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Rearing Condition of Female Zebra Finch Influence on Song Preference in Adulthood

By

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Abstract

Song in zebra finches is an important mating signal- males learn a specific song from a "tutor" and eventually use it to attract a female, while females choose a mate based on their song preferences. Given that singing is a male-specific behavior, there is limited knowledge surrounding song learning ability in female zebra finches, as it was presumed that females are not receptive to song exposure during development. This study utilized fourteen female zebra finches to address whether early rearing conditions influence song preference in adulthood. Females were semi-randomly assigned to one of two conditions- "tutored" (exposure to the song of an unrelated male) or "isolated" (completely isolated from song). Song preference was tested with an operant conditioning perch-trigger assay, in which hopping on a perch triggered the playback of one of two songs. Results indicated that all "tutored" females (n = 7) significantly preferred the "familiar" song, or the song of the male that they had been exposed to during development, over an "unfamiliar" song of similar structure. The group of "isolated" females (n = 7) did not form a specific preference for one song over the other, and individually, they displayed varied preferences. These findings suggest that females are sensitive to song exposure throughout development, thus having implications for early song learning on mate choice in adulthood.

Introduction

  While our genetic code provides the foundation for development, it is simultaneously recognized that early life experiences are influential on future behavior. As learning occurs, neural circuits are remodeled to reflect the information from experiences that has been processed. Behavioral studies of animal models are profoundly helpful in understanding the formation and plasticity of behavioral patterns. This research project explored how early rearing conditions contribute to the emergence of learned behavioral patterns, specifically studying a prominent animal model of this, the zebra finch songbird (*Taeniopygia guttata*).

  As juveniles, male songbirds are exposed to an adult "tutor" song, which serves as the model for learning and eventual production of their own unique song (Eales, L., 1985). Song learning is a male-specific behavior that can only be acquired during the "critical period," a phase of development in which, similar to humans, experiences remodel neural circuits and create lasting behavioral patterns (Hess, E., 1959). This critical period for memorization of tutor song occurs from post-hatch day 30 (P30) to P65 and is closed exclusively by tutor song experience; it is not contingent on age (Price, P., 1979). As previously demonstrated, the absence of a song model for a juvenile appears to postpone its critical period and ultimately results in song production that is abnormal in both content and structure until a model becomes available (Eales, L., 1985).

  Given that singing is a sexually dimorphic behavior, researchers originally neglected studies involving female zebra finches and centered their focus toward variables impacting male song production. It was erroneously presumed that differential neuroanatomy of males and females likely equated to dissimilar learned song preferences (Cynx et al., 1990). More recently, there is sufficient evidence that females are similarly receptive to their father's song during the

sensitive phase of development (Miller, D.B., 1979; Clayton N.S., 1988 Jennions & Petrie, 1997; Braaten & Reynolds, 1999; Riebel K., 2000; Riebel K., et al., 2002; Riebel K., 2003; Lauay et al., 2004; Tomaszycki et al., 2006; Woolley & Doupe, 2008; Braaten et al., 2008; Honarmand, M., 2009; Campbell & Hauber, 2009). In a study comparing preference learning among siblings, both males and females freely chose the playback of their father's song over an unfamiliar one after having exposure during early development. Additionally, males and females displayed no differences in activity levels, i.e. mean number of key pecks, indicating that the father's song was equally preferential to both (Riebel, K. et al., 2002).

Being that male zebra finch song is an important mating signal for the attraction of females (Morris, D., 1954), and females evidently show preferences for learned songs as discussed prior, studies have begun demonstrating that females' experiences as juveniles likely contribute to mating decisions in adulthood. Before more recent efforts, the quality and condition of male/male song was the focal point of female mate-choice studies, and it was widely held that all females equally valued characteristics of song structure like duration, repertoire, syntax, and other special elements (Clayton & Pröve, 1989; Neubauer, 1999; Collins, 1999; Tomaszycki & Adkins-Regan, 2005), as well as components of song performance like song rate, amplitude, directed vs. undirected song, and physical appearance (Woolley & Doupe, 2008; Sossinka & Böhner, 1980; Burley & Coopersmith, 1987) as the sole determinants in choosing mates. As of late, there is much greater recognition given to the impacts of female early living conditions on mate choice, including evidence that variation in rearing environments can significantly influence female preferences in adulthood (Riebel, K., 2000; Lauay et al., 2004; Campbell & Hauber, 2009; Riebel, K. et al., 2009). Much is still to be discovered about what specific contexts and conditions interact with learned preferences.

Thus far, investigations of female song preferences have produced data in support of the theory that preference for specific elements of song may be experience-dependent rather than generalizable to the species as a whole (Neubauer R., 1999; Lauay et al., 2004). One investigation utilized a 2-way testing arena in which speakers located on both ends played either a high-quality song or a low-quality song. Only female zebra finches with prior exposure to adult song as juveniles approached the speaker playing high-quality song significantly more than the speaker playing low-quality song (Lauay et al., 2004). In conducting additional tests for preference of familiar vs. unfamiliar song, females reared without exposure to their father's song chose tutored vs. untutored song equally as often as females with exposure during their juvenile phase; however, the song stimuli varied in structure, which is a possible confound. This present study controlled for differences in stimuli complexity and focused solely on preference for "familiar" vs. "unfamiliar song" in females had been raised either exposed to or isolated from adult song as juveniles.

A common paradigm in the literature of female preferences, which mimics prior work carried out with males, has become testing song preference after exposure to an unrelated tutor when the father's song is not available. In a female demonstration of this, all zebra finches were raised apart from their fathers for a time which included the critical period and were either tape-tutored with a song belonging to another adult male or reared in isolation from song completely (Riebel K., 2000). An operant conditioning task was utilized in which playback of a song served as the "reward." Several landing perches were each assigned to one of two songs- either the taped song or an unfamiliar one. In a series of tests, hopping on a perch and pecking a key triggered the playback of the song, thus signaling to researchers which one the female actively preferred to hear. The findings from this study indicated that not only does mere exposure to a

tape-recorded song (no physical presence of the model) elicit preferences, but furthermore, females will learn the song of an unrelated tutor model during the critical period when the father is not readily available. This present experiment similarly utilized an operant, perch-hopping task to assess preference; however, birds were not required to peck a key. Merely landing on the perch triggered the playback of a song, as eliminating that extra step reduced the need for additional training. Furthermore, this project housed female juveniles with live, "foster" (unrelated) adults rather than exposing them to the taped song of an unrelated male.

There continues to be strong evidence for learned preferences of females raised by unrelated tutors, or "fosters." One study explored the limits of such learning and cross-fostered both male and female zebra finch juveniles into Bengalese nests (Campbell & Hauber, 2009). Utilizing a playback procedure, researchers investigated the song preferences of subjects raised in either conspecific (same-species) or heterospecific (different-species) nests. Significantly, all birds preferred the song of the species from which they were reared, including zebra finches raised by Bengalese finches, reinforcing that rearing environment influences song preference. This present experiment employed the use of "foster" adults in the sense that juveniles were reared by unrelated adults, but all birds were from the same species. Furthermore, only females were utilized as experimental subjects, as there is less known about the formation of their song preferences.

Research is continuing to develop in support of female experience-dependent song preferences. There is evidence that these preferences may reflect the context in which the female had been reared, as mentioned previously by studies comparing song-isolated and song-exposed juveniles, and independently, juveniles exposed to the song of "foster" parents. This present research integrated both constructs, comparing the preferences of females reared in isolation

from song to those reared with an unrelated "tutor" male. My hypothesis argues that the females who experienced song from an unrelated "tutor" during development would form learned preferences for their respective "familiar" song, while "isolated" females would not express a definite inclination toward one type of song.

Methods

Bird Care

All procedures were approved by the University of Chicago Institutional Animal Care and Use Committee and followed NIH Guidelines for the Care and Use of Laboratory Animals. All birds were maintained in a 14:10-hour light:dark period for the duration of the experiment. Food and water were provided *ad libitum.* The temperature in the experimental room was maintained at approximately 77°F, and an extra heat source was provided as necessary.

Controlled Rearing of Experimental Subjects

14 female zebra finches were used as subjects. All birds were raised in laboratory aviaries until the day after they fledge (~P23), at which point they were moved to a separate room and placed in individual cages with 2 adult female companions and 1-2 other juveniles. In order to isolate each bird from all sounds other than those produced from within their cage, each cage was also placed inside of a cooler with the lid closed. Additionally, white noise was played at all times in the room. At P30, by which age females are sensitive to social learning, each juvenile was semi-randomly assigned into one of two living conditions ("tutored" or "isolated") and moved into new, individual cooler cages with two foster parents, or, adult zebra finches who are not biologically related to the subjects but will rear them. Siblings were assigned to opposite

conditions. Juveniles assigned to the "tutored" condition were housed with one adult foster male and one adult foster female, in which living with an adult male exposed them to song. The "isolated" birds were housed with two adult foster females in order to standardize the social complexity between the two conditions, maintaining a triad. Female adults produce vocalizations, or 'calls,' but do not sing, and therefore, "isolate" birds were not exposed to song. These vocalizations however serve as early social contact for juveniles to become familiar with sound and receive feedback. At P65, all adult foster males placed with the "tutored" birds were removed from the cooler cages and replaced with a new adult female so that each bird, regardless of assigned condition, was living with two foster females. In order to normalize this shift across conditions, one foster female housed with each of the "isolated" birds was similarly replaced with a new foster female. The foster adults remained in these cages for the duration of the experiment. P65 was designated for this experiment to be the end of exposure to the "tutor" because it parallels the age in males at which the critical period closes, although this has not yet been confirmed in females.

Song Stimuli

The songs of 7 adult male zebra finches were individually recorded in sound-attenuated chambers using SAP2011, an open code software which performs automated recording and analysis of animal vocalization. A female adult zebra finch was placed in the cage with each male to increase the likelihood of recording a female-directed song. Birds were left in the chamber until the males produced at least 10 variations of song. Only song produced after at least 3 h post-lights on was utilized, as male song is more variable during the morning "rehearsal" period (Ollason & Slater, 1973; Johnson et al., 2002; Derégnaucourt et al., 2005).

The "Explore & Score" feature was used on Sound Analysis Pro to examine the acoustic

characteristics of song and classify a singular song motif, or phrase, based on the physical

representation of syllables. The primary song of each male was further verified by viewing side-

by-side sonograms of at least two song recordings and matching syllables. Since males typically

sing several repetitions of the motif in a song bout, the middle motif, in particular, was manually

extracted and utilized for playbacks (*See Figure 1*). This middle repetition is typically the most

stable and consistent song, void of introductory notes and additives at the tail-end of a bout

(Bruno & Tchernichovski, 2019).
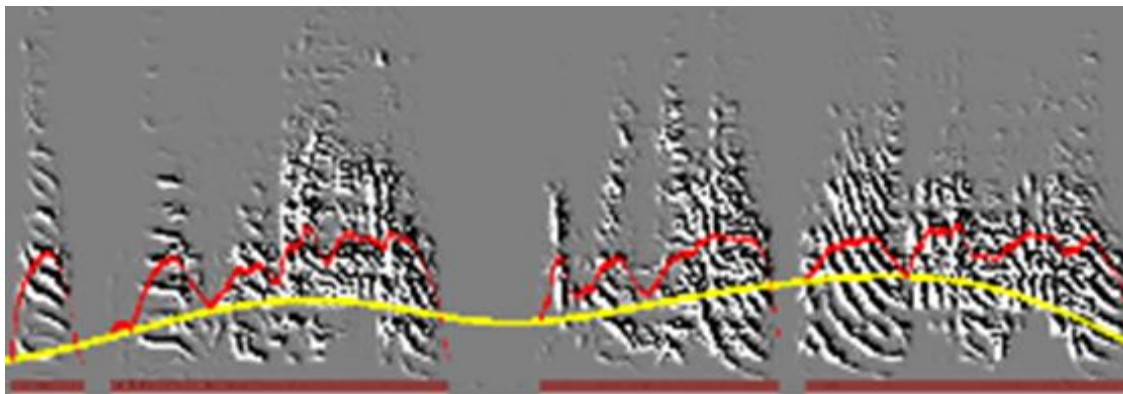
Figure 1. Example sonogram of middle motif from "tutor" Y220. Red lines depict 4 segmented syllables. Time is x-axis and frequency modulation is y-axis.

Choosing "Unfamiliar" Song Match for "Tutor" Song

An unfamiliar song, or, one that the experimental bird had not been exposed to at any

point while being reared, was matched to its respective "tutor" song, primarily according to

duration, syllable number, and syllable complexity (*See Figure 2*). This unfamiliar song served

as the second stimulus option in the operant perch-trigger setup described elsewhere. All

unfamiliar songs derived from the London Lab archives of previously recorded male song at the

University of Chicago.

Similar to the aforementioned process for obtaining the primary "tutor" song, the "Explore & Score" feature of Sound Analysis Pro allowed for the visualization of characteristics of the unfamiliar song. An unfamiliar song was qualified to be an adequate match for the "tutor" song initially after equating syllable number and duration of the primary motif, and secondarily, performing a 1x1 "symmetric" similarity measurement which confirmed alike complexity (*See Table 1*). This "symmetric" measurement option on SAP2011 assesses the similarity, in clusters, between two songs from unrelated models. Further, there are two methods of comparison available for symmetric analysis- time-course and mean values. The time-course method is more indicative of sections of sequential match, whereas the mean values method identifies large areas of similarity. Quantitative results from both methods were obtained. The similarity measurements produced a quantitative results table comprised of several outputs, but % similarity and % sequential match were utilized as the primary criteria for these song pairings. A comparison image illustrated using color, sections of high and low similarity.

A satisfactory % similarity between the unfamiliar and "tutor" songs was established by first obtaining those results from a 1x1 comparison of two songs from the same "tutor," which was interpreted to represent approximately the highest possible similarity scores between two songs, and conversely, a 1x1 comparison of a "tutor" song versus an "isolate" song, which was interpreted to represent low-end similarity scores given that an "isolate" male is one that had not been exposed to a song model during its critical period, and therefore, has a song that is abnormal is structure. Additionally, the nature of an "isolate" song indicates that is completely unrelated to that of a "tutor" song, and thus represents the similarity of two zebra finches having no relatedness from learning. An unfamiliar song was regarded as an adequate match for a "tutor" song if its results of the 1x1 comparison with the "tutor" song fell within the range of and

existed at least 10% away from both its respective "tutor's" highest and lowest similarity scores, ensuring that it was neither too similar nor dissimilar.

Each isolate bird was "paired" with a tutored bird in terms of perch song assignments, meaning that every set of tutored-isolate bird pairing was tested using the recording of the adult tutor song that the tutored bird had been exposed to during its "sensitive phase." Birds were matched according to age.
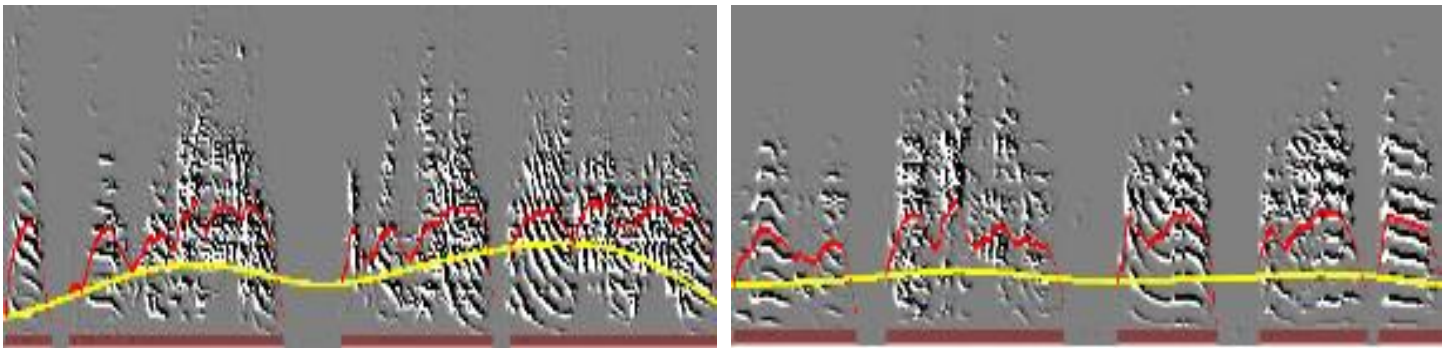

Figure 2. Sonograms of Y220 "tutor" song (left) and Y125 "unfamiliar" match (right)

Tutor	Unfam. Match	%Similarity	%Sequential	Accuracy	Pitch diff	FM diff	Entropy diff	Goodness diff	AM Diff	Tutor Duration(ms)	Unfam Duration(ms)
Y220	Y125	72.24	83	74.63	1.765	1.064	1.465	2.211	2.396	700	600
GR1115	OR268BK100	69.47	97.81	76.99	2.12	1.348	1.731	1.95	1.923	590	570
SK2	VT40	55.78	99.18	71.79	2.551	1.146	3.097	1.267	2.109	350	370
GR111	GR587	54.28	90.89	59.32	1.54	1.963	2.094	3.618	2.56	1200	1300
OR81	Y224	60.76	84.39	60.94	1.563	1.841	2.586	3.05	2.527	900	800
OR74	VT40	65.81	89.78	71.1	2.545	1.374	2.182	2.44	2.064	400	370
OR104	BG62	60.1	97.96	66.9	2.391	1.511	2.792	3.175	2.696	500	490

Table 1. Time-course data from symmetric 1x1 analyses, comparing "tutor" song with "unfamiliar" matches.

Tutor Song 1	Tutor Song 2	%Similarity	%Sequential	Accuracy	Pitch diff	FM diff	Entropy diff	Goodness diff	AM Diff
OR104	OR104	97.67	95.23	91.68	2.948	1.774	3.069	2.34	2.784
GR111	GR111	97.51	99.35	92.81	2.095	2.367	2.382	3.732	2.763
GR1115	GR1115	90.89	99.83	86.83	1.925	1.271	2.197	2.251	1.591
OR74	OR74	99.53	98.1	94.72	1.279	1.585	2.151	3.193	1.714
OR81	OR81	98.91	99.24	94.42	2.246	1.569	2.869	2.533	2.176
SK2	SK2	94.56	99.72	91.29	2.329	1.293	3.176	2.059	2.014
Y220	Y220	94.81	98	87.86	1.985	1.02	1.72	1.951	2.161

Table 2. Time-course data from symmetric 1x1 analyses, comparing two variations of "tutor" songs.

Tutor Song	Isolate Song	%Similarity	%Sequential	Accuracy	Pitch diff	FM diff	Entropy diff	Goodness diff	AM Diff
GR111	Isolate	48.39	45.72	58.99	1.692	2.057	1.928	3.382	2.29
OR104	Isolate	44.95	88.53	55.41	2.727	1.912	2.334	2.508	2.301
OR74	Isolate	54.34	26.85	66.75	1.208	1.633	1.922	3.594	1.67
SK2	Isolate	33.42	23.2	55.36	2.486	1.923	3.622	2.73	1.843
GR1115	Isolate	56.61	42.71	64.5	2.352	1.187	1.932	1.744	1.433
OR81	Isolate	48.22	62.02	66.36	2.139	1.63	2.155	2.789	1.898
Y220	Isolate	50.97	47.95	69.49	1.946	1.491	1.492	2.621	1.81

Table 3. Time-course data from symmetric 1x1 analyses, comparing "tutor" with "isolate" songs.

Apparatus

The test apparatus consisted of a 17 x 12 x 14 in. cage containing four landing perches, protruding perpendicular to the cage walls. The two walls opposite each other and adjacent to the food and water dishes were utilized, with two perches fastened to each with zip ties. Perches were placed at a height of 8 inches from the cage floor. The two perches occupying the same wall were spaced 5 inches apart from each other. Perches were slightly staggered to avoid close contact with the ones on the opposite wall. Balsa wood dowels (1/8") were used for perches. Two speakers were used for song playback, both located outside of the cage. One of each was placed directly behind either of the two perches designated for song playback. Food and water dishes were centered on the two walls opposite each other and without perches to lessen the chance of either influencing perch hopping tendencies.

Experimental Room Set-Up

The room in which testing took place was set up with wire racks along three different walls. Two birds were simultaneously tested every session time, each in an individual cage placed on its own rack on a separate wall in order to minimize interference of each other. In order to expose the experimental birds to a more natural social environment and therefore facilitate perch hopping activity, several female zebra finches unassociated with the experiment

were housed inside of the testing room on a lower level of wire racks, one cage directly

underneath each of the three experimental cages. These female cages were out of sight of the

experimental cages, and thus provided social contact for the experimental birds that was purely

auditory. This lack of visual stimulus prevented the possibility for the physical placement of the

females to create a bias for the experimental bird toward one side of the cage. The purpose of

housing only females in the experimental room rather than males was to ensure that the only

male songs the experimental birds were exposed to was that of the designated song stimuli.

While there is potential for the experimental birds to have heard the song stimuli of other birds

simultaneously being tested, it is likely that the proximity of one's own playbacks elicited a

higher degree of attention (Hauber et al., 2010; Ritschard et al., 2010; Loning et al., 2022).

Adult Song Preference Tests

Testing began after each bird reached sexual maturity; past P90. The birds were

individually tested in experimental cages utilizing an operant-conditioning perch-trigger assay, in

which landing on a perch triggered the playback of a song. Two of the perches were

predetermined to play one of two songs designated for each pair- either the foster male "tutor"

song that had been heard by the "tutored" bird of each pairing or an "unfamiliar" song of similar

complexity. Landing on the other two perches resulted in no song. The perches were connected

to Raspberry Pi devices, which triggered the playback of the pre-recorded songs through a

speaker and simultaneously saved a log, keeping record of every perch hop.

Two hour testing sessions occurred from either 2-4 h or 5-7 h post-lights on, both of

which take place in the first half of the day in a 14:10 h light:dark cycle. Each bird underwent 3

testing sessions during both time slots, for a total of six sessions over six consecutive days.

Individual birds were transferred to an experimental cage located in the testing room 1 h prior to

the beginning of each session, which served as an acclimation period with no song. Song

assignment to perches was rotated halfway through the six sessions to avoid perch placement

bias. At the end of every session, birds were returned to their cooler cages.

Preliminary Tests

A set of preliminary investigations were necessary to conduct for addressing uncertainties

surrounding the most beneficial experimental set-up for the primary song preference test, given

the limited number of prior female zebra finch behavioral studies. All preliminary behavioral

observations and recordings took place during the first half of the day in a 14:10 h light:dark

cycle. Each preliminary test occurred over the course of six days- one session per day per

individual bird. Unless otherwise stated, all tests took place in experimental cages in the primary

experimental room surrounded by other zebra finches. All perch-hopping behavior was recorded

by the primary researcher using Behavioral Observation Research Interactive Software (BORIS,

2012).

The first preliminary experiment (*Experiment 1*) investigated whether varying perch

heights influenced perch-hopping patterns, as zebra finches are highly active perch hoppers and

prefer to be above ground-level (Zann, 1996). Given this characteristic, it was critical to

determine whether specific perch heights would alter perch-hopping tendencies independent

from the influence of a song stimulus. In the interest of preventing a possible impact of perch

height on perch-hopping behavior, three adult female zebra finches uninvolved with the primary

experiment were individually tested. Four perches were placed side-by-side on one of the cage

walls, staggered so that two perches were at a low height, 5 inches from the ground, and the

other two were placed higher, 8 inches from the ground. Approximately 2.5 inches of space separated each perch. The primary researcher recorded the number of times each individual bird hopped to each perch, without the presence of a song reward.

The second preliminary test to take place focused on identifying what time of day would reliably produce a stable amount of perch-hopping activity (*Experiment 2*). It has been previously observed that zebra finches go through "lulls" in activity levels during certain times of the day (Ollason & Slater, 1973; Williams & Ternan, 1999). The three aforementioned, non-experimental adult females were observed and recorded for perch-hopping activity between 1-h and 7-h post-lights on, which encompasses the entirety of the first half of the typical 14-h lights-on period. All four perches protruded from a singular cage wall, lined side-by-side at 8 inches from the ground with 2.5 inches of space left between each. The primary researcher recorded the number of times each individual bird hopped to a perch, without the presence of a song reward.

Another preliminary experiment was necessary for determining what type of environment would facilitate perch-hopping activity (*Experiment 3*). Previous female behavioral studies have taken advantage of sound-attenuated chambers, as they are sufficient in isolating the experimental subjects from all external auditory and visual stimuli outside of the experimental song stimuli; however, there is no firm evidence to support that this type of setting is most appropriate for eliciting normal perch-hopping activity, because zebra finches are naturally social beings. For this reason, the primary researcher recorded perch-hopping activity of the same three adult females in two separate locations- sound-attenuated chambers in an isolated room (confined from external stimuli), or placed on a wire rack in an open room housing several other cages of zebra finches (exposed to external stimuli). This preliminary experiment occurred twice- the first set of sessions utilized no song reward in either location and merely recorded

perch-hopping activity as stimulated by the surrounding environment, and as a follow-up, a second round that did assign a song reward to specific perches in both locations.

Described in more detail in the subsequent results section, this second round employing a sound reward was carried out upon analyzing the data from the first set of sessions without a sound reward, as findings indicated that the isolated environment of the chambers induced very little to no movement whatsoever, and it was necessary to establish whether any changes would occur in the presence of a song stimulus, as would be present during the primary song preference experiment. Because the focus of this second round of sessions was merely recording activity in response to a song stimulus, not investigating song preference between different song stimuli, the researcher utilized a singular song recording for the assigned playback perches. The song recording for this preliminary test was a bout from one of the experimental tutor birds, with a duration of 2500 milliseconds. The playback of this recording was assigned to two of the four perches, in correspondence with the set-up of the primary song preference experiment.

Although it had been demonstrated that the adult females were more active in a social environment given the ability to engage with others, it was evident that this consequently allowed for exposure to the songs of other male zebra finches present in the room. In order to ensure that the only male song the adult females were exposed to was that of the current song stimulus, all males were removed from the room. Perch-hopping activity in the presence of a song reward was again recorded by the researcher before moving forward with other tests in pursuance of whether adult females were equally active with solely other females in the room.

Upon empirically confirming that the adult females were significantly more active in an open room housing several other cages of zebra finches and that the presence of only females provided ample social contact, the successive preliminary tests were conducted in that

environment. The remaining tests utilized a song reward, the song recording of an experimental

"tutor," assigned to two of the four perches. Three new adult females were tested in addition to

the original ones.

First, it had been observed during previous sessions which included the presence of a

song reward that a full bout duration of 2500ms was extensive in consideration of the rapid pace

at which zebra finches perch-hop. The primary researcher noted that the subjects, upon landing

on a song-assigned perch and triggering a playback, continued to perch-hop, often reaching a

control perch before the playback from the preceding perch had ended. It raised concerns

regarding the potential for experimental subjects to associate control perches with the song

reward. Although female zebra finches regularly hear a male song bout consisting of several

repeated motifs, for the validity of the song preference tests, the playback was shortened to a

singular song motif. The duration of the motif utilized for this preliminary test was

approximately 400ms. The number of perch hops to each individual perch was recorded with the

newly shortened playback to test whether this was an appropriate solution (*Experiment 4*).

The final preliminary experiment to occur addressed perch placement (*Experiment 5*). Up

until this point, all four perches were lined in a row on a singular cage wall; however, it was

speculated that utilizing two cages walls opposite each other and spacing two perches on both

would be beneficial for two reasons- first, this layout lends itself to a natural arrangement that

subjects would encounter in the wild and therefore coincides a familiar direction of movement,

and secondly, it provides additional spacing between perches, which further mitigates the above-

mentioned concern resulting from playback duration and subjects' rapid perch-hopping

tendencies. Perches were slightly staggered from those on the opposite wall to avoid otherwise

close proximity in being directly across from one another. Furthermore, the cages housing other

female zebra finches designated for social contact were placed directly underneath each of the experimental cages. This was done to prevent the visual stimuli of other females from evoking bias for one cage wall over the other, as is likely if the female cages were placed on the sides of the experimental cage walls holding the perches. The number of perch hops made to each individual perch was recorded. The recording for playbacks was the truncated, single 400ms motif from the preliminary experiment prior.

Results

Experiment 1- Preliminary Test for Perch Height (No Song Reward)

The first preliminary experiment tested whether having perches positioned at varying heights created a perch-placement bias prior to the assignment of a song reward. The total percentage of hops made to perches at the higher height (83.6%) was overwhelmingly more than the total percentage of hops made to perches at the lower height (16.4%), indicating an overall preference for the perches further from ground level as opposed to those closer to ground level (*see Figure 3*). These findings were consistent across all three females that had been tested, indicating that this preference for higher positioned perches is likely common among zebra finches, or at the very least, adult females. In developing the set-up for the primary song preference test, these results highly suggested that arranging perches at equal heights would be most beneficial in reducing perch-placement bias, and secondarily, that perches further from ground level may facilitate a higher level of perch-hopping activity.
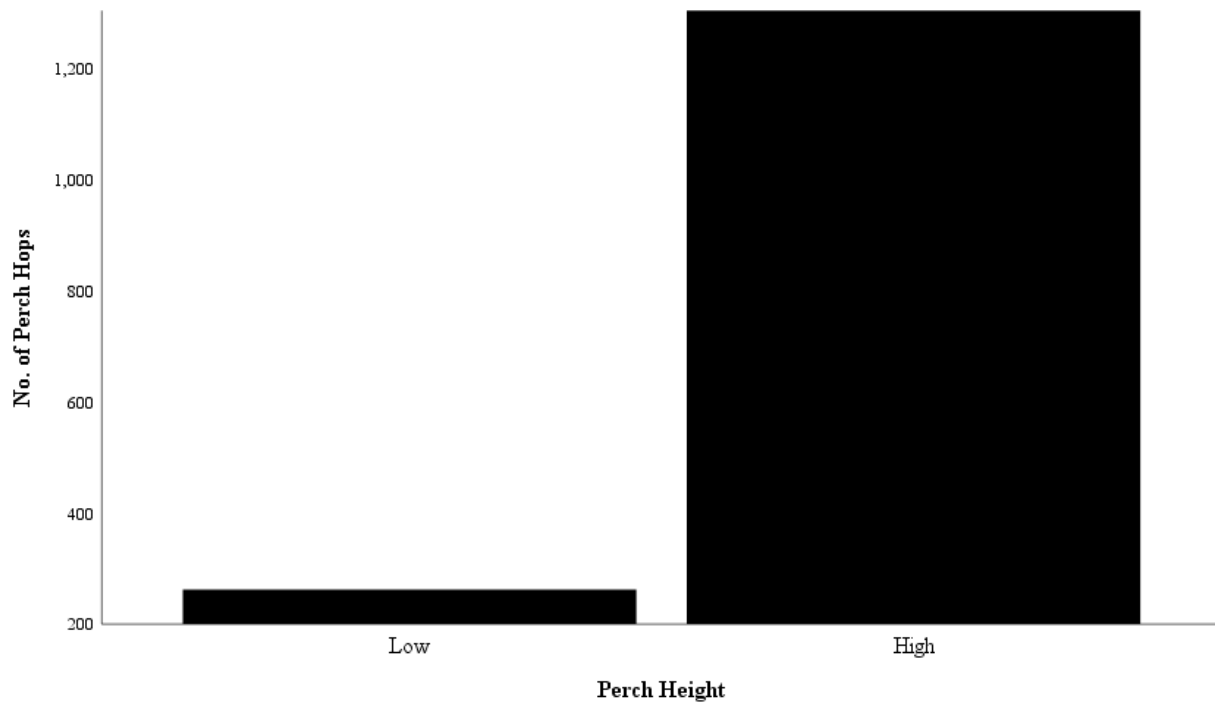
Figure 3. Total frequency of perch hops to high vs. low perches.

Experiment 2- Preliminary Test for Time of Day (No Song Reward)

This preliminary experiment investigated what time of day would capture an ample amount of perch-hopping activity, strengthening the validity of the primary song preference test. Females were tested between 1-h and 7-h post-lights on, all encompassed within the first half of the 14-h lights on period. The decision to stay within the first half of the lights-on period was based on previously cited studies that locomotor activity progressively decreases into the late afternoon/early evening. Results of this test indicated that while perching-hopping activity of individual females is distributed variously throughout different hours, a large percentage of perch-hopping occurs between 3 h (28.2%), 4 h (21.6%), and 5 h (20.5%) post-lights on (*see Figure 4*).
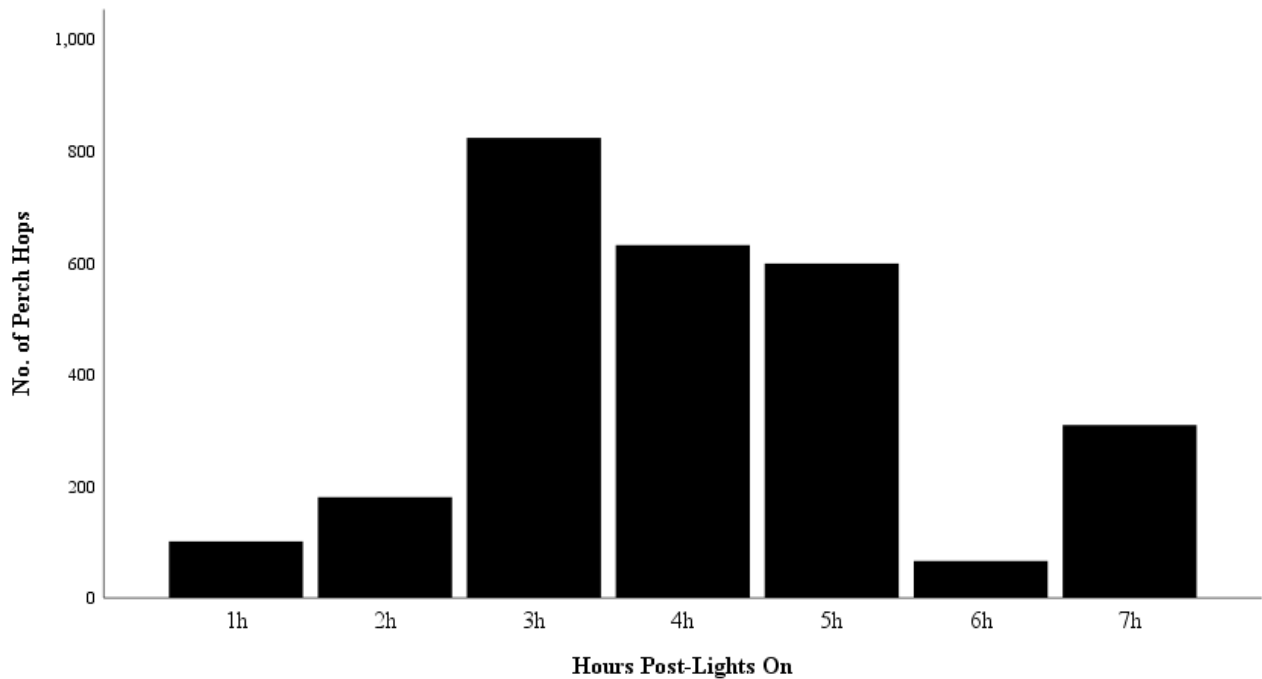
Figure 4. Distribution of perch hops made each hour from 1h - 7hr post-lights on.

Experiment 3- Preliminary Test for Location

This preliminary test compared levels of perch-hopping activity in sound-attenuated chambers in a secluded room, isolated from all external stimuli versus a room housing several other cages of zebra finches and thus exposed to all environmental stimuli within the room. Results of this test indicated that females hopped significantly more when placed in a room with other zebra finches (*no. of perch hops* = 2906) compared to being isolated in a chamber (*no. of perch hops* = 10), signifying that having the ability to engage socially with other finches promotes perch-hopping behavior (*see Figure 5*). These findings were consistent across each of the female subjects, further emphasizing that an experimental set-up in a location that provides

social contact is favorable, as it appears to motivate perch-hopping activity. This is likely

because it coincides with a zebra finch's natural inclination to reside in social environments.
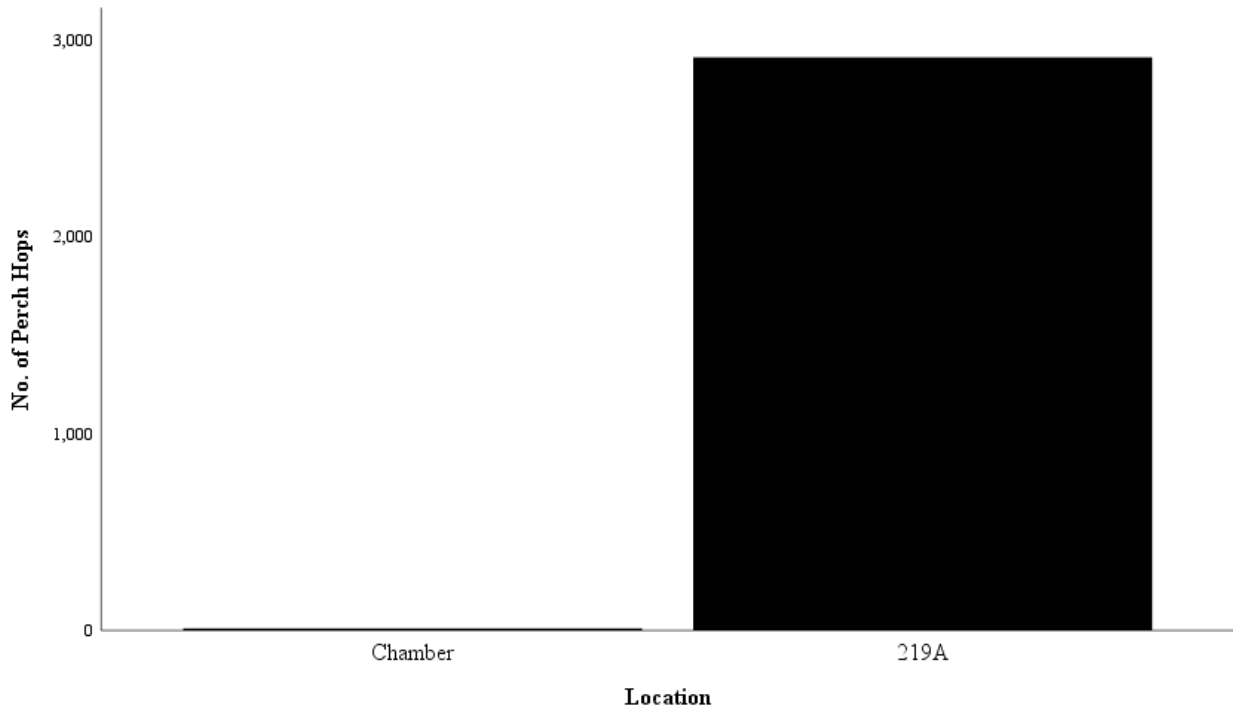
Figure 5. Total no. of perch hops made in an open room housing other zebra finches (219A) vs. sound-attenuated chambers.

**Researcher conducted an additional test with the presence of a song reward to determine

whether that might motivate perch-hopping activity levels in isolated chambers. Results were

comparable to the ones above (little to no activity).

Experiment 4- Preliminary Test for Duration of Song Reward

This preliminary test was conducted because the primary researcher observed that the

duration of the recorded bout utilized as the song reward extended past the length of time that the

female remained on its assigned perch, continuing to play once the female reached a different

perch. Findings of this test utilizing a singular song motif for a shorter playback reward not only demonstrated that females overall hopped significantly more to song-assigned perches than control perches (m_{song} = 953.2, s.d = 179.9 ; $m_{control}$ = 447.0, s.d. = 65.4; m_{diff} = 506.2, 95% *CI* [363.0, 649.3.3]) (*See Table 4, Pair 2*), but it also reduced the number of hops made to control perches, compared to the previous test which used a longer bout ($m_{control1}$ = 565.8, s.d. = 128.3; $m_{control2}$ = 447.0, s.d. = 65.4 ; m_{diff}= 118.8 , 95% *CI* = [30.9, 206.6]).

Experiment 5- Preliminary Test for Perch Placement (with Song Reward)

This final preliminary experiment was completed for the purpose of testing perch placement- utilizing the two cage walls opposite each other and positioning two perches staggered on each, rather than the previous set-up of four perches lined in a row on a singular cage wall. Results of this test displayed that females hopped significantly more to song-assigned perches than control perches (m_{song} = 1015.3, s.d. = 214.5 ; $m_{control}$ = 346.8 , s.d. = 43.1; m_{diff}= 668.5, 95% *CI* [467.5, 869.5]) (*See Table 4, Pair 3*) and further reduced the proportion of hops made to control perches, compared to the proportion of hops made to control perches in the previous preliminary test ($m_{control2}$ = 447.0, s.d. = 65.4; $m_{control3}$ = 346.8 , s.d. = 43.1; m_{diff}= 100.1, 95% *CI* [52.5, 147.8]).

Paired Samples Test

| | | Paired Differences[a] | | | |
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	95% Confidence Interval of the Difference Upper	t	df	Sig. (2-tailed)
Pair 1	Song - Control	245.16667	51.60006	21.06564	191.01572	299.31761	11.638	5	.000
Pair 2	Song - Control	506.16667	136.39563	55.68328	363.02824	649.30510	9.090	5	.000
Pair 3	Song - Control	668.50000	191.53250	78.19282	467.49896	869.50104	8.549	5	.000

Table 4. Mean differences between no. of perch hops made to song vs. control perches. Pair 1: long bout; Pair 2: short bout; Pair 3: staggered perches.

Primary Adult Song Preference Test

Data from the primary preference test indicated first and foremost no difference in activity levels between the two conditions, as the mean number of key pecks per session between the "tutored" and "isolated" groups were nearly equal ($m_{tutored}$ = 105.21, *s.d.* = 58.22; $m_{isolate}$ = 103.24, *s.d.* = 54.23) (*See Figure 6*).
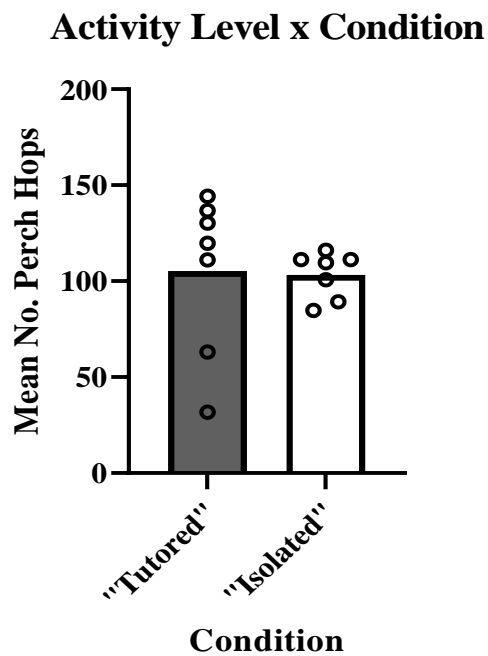
Activity Level x Condition

Figure 6. Mean number of perch hops by assigned condition. Circles represent the means of individual subjects.

Secondarily, the mean number of perch hops made to song-assigned perches exceeded the mean number of perch hops made to control (no song) perches (m_{song} = 77.70, *s.d.* = 42.47; $m_{control}$ = 26.52, *s.d.* = 13.99), strengthening the validity of the stimuli as a song reward for this preference test. This difference remained persistent when separated into condition (*see Table 5 and Figure 7).*

Mean No. of Perch Hops to Song and Control Perches

Condition		Song Perch	Control Perch
"Tutored"	Mean	77.9048	27.3095
	No. of Sessions	42	42
	Std. Deviation	43.69618	15.09884
"Isolate"	Mean	77.5000	25.7381
	No. of Sessions	42	42
	Std. Deviation	41.74443	12.92956
Total	Mean	77.7024	26.5238
	No. of Sessions	84	84
	Std. Deviation	42.47374	13.99352

Table 5. Mean and s.d. of perch hops to different perch types (control or song) by assigned condition.

Perch Hops to Control and Song Perches x Condition
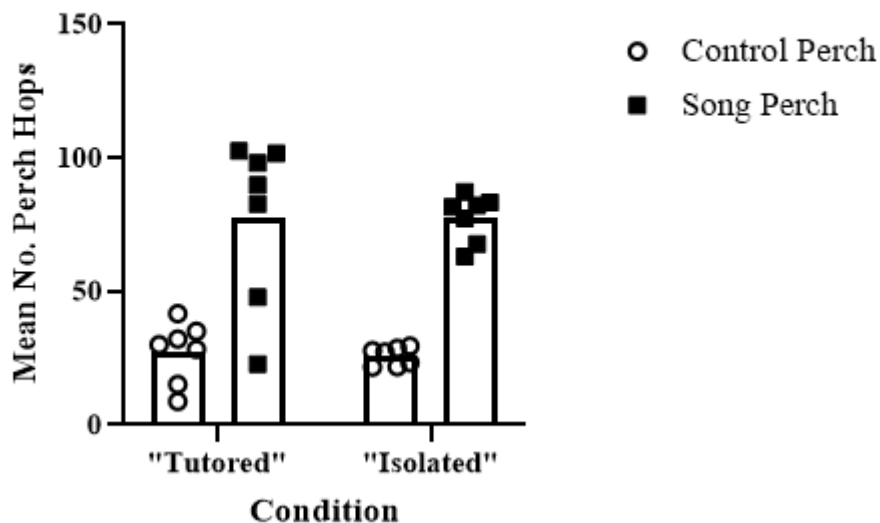
Figure 7. Mean number of perch hops to different perch types (control or song) by assigned condition. Circles/squares represent the means of individual subjects.

In terms of song preference, the group of "tutored" birds hopped significantly more to the perch playing back their respective "familiar" song compared to the perch with the "unfamiliar" song ($m_{familiar}$ = 45.05, $s.d.$ = 25.08; $m_{unfamiliar}$ = 32.86, m_{diff} = 12.19, $s.d.$ = 8.78, 95% CI [9.45,

14.93], $p < 0.001$), indicating a preference to hear the "familiar" song over the "unfamiliar" one.

The group of "isolated" birds displayed no significant preference between either song of their

respective stimuli pairs. ($m_{familiar} = 39.02$, s.d. $= 19.82$; $m_{unfamiliar} = 38.48$, s.d. $= 22.48$, $m_{diff} = .55$,

s.d. $= 7.33$, 95% *CI* [-1.74, 2.83], $p = .631$) (*See Table 6*).

				Paired Differences					
					95% Confidence Interval of the Difference				
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper	t	df	Sig. (2-tailed)
"Tutored"	Familiar Song - Unfamiliar Song	12.19048	8.78257	1.35518	9.45363	14.92732	8.995	41	.000
"Isolate"	Familiar Song - Unfamiliar Song	.54762	7.33253	1.13143	-1.73736	2.83260	.484	41	.631

Table 6. Paired t-test displaying mean differences of Familiar Song-Unfamiliar Song perch hops by assigned condition.

A within-subjects comparison of each bird's mean perch hops between the two song

stimuli of their respective pairings further revealed variation in song preference on an individual

level and additionally allowed for analysis of each "tutor"-"isolate" bird pair (see Figure 8).

Notably, all 7 "tutored" subjects were found to have individual song preferences for their

respective "familiar" song in particular, and 5 of 7 "isolated" subjects were also found to have

individual song preferences- 3 for the designated "familiar" song and 2 for the designated

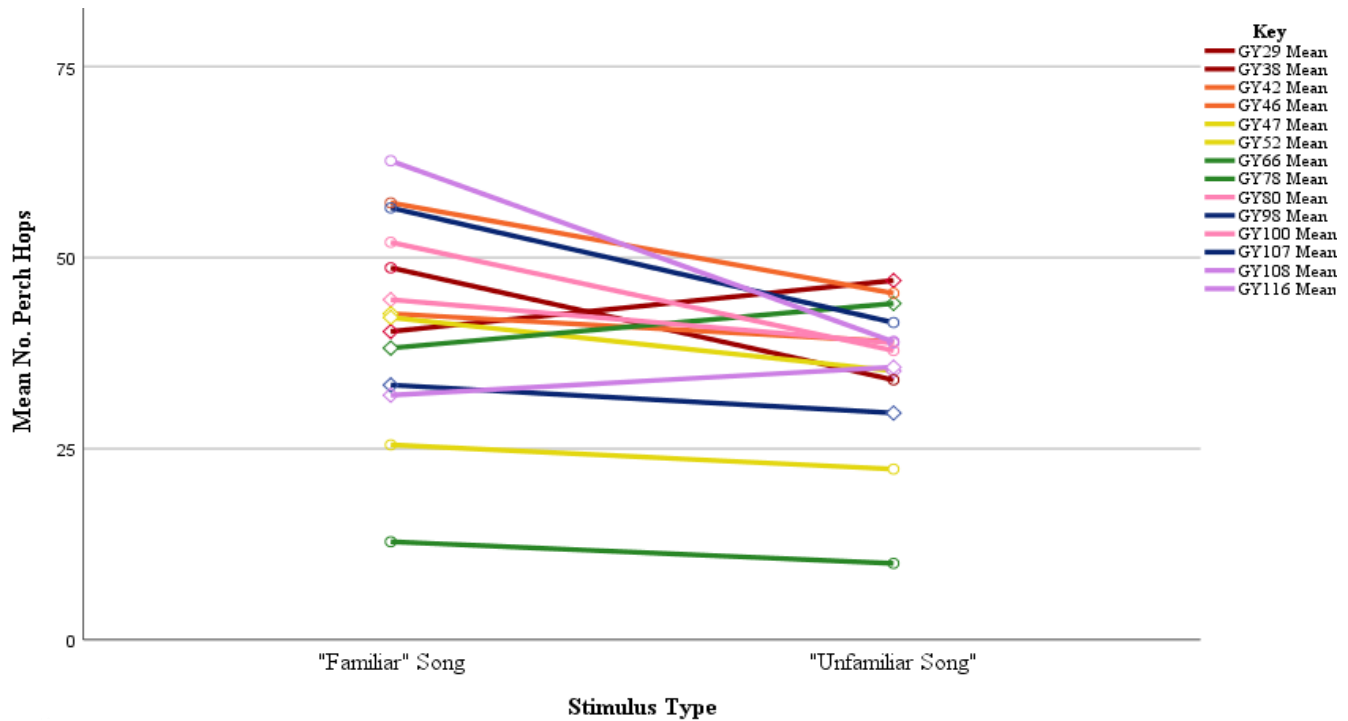"unfamiliar" song of their specific pairings. (see Table 7).

Figure 8. Within-subjects comparison of individual subject mean perch hops to Familiar/Unfamiliar songs. "Tutor"-"Isolate" pairs are illustrated in the same color. Circles represent "tutored," diamonds represent "isolated"

		Paired Differences							
				Std. Error Mean	95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
		Mean	Std. Deviation		Lower	Upper			
GY29	Familiar Song - Unfamiliar Song	14.66667	4.27395	1.74483	10.18143	19.15190	8.406	5	.000
GY38	Familiar Song - Unfamiliar Song	-6.66667	6.28225	2.56472	-13.25948	-.07385	-2.599	5	.048
GY42	Familiar Song - Unfamiliar Song	3.66667	6.15359	2.51219	-2.79113	10.12446	1.460	5	.204
GY46	Familiar Song - Unfamiliar Song	11.83333	6.55490	2.67603	4.95439	18.71228	4.422	5	.007
GY47	Familiar Song - Unfamiliar Song	7.00000	4.60435	1.87972	2.16804	11.83196	3.724	5	.014
GY52	Familiar Song - Unfamiliar Song	3.16667	2.22860	.90982	.82789	5.50544	3.481	5	.018
GY66	Familiar Song - Unfamiliar Song	-5.83333	5.60060	2.28643	-11.71080	.04413	-2.551	5	.051
GY78	Familiar Song - Unfamiliar Song	2.83333	2.63944	1.07755	.06341	5.60326	2.629	5	.047
GY80	Familiar Song - Unfamiliar Song	14.16667	6.11283	2.49555	7.75165	20.58169	5.677	5	.002
GY98	Familiar Song - Unfamiliar Song	15.00000	4.85798	1.98326	9.90186	20.09814	7.563	5	.001
GY100	Familiar Song - Unfamiliar Song	5.66667	2.80476	1.14504	2.72325	8.61008	4.949	5	.004
GY107	Familiar Song - Unfamiliar Song	3.66667	3.50238	1.42984	-.00886	7.34219	2.564	5	.050
GY108	Familiar Song - Unfamiliar Song	-3.66667	7.31209	2.98515	-11.34023	4.00690	-1.228	5	.274
GY116	Familiar Song - Unfamiliar Song	23.66667	10.76414	4.39444	12.37040	34.96294	5.386	5	.003

Table 7. Paired t-test results displaying individual subject mean differences of perch hops from Familiar-Unfamiliar songs. Positive values indicate preference for "familiar" song. Negative values indicate preference for "unfamiliar" song.

All 7 "tutored" subjects maintained an equal preference ratio (perch hops to "familiar" song divided by total perch hops) across the first three sessions and last three sessions ($m_{ratio1-3} = 0.42$, $m_{ratio4-6} = 0.42$; $m_{diff} = 0.00$, $p = 0.111$). The 3 "isolated" subjects who similarly favored the designated "familiar" song of their pairs demonstrated an increased preference in the last three sessions ($m_{ratio1-3} = 0.38$, $m_{ratio4-6} = 0.42$, $m_{diff} = -0.04$, $p = 0.04$), indicating that the difference in preference ratio of the "isolates" between the first and second halves of testing is statistically significant. Furthermore, the 2 "isolated" subjects who favored the designated "unfamiliar" song of their pairs similarly exhibited an increased preference ratio in the second half of testing, equivalent to that of the "isolates" who favored the "familiar" song ($m_{ratio1-3} = 0.38$, $m_{ratio4-6} = 0.42$, $m_{diff} = 0.04$, $p = 0.05$) (*See Table 8*).

SUBJECT	SESSIONS 1-3	SESSIONS 4-6	DIFFERENCE
GY29 (T)	0.45	0.44	0.01
GY38 (I)	0.38	0.42	-0.04
GY46 (T)	0.39	0.40	-0.01
GY47 (I)*	0.39	0.44	-0.05
GY52 (T)	0.42	0.41	0.01
GY66 (I)	0.38	0.41	-0.03
GY78 (T)	0.41	0.39	0.02
GY80 (T)	0.43	0.43	0.00
GY98 (T)	0.43	0.43	0.00
GY100 (I)*	0.38	0.42	-0.04
GY107 (I)*	0.37	0.41	-0.04
GY116 (T)	0.47	0.45	0.02

Table 8. Individual subject preference ratios from first half of testing (sessions 1-3) to last half of testing (sessions 4-6). (T) next to individual subject indicates "tutored," (I) indicates "isolated," (*) placed next to "isolated" with "familiar" song preference.

Discussion

Song learning in females after exposure to a "tutor" at a young age (~P30-P65) has been demonstrated to contribute to specific song preferences for that "familiar" song over an

"unfamiliar" song in adulthood. While the exact structure, i.e. timing, of a likely sensitive phase in females is yet to be established, this study contributes to the foundation of research which supports early learning ability in females (Miller, D.B., 1979; Clayton N.S., 1988 Jennions & Petrie, 1997; Braaten & Reynolds, 1999; Riebel K., 2000; Riebel K., et al., 2002; Riebel K., 2003; Lauay et al., 2004; Tomaszycki et al., 2006; Woolley & Doupe, 2008; Braaten et al., 2008; Honarmand, M., 2009; Campbell & Hauber, 2009). All adult "tutored" females, both on a group level and independent of one another, freely chose the song of their foster father over an unfamiliar one consistently throughout six days, or six sessions, via perch hopping to trigger the playback of two possible song rewards (or no song). Comparatively, the collective "isolated" subjects, for whom a song model was not available at a young age, displayed no specific preference to hear one song over the other, thus indicating a reliable difference in perch-hopping behavior between the two groups as a result of their assigned condition.

Upon further examination of each "isolate" individually, it was found that 5 of 7 subjects had formed a specific preference for one of the song stimuli in their respective pairing; however, the variability in song choice suggests the development of this inclination due to chance, or, reasons unrelated to the experiment. A possibility that was considered during analysis of the results was the natural tendency of females to gradually with time form a preference for one song over the other after repeated exposure, which parallels the intrinsic behavior of female zebra finches to select a mate based on song (Riebel K., 2009; Morris D., 1954). This was investigated by comparing the results of the first half of testing (sessions 1-3) with the second half of testing (sessions 4-6) between the "tutored" group and initially only the "isolates" who had a similar preference for the same "familiar" song. It was noticed that the preference ratio of the "tutored" subjects to hear the "familiar" song remained consistent from the beginning to end of testing,

supporting an immediate and fixed desire. Dissimilarly, the preference ratio of the "isolates" who also chose to hear the designated "familiar" song of their pairings significantly increased to reach the degree that the "tutored" displayed only in the latter half of testing, proposing a plausible difference in nature between the preferences of the two conditions. As an extension of this, the preference ratios between the "isolates" who desired the pre-defined "familiar" versus "unfamiliar" songs according to their tutor-pairings was additionally compared, and it was demonstrated that this same inclination in the second half of testing was observed for the subjects who preferred the "unfamiliar" song. These findings therefore offer up a potential explanation that on an individual level, the preferences of the "isolates" result from an instinctive and fundamental behavior of female zebra finches to eventually select a mate. This however cannot be confirmed solely based on these results, as comparing within this group of "isolates" after stratifying based on song preference largely reduces the power, and thus this claim requires additional investigation. Nonetheless, the validity of the primary findings between the two conditions as a whole- "tutored" and "isolated"- is reinforced by the equivalence of overall activity levels and preference to hear song over silence between the "tutored" and "isolated" groups.

In addition to these primary findings, this study secondarily reinforces the argument of past research demonstrating the ability of female zebra finches to learn song from an unrelated "tutor," given that the females exposed to song in this experiment were reared by a foster male (Campbell & Hauber, 2009; Riebel K., 2000; Riebel & Smallegange, 2003). It could be argued that the "tutored" females preferred the "familiar" song due to its element composition and complexity rather than its prior engagement, as it has been discovered that females value specific characteristics of song structure for their indication of the fitness of the male himself (Boogert et

al., 2008; Gil & Gahr, 2002). This, however, is highly unlikely due to the experimental control over song stimuli, as the "familiar"-"unfamiliar" songs of each bird pair were matched according bout duration, syllable number, and complexity/similarity score. Furthermore, the variation in song preference of the birds who were "isolated" from song signifies that neither song in the pair is superior over another in terms of song structure.

The present findings reaffirm the sensitivity of female zebra finches to song exposure during development, that of which is similar to males but has been previously dismissed due to differential neuroanatomy (Cynx et al., 1990). Given that song is an important mating signal for females, future research would benefit from utilizing behavioral paradigms to study the influences of learning on mate choice. While there is substantial research displaying numerous facets of mate choice and characteristics that are valued by females, continued efforts should investigate the hierarchy of such aspects, i.e. "tutor" song versus higher complexity "unfamiliar" song. Of equal importance would be exploring the nature of female learning, i.e. timing of a "sensitive phase," given the results of this study and previous literature. It cannot be assumed that it operates similar to that of a male's "critical period," as the neuroanatomy of zebra finches contributing to song learning is sexually dimorphic, and thus likely results in differential learning patterns to some extent.

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