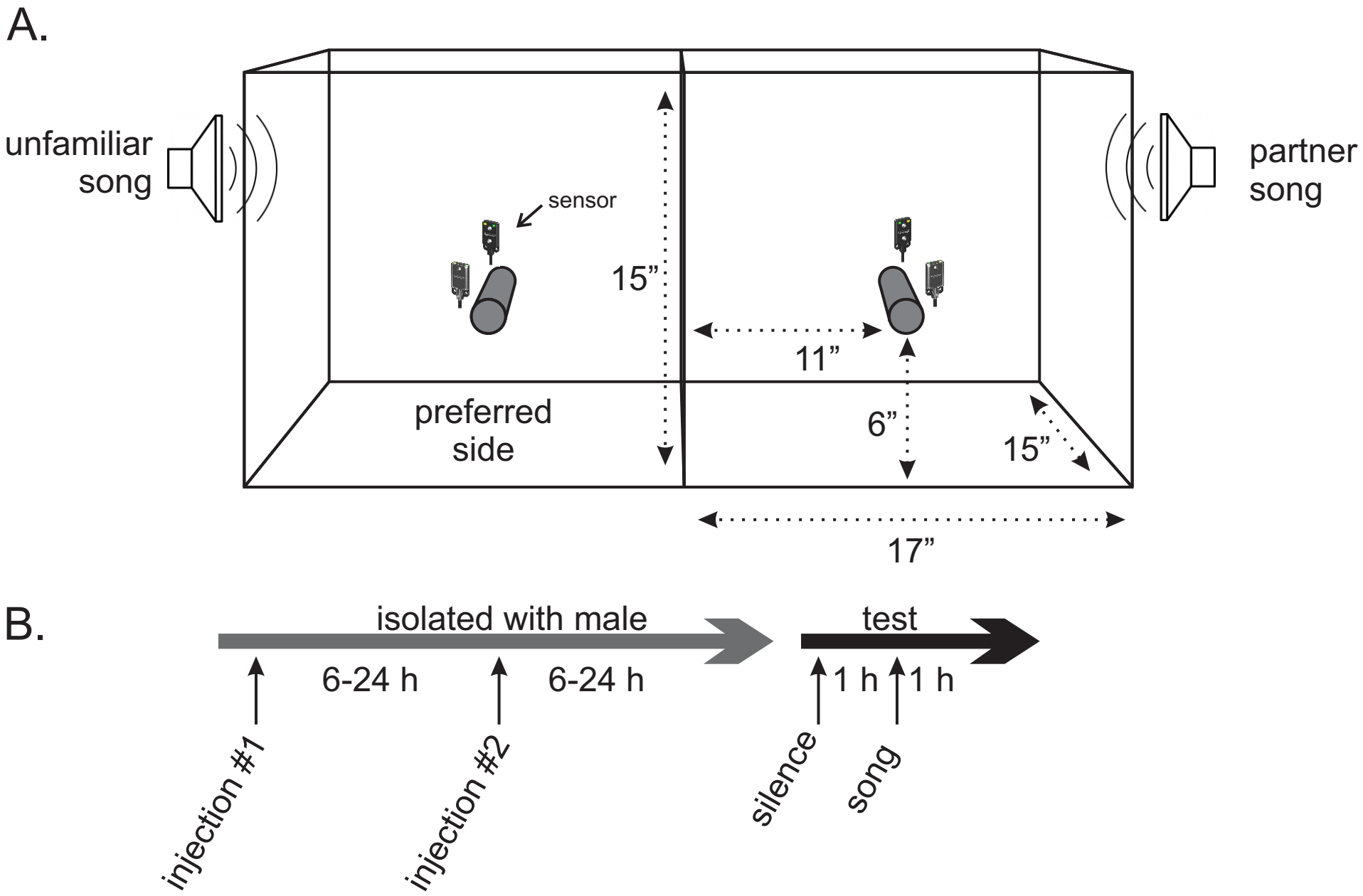
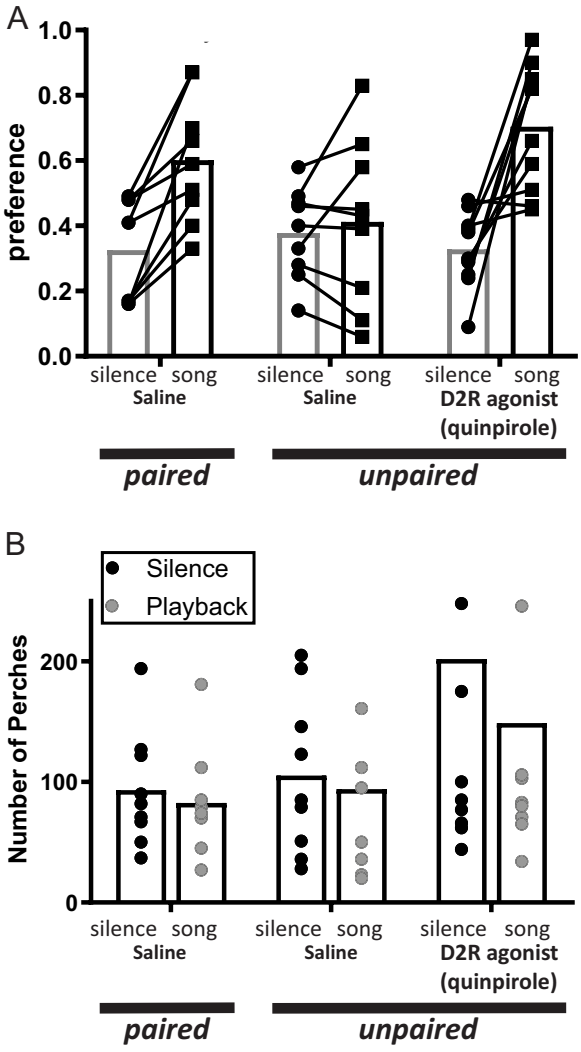


Figure 2



Name of Material/Equipment	Company	Catalog Number	Comments/Description
(-)-Quinpirole	sigmaaldrich	Q102	
acoustic foam	Coulbourn Instruments, Allentown, PA	H10-24A	
audio amplifier - 2 channel	Amazon	Pyle PCAU46A	any small audio amplifier should work
Banner Engineering Q08 Series, emitter. SO60-Q08	https://www.alliedelec.com	Stock #: 70659809	photoelectric sensor
Banner Engineering Q08 series, receiver. EO60-Q08-AN6X	https://www.alliedelec.com	Stock #: 70699384	photoelectric sensor
Car stereo speakers	Amazon		Pioneer TS - F1643R
Digital I/O card	National Instruments	PCI-6503 or USB-6501	
LED lights - under-counter	Amazon		
multifunctional ribbon cable	National Instruments	180524-20	
sound pressure level meter	Amazon		
terminal block	National Instruments	777101-1	

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Please revise lines 208-211 to avoid textual overlap with previously published work.

Done.
3. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

We have included a statement at the beginning of the protocol section.

4. National Instruments

We are not sure what this means.

5. Please add more details 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. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
6. Section 1: A schematic of the chamber setup would be helpful to guide the readers.

We have added much more detail in the protocol.

Figure 1 is a schematic of the chamber setup. We have added the dimensions of the cage to Figure 1, we also added a timeline to the figure (now Figure 1B).

7. 1.1: Please specify the material used to build the cage.

This has been added
8. 1.2: Please specify the type/material of the window.

The window is a cut-out of the cage. A short description stating the window is cut from the cage has been added.

9. 1.3: Please specify the type/size of the perch.

Any perch can be used. We used a regular wooden dowel, which we added to the protocol. We prefer not to give a detailed description as this is something generic.

10. 1.4: Please describe how to place the sensors. How they are fixed/attached to the perch?

We have added this description. They are zip-tied onto the cage above the perch.

11. 1.5: Please describe the type of the LED light (e.g., light bulb or tube?).

Added. These are basic under-cabinet LED light strips.

12. 1.8: Where exactly is the anechoic foam placed?

Added to the protocol. This is added to the outside of the cage.

13. 2.1.5 and 2.1.6: Please describe how to record the name and configure operant playbacks. Please ensure that the protocol here can stand alone. As currently written, users must refer to another protocol and refer back and forth in order to complete this protocol. Please remove the references to the specific steps of the other manual.

Some of this information was already in the protocol. We have added a more complete description of the steps.

14. 3.4: Please specify the drug given here.

Done

15. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

16. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

17. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the

editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
18. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.
19. References: Please do not abbreviate journal titles; use full journal name.

We apologize for this error. We did use the JoVE template for Endnote and cannot understand why this was not formatted correctly.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The protocol is for a behavioral paradigm aimed to determine song discrimination properties of adult songbirds, here: zebra finches. The basic idea is to use a 2-chamber system with perch-activated audio playbacks to compare the number of instances when the bird triggers playbacks on either side of the cage. The paradigm is nice in that the song is triggered by the bird's behavior, which can strengthen interpretation of stimulus preference, i.e. the bird is motivated to perch in one chamber versus the other. Song is an important social stimulus for males and females across development and across species. This paradigm could therefore be of use for simple yet informative discrimination testing beyond the adult female zebra finch subjects used here.

Major Concerns:
There are several major concerns.

First, not enough information is provided given to construct the equipment from scratch. Given that undergraduates were referenced several times, the instructions should be detailed enough for a relatively inexperienced person to build a working system.

We have included more detail of the construction of the cage, connecting the sensors, and using the software.

In the chamber section alone, addition of such details as the materials used to build the chamber, the height and material of the window, the specs of the songs that need to be considered, how to test for "correct alignment", exact positions of food/water and perches etc would transform this description into a useable protocol but none of this information is currently provided.
The same level of detail is lacking from the other Protocol sections. Some better explanations of the connectors and chapters of the SAP manual, what to look for in the software, how the perch landings were scored (it says "record the number of times" is this manually? In SAP? In some other behavioral scoring software?), how one "ensures" that the bird explores both chambers, how a student would measure for 70dB playback pressures, are needed for someone to follow the procedure. Diagrams might be particularly useful to show the setup.

Figure 1 is a diagram of the set-up. We have added the dimensions of the cage to this figure. We have added more details about where the materials can be found. We do not feel that the exact location of the food and water are important, only that they are mirrored in each side of the cage. The window is simply a section of the cage that has been cut out that allows the bird to move from one chamber to the other. We have added this detail to the protocol.

Song amplitude is measured with a sound pressure meter (available from Amazon), which we have added to the protocol. SAP collects all the data and displays the number of triggers in the software.

Second, not enough behavioral information to assess how rigorous the paradigm is or how reliable the outcomes are.

For example: What is the similarity between the two songs that are played? What time of day are the sessions performed? Is there more than one session per bird, and if so, what is the scheduling of those? Were any behavioral metrics used to confirm social bonding beyond the co-habitation time to take into account individual differences in this complex behavior? What are the trajectories towards learning which chamber has which song since there is no prior training for the birds to learn that perch-hopping would trigger any sound? Relatedly, what are the raw numbers, not just proportions of perch-hops that occur in one hour? Is the quantity sufficient for robust examination and consistent results? Is one hour the optimal period of time to assess the behavior? How many times does the co-housed male need to sing to have an effect?

We agree. We tried to use data that was collected after the paper describing the results was published to avoid copywrite permissions. We realize that this does not provide enough data for statistical analysis and have added the data from the previous publication, in addition to a little more data that was more recently collected to the current manuscript.

Many of these questions are very interesting and can be addressed in future experiments using the procedure described. We have simply described a method to assess song preference.

We have added a figure with the raw number of triggers. We did not include birds in our analysis that do not move sufficiently between chambers to give us confidence in the choice of preference. A bird must land on a perch as least 12 times in an hour for us to include the data.

Third, by what metric can this task be considered "more reliable than related methods"?

We have changed this paragraph.

Fourth, the explanation of the timing of the drug administration is misleading, as this would depend on the activation time and half-life of whatever drug was used, and would depend on the relationship to the time spent in the chamber.

We have added a time-line of the drug delivery that we hope helps understand the timing of drug delivery. We did not test the half-life of the drug.

Fifth, the Representative Results section is massively redundant with the Protocol section. Further, there are some method details, e.g. how to administer the drug, that are only in the Results section but missing from the Protocol section.

Excellent point. We re-wrote the results section and think it is much improved and more focused (and less redundant).

Minor Concerns:
Dose, vehicle, source of drug is not included.

Included.

The results in lines 186-187 are confusing with respect to the time scales - how is 24hr comparable to 24-48hr plus drug? That is two factors that are different, not one.

As stated above, we hope the addition of the timeline to Figure 1 will help reduce the confusion.

The perch positions are described differently in line 88 and line 161

Thank you for finding this mistake. We have included the placement of the perches in Figure 1.

Reviewer #2:
Manuscript Summary:
The authors describe an operant conditioning method that can be used to assess female song preferences. The study system the authors used is pair bonding in the monogamous zebra finch. Understanding the behavioral and neurobiological mechanisms underlying pair bonding is a fundamental question in biology. Additionally, this is a valuable behavioral assay for assessing female song preference which can be applied to a variety of study species/systems.

Major Concerns:
I have no major concerns.

Minor Concerns:
1. Ln 60-61; Given the citations here, it may be more appropriate to say "...in general, across many species songbirds, male song is thought to serve as an honest indicator in male fitness" and/or "in zebra finches, male song is critically involved in pair bond formation". Another possible reference here is Woodgate et al., 2012 Animal Behavior.

Thank you for the suggestions. We have changed the wording and added the reference.

2. In the discussion, it comes across that only one other published study has used similar methods to assess female song preference. Importantly, this paradigm has been used in other bird

species/study systems (e.g. Leito et al., 2006 Anim Behav; Hernandez et al., 2017 J Comp Psychology; Hahn et al., 2017 Anim Behav). This makes the authors manuscript and description of the female preference assay even more valuable and applicable to a greater range of researchers.

Thank you for pointing this out. We have added these references and agree that the use of similar methods in other bird species increases the value of this assay. We could not find Hernandez et al 2017 and are unsure of the manuscript to which you are referring.

Reviewer #3:

Manuscript Summary:

In this study, Coleman et al., describe an operant conditioning method to test the song preference of female zebra finches. Although song preference assays have been employed in the songbird field for decades, the authors detail a method that employs freeware and relatively cheap technology to gauge song preference of female zebra finches. Given the ease of use, and a setup that is amenable for use by undergraduates, this protocol is likely to be helpful to the songbird community, however there are a few major concerns that need to be addressed and clarifications that would significantly improve this submission.

Major Concerns:

1.My major concern is that as stands, the sample sizes are too small in Figure 2A to demonstrate that their protocol is a functional means of testing song preference. The authors have a sample size of 3 in the paired condition, of which ⅓ animals do not show a preference for the partner's song. I recommend that the authors revise the figure to include a larger n and stats to demonstrate that the protocol in fact works.

We agree. We tried to use data that was collected after the paper describing the results was published to avoid copywrite permissions. We realize that this does not provide enough data for statistical analysis. We have added the data and statistical analysis from our published paper (and added a couple more points) in this submission.

2. The authors use calculate preference differently through the manuscript.

Line 155 -

4.4 Calculate chamber and song preference by dividing the number of triggers on the partner's side of the cage was divided by the total number of triggers.

Line 173 - To calculate the song preference, the number of times the female triggered her partner's song is subtracted from the number of times she triggered the unfamiliar male song, which is all divided by the total number of triggers

Would be better to stick to one method, that matches the one used to generate the figure.

Another reviewer also noted this. We have changed our description of preference and hope we are now consistent throughout the manuscript.

3. It is not clear from the protocol how the authors deal with false positives and false negatives from incorrect sensor triggers or faulty placement? (Section 1.4)
Suggestions on how to determine that the sensor has in fact been placed correctly would be very helpful to readers (Section 1.5).

Agreed. We test the sensors each time to run an experiment by manually breaking each IR beam. We have added this to the protocol.

4. Do authors exclude female birds with large side biases in the silence condition ? If yes, including exclusion criteria information would be handy.

As shown on Figure 2, we did not. There were some females with a very large side bias. We did exclude females that were not active enough; those females that perched fewer than 12 times in an hour. We have added this to the protocol.

5. Line 124: The authors could provide a range that they consider to be an active female ?

See above.

6. Section 2.3.2: Any criteria for choosing an unfamiliar song, do you have to check for songs that dissimilar to the mate's song?

We used the same unfamiliar song for all experiments to avoid concerns about how similar the song is to the mate's song. Including a quantification of similarity in partner song and unfamiliar song is an interesting test for future experiments.

7. A flowchart of the experimental timeline would be helpful
Added to Figure 1.

8. The authors use novel male, unfamiliar male, partner song - sometimes interchangeably. Also, the authors use the term unfamiliar male song to mean different things in different portions of the manuscript. I would use consistent terminology.

We have gone through the manuscript and used unfamiliar male and partner male.

9. Mention age of females and males used in the assay.

We get our birds from a local supplier do not know the age of the birds. We do know they are all adults (> 90 dph) based on beak color. We have added a statement about the age of the birds to the beginning of the protocol section.

10. Line 185: Do the authors have a recommended cutoff for how much the male should sing.

Does how much male sings matter, is there a correlation between preference and how much the male song during the 24-48h period ? Perhaps, the authors can address this in their discussion.

These are interesting questions that we have not addressed. We mention this because we had one example of a female that did not prefer her 'partner's song' after 24 hours (+ quinpirole), only to realize the male had not sung at all (not included in this analysis).

Minor Concerns:
Line 51: I think the authors mean "bonds" instead of "forms" .

Thank you for the correction.

Reviewer #4:
Manuscript Summary:
The methodology manuscript provides a nice overview of song preference testing in captive, domesticated songbirds. The goal of this line of research is to elucidate the behavioral and neural mechanisms of affiliative bond formation. Songibrds are a widely studied animal model for exploring this scientific question because many species are relatively monogomous. The ease of testing song preference is nicely illustrated in this manuscript and will be of value for animal research programs both for research programs and educational experience in laboratory techniques courses.

Major Concerns:
I suggest four additions to the manuscript to enhance the reproducibility of the approach:

1. I would like the authors to describe the testing room that the anechoic chamber is contained within. How many other testing chambers are kept in the room? How much sound transmission occurs between the anechoic chambers? In methodology approaches such as these, the testing conditions for the chamber are described, but the "meta-data" for the location and salience of other animals in the testing room are not typically discussed but may be critical determinants of behavioral variability.

We have one chamber in the lab and can only do one experiment at a time. The anechoic foam is used to help with sound refraction within the chamber. Indeed, having other animals in the testing room would be problematic.

2. Timelines for the experimental protocol would be helpful. The timeline for the "paired" condition was vague. Greater than 2 weeks of paired housing could include up to 7 years of paired housing and that may not be the same as when birds are paired for only 2 weeks. Are there nest boxes in the paired conditions? Is nesting necessary? What about egg laying and raising of young? Describing this portion of the pairing is essential for replication of the results. Additionally, the timeline for initial side bias testing (playing silence) relative to the side preference testing was not clear to me. I believe it is 48h total for the side bias and preference

test but this is not well specified within the methodology. Here a timeline should be straightforward to include with Fig. 1 showing the cage design. The timeline should also include information about time of day for song preference relative to light cycle. The light cycle should also be included in the methods.

We agree with the reviewer that these are interesting questions to be addressed with this and other methods. We used 2 weeks for pairing as that is what has been published (see Zann, 1994). We did not test for the necessity of nesting, egg-laying, or raising young. We did not control for the time of day, although we did not extend the experiments past the normal 'lights out' for the birds.

We have added a time-line to Figure 1.

3. I do not think that there are enough birds shown in the paired group and the unpaired, no drug treatment groups. The reason for this is that 2 of 3 birds in the paired group switch their perch preference, but one does not. I would like to see more data here to see how many paired females show a preference for their partner's song to be convinced that the approach works as advertised. My concern is to ensure that the methodology is highly reporducible across laboratories.

Most of the reviewers brought up this point. We have included additional data from the published manuscript (Day et al., 2019).

4. More information about the song selection process should be included as this is central to the methodology. For example, do songs include introductory notes? If so, how many and what is the inter-note interval? Every song rendition varies in the number and tempo of introductory note production. Additionally, how do you select an unfamiliar song that matches the partner song in terms of song duration, sound density, harmonic emphasis, etc. These details of the challenges of matching acoustic features of songs should be discussed in the discussion section of the manuscript.

We used the same 'unfamiliar' male song for all experiments to avoid complications of matching acoustic features and a song that was more or less complicated than others. This is an excellent experimental set-up to test female perceptual differences in tempo, duration, etc.

Minor Concerns:
Introduction:
Lines 74-75 - I suggest that you specify that one is calculating a ratio to assess female preference.
As a small caveat, how does one know that side preference doesn't shift with time and exposure to sound if one does not run the control condition of playing to the preferred side to demonstrate that a side preference does not switch with time.

We did not control for a changing side preference.

1.3: "place perch toward end of chamber" is too vague. Please give a specific location. It should be the same for every cage if the cages are identical.

1.5: It would be helpful to measure the level of illumination (if you have access to a light meter). There are different types of led lights (warm, bright light, etc); the type and whether they are full spectrum should be specified.
2.2.5: What is the minimum number of triggers to count the trial? Give a range based on your experience to guide the user.

We used a threshold of 8 or more perches on one side of the cage, or at least 12 perches total.

3.4: 50 ul of drug administered how? IM? SC? As it is written here and on page 183, I'm not totally clear. It seems like SC. Regardless, please specify route of administration.

We have added this information; we used a subQ injection.

3.5: ~70 db at what distance/location in the perch? Range? Peak? Average?

We measure the average amplitude at the perch. We added this to the protocol.

Discussion
Small correction -- The most expensive components are the computer and A/D board not the photo-electric sensors. These sensors can be found even cheaper than described in the ms (<$20).

Good point. We changed the sentence to state that the set-up is relatively inexpensive.

Figure 1. Stranger song should be unpaired song.

We changed this to unfamiliar song.