Sexual Preference in Female Singing Mice (*Scotinomys Teguina)* Based on Male Song Effort

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Honors Research Thesis

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**Abstract**

Females may choose males whose traits signal better genes, as well as those who demonstrate trait differences from the males a female was raised with. Additionally, female sexual preference across species varies with hormonal levels and behavioral estrus cycles. Sexual preference in females has been neglected relative to male preference, which is more robustly expressed and easier to measure in most species. Evidence on the specifics of how females choose mates is sparse, but extravagant male signaling has long been a suspected factor. Male Alston's singing mice (*Scotinomys Teguina*) produce loud and elaborate songs, which they use most frequently in sexual scenarios. These songs vary in complexity: while some males are able to produce high-effort songs, others can't afford the energy expense or predator exposure and thereby produce lower-effort songs. This study examined female preference for high or low-effort songs produced by a male. The most effective method of estrus induction was determined by varying hormone treatment methods. Young, estrus induced and validated females were tested by measuring approach and investigation behavior to high and low effort songs in a two choice test. Preference test results display strong female preference for the high effort stimuli, and may indicate a trend of females selecting "superior" males with higher energy reserves and stronger virility based on male signaling characteristics.

Female sexual preference is credited with the capacity to disrupt the net direction of evolution (Darwin, 1871; Fisher 1915; O'Donald, 1962; Williams, 1966; Zahavi, 1975; Lande, 1981; Kirkpatrick, 1982; Byers & Waits, 2006). However, the mechanisms underlying female sexual behavior remain elusive. Sexual preference research has been heavily male focused, and though there is ample evidence of female selection resulting in heritability of selected traits, female sexual preference remains to be fully elucidated. Understanding of the sexual psychology of females generally is of great importance  – do females select for mates with more impressive traits, and are those traits indicative of a superior partnership? Can female behavior be predicted by the behaviors of those they consider potential mates?

This study is an investigation of female sexual preference and the mechanisms behind it using singing mice, a small rodent species native to the montane cloud forests of Central America. Young female singing mice (*Scotinomys Teguina)* will be used to determine whether females will select for more "impressive" traits in a mate, in this case, male song complexity. Singing mice use their trills to converse socially, but most sing frequently to signal sexual interest or pleasure (Asaba, Kato, Koshida & Kikusui, 2015; Hooper & Carleton, 1970). Song complexity varies by mouse; some mice produce more elaborate, higher-effort trills than others (Fernandez-Vargas, Tang-Martinez & Phelps, 2011; Miller & Engstrom, 2007). Burkhard, Westwick and Phelps found higher song effort to be significantly correlated with increased levels of the hormone leptin, which is involved in long-term energy storage and cost-management (2018). The varying elaborateness of the male singing mouse's trill provides an unambiguous, precise, and easily manipulable trait that allows exploration of female sexual psychology. Young female mice will be exposed to a choice test between "high effort" and "low effort" songs – characterized by being more and less difficult to produce, and the strength of female preference

for varying song efforts will be measured. Additionally, anogenital distance, which predicts

relative masculinity and femininity, will be measured as an indicator of hormone levels.

Females demonstrate most sexual interest when "in estrus," a biological and behavioral

state of fertility (Becker, Breedlove, Crews & McCarthy 2002). A correlated experiment on

estrus induction using estrogen injections is being piloted, with the goal of priming the

hypothalamus for hormone release for more convenient and controlled preference testing

(Edwards, 1970).

I hypothesize that females will select for the high effort songs by spending more time

near and more frequently approaching the high-effort stimulus,  indicating preference for mates

of superior condition based on song complexity.

**Signaling: Costs and Benefits**

Signaling theory holds that animals use morphological, chemical, and behavioral signals

to communicate. A "signal" is a behavior or structure that has evolved to communicate

information with a recipient, evoke an appropriate response from the recipient, and cultivate a

positive effect on the fitness of the signaler and often the recipient as well (Laidre & Johnstone,

2013). These signals communicate both internal information, such as identity, condition,

location, and affective state, and external information, such as threat presence and food sources

(Laidre & Johnstone, 2013).

Use of animal signaling to indicate condition and sexual interest is of particular relevance

to this study. Signals operate on a cost-benefit ratio (Zahavi, 1975), particularly in cases of

sexual selection. Elaborate signals use more energy and in the case of morphological or

behavioral signals, can expose the signaler to predators and other threats. Thereby extravagant

mating signals of a higher cost can be prohibitive to weaker, smaller, or sicklier males, and females can infer higher mate quality from signal quality (Zahavi, 1975).

**Songs as a Signal**

Alston's singing mice (*Scotinomys Teguina*) perform individually unique songs of varying complexity. The songs are used to converse socially in a way scientists believe to be predictive of our own speech patterns (Okobi et al., 2019). Males perform most frequently in proximity of a potential mate, indicating that songs may sometimes be a sexual behavior (Asaba, Kato, Koshida & Kikusui, 2015; Hooper & Carleton, 1967). Song progression and singing behavior are significantly modified by social interaction of both platonic and sexual nature (Okobi et al. 2019; Fernández-Vargas, Tang-Martínez & Phelps, 2011). Although singing mouse pups produce ultrasonic vocalizations, first sonic advertisement songs do not occur until sexual maturity (Campbell et al., 2014). Male mice are particularly inspired to sing in the presence of a female or immediately after intersexual pairings (Hooper & Carleton, 1976).

Singing mouse songs vary on scales of frequency, amplitude, and duration, and can be analyzed reliably in terms of complexity and the effort required to produce them (Miller & Engstrom, 2007). These songs may be signals, particularly male signals of sexual advertisement directed at females, due to the social context in which they are most frequently used. Most importantly, male songs have been found to be a predictor of physiological condition.

High song effort in males is characterized by high rates of singing, as well as longer, louder, and higher frequency songs. High song effort is positively correlated with levels of the hormone leptin (Burkhard, Westwick & Phelps, 2018). Leptin is established as a hypothalamic regulator of food intake and long-term energy expenditure, and leptin signaling pathways are crucial to maintaining a healthy energy level (Pandit, Beerens & Adan, 2017). This correlation

suggests that mice capable of producing energy-costly, high effort songs are of higher quality, meaning they may have higher energy stores, better nutritive health, superior fitness, or more genetic immunity to parasites. Song characteristics are also a proxy for testosterone levels; males who sing more frequent, higher-effort songs have higher androgen concentrations than those who sing low effort songs (Pasch, George, Hamlin, Guillette & Phelps, 2011). By singing elaborate, costly songs, males may indicate to females not only that they are interested in sex, but that they are a physiologically and genetically preferable mate. The present study aims to investigate whether females are responsive to this signal, and thereby select for a higher quality mate based on song characteristics.

**"Good Genes" Hypothesis**

Hamilton and Zuk (1982) proposed the "good genes" hypothesis, one of the most popular explanations for how females select between potential mates. The theory suggests that females select for male traits that signal genetic superiority, and thereby greater viability in her offspring. Genetic superiority might entail physiological advantages, larger body mass, or immunity to a parasitic threat (Hamilton & Zuk 1982).

The good genes theory is subject to debate. Some scientists deem it completely invalid or at least insignificant in real-world impact (Moller & Alatalo, 1999; Howard, Whiteman & Schueller, 1994). Nonetheless, there is consistent evidence in support of the good genes theory: Byers and Waits (2006) found that in the wild, male "attractiveness" as quantified by total number of past offspring sired, is highly correlated with offspring survival. According to Jaquiery et al. (2009), male attractiveness is also significantly correlated with offspring size and growth rate. Thus, female singing mice may be inferring genetic superiority in potential mates from their songs.

**Sexual Preference Behavior in Females**

In most species, sexual encounters are characterized by male competition for female attention, with subsequent female behavioral preference deciding who gets to mate with whom. Preference is often measured by female approach, meaning that the female advances toward the male or male-related stimulus presented to her (Becker, Breedlove, Crews & McCarthy, 2002, Beach, 1976; Page, Kuti, & Sur, 2009; Wills et al., 1983; Blanchard & Blanchard, 2003), as well as duration of time spent in proximity of the stimuli (Becker et al., 2002; Winslow, 2003; Crawley et al., 2007; Moy et al., 2007; Ferguson et al., 2000).

Intersexual allogrooming, social grooming between members of the same species, has been observed between male and female singing mice (Fernandez-Vargas, Tang-Martinez, Phelps ). Significantly more allogrooming occurs between males and females in estrus than any other pairing, signifying that allogrooming is a sexual behavior (Fernandez-Vargas, Tang-Martinez, Phelps, 2011). High levels of genital sniffing are related with sexual interest, particularly in females during the estrus phase (Weinhold and Ingersoll, 1988; Liu et al., 2020). The paramount event of female preference behavior in rodents is lordosis, by which the female identifies herself as sexually receptive to male advances and makes herself physically available to mate by arching her back and lifting her tail. Female sexual behavior research specific to singing mice is sparse, and this study aims to identify and catalogue female sexual behaviors in addition to addressing its main concerns.

**Female Ability to Distinguish Between Songs**

The ability of females to distinguish between songs generally is tantamount to the idea of female sexual response to song effort. Recently, singing mice have been discovered to recognize calls and respond in a sophisticated turn-taking dynamic (Oboki et al, 2019). Oboki et al. (2019)

found that male songs are modulated and enhanced by social context, which suggests that females likely respond favorably to such modulations in a sexual context.

Singing mice are not the only rodents that vocalize. Lab mice (*Mus musculus*) do not sing with the same complexity, though they do produce more elementary ultrasonic vocalizations (USVs). They vocalize differently in response to female urine, male urine, presence of a male, presence of an anesthetized female, and presence of an active, behaving female (Chabout, Sarkar, Dunson & Jarvis, 2015). In response, female lab mice display preference for songs elicited by a specific context, demonstrating ability to distinguish between songs (Chabout, Sarkar, Dunson & Jarvis, 2015). Female mice raised with a father also demonstrate a preference for songs produced by males from genetic strains different from their own, particularly when stimulated by male sexual chemosignals (Asaba et al., 2014). Such evidence suggests that female mice can indeed distinguish between male vocalizations.

**Female Hormones and Estrus Behavior**

Many female mammals, including singing mice, must be in some state of biological reproductive receptivity to display interest in a mate (Becker et al., 2002). In female rodents, this is known as the "estrus cycle" (Caligioni 2010; Byers, Wiles, Dunn & Taft 2012). The estrus cycle is a behavioral, chemical, and morphological fertility cycle which in singing mice lasts 4-5 days. It has four distinct stages: proestrus, estrus, metestrus, and diestrus. Each stage is characterized by different levels of luteinizing hormone and follicle stimulating hormone, which are released by the anterior pituitary gland of the brain (Biles et al. 2012). This is in turn regulated by levels of gonadotropin-releasing hormone, secreted by the hypothalamus. Luteinizing hormone and follicle stimulating hormone stimulate ovarian cells, leading to ovulation and estrogen production (Caligioni 2010). Females are only able to reproduce during

the estrus stage, characterized by recently peaked estrogen levels and ovulation. Behavioral

receptivity, however, is more transient. Female interest lasts only a few hours, timed to coincide

with ovulation by a rush of estrogen to the brain (Becker et al., 2002).

Sanchez-Andrade et al. (2005) found that social recognition in female mice fluctuated

throughout the estrus cycle, with enhanced social recognition during estrogen-heavy stages.

Hanson and Hurley (2012) found that the duration, frequency, and bandwidth of USVs in male

lab mice were modulated by the estrus stage of the female recipient, illustrating vocalization

context-sensitivity and the widespread significance of the estrus cycle.

In an effort to make preference testing with female singing mice more controlled and

convenient, I am investigating approaches for inducing estrus. Though it has never before been

accomplished in singing mice, there are well established procedures for inducing estrus in

laboratory mice. Typical treatments involve a treatment of pregnant mare serum gonadotropin

(PMSG) and human chorionic gonadotropin (HCG) injections (Merkwitz et al., 2016), or

estradiol benzoate and progesterone injections (Edwards, 1970). Singing mice are somewhat

distantly related to lab mice, so hormone treatments will likely differ from traditional protocols.

Nonetheless, a reliable way to induce and control estrus in the female singing mouse would

facilitate improved female choice research.

**Anogenital Distance as a Measure for Hormone Level**

Anogenital distance (AGD) is the distance between the anus and the genitals in

mammals. AGD is sexually dimorphic, meaning it is markedly different in the two sexes:

females have a short AGD, while males have a noticeably longer one. AGD is a proxy for

developmental hormones in mice and some other mammals; males with longer AGDs have

higher testosterone levels, and display more masculine traits (Manno, 2008; vom Saal & Bronson

1980; Schwartz, Christianson, Vinggaard et al., 2019). In male singing mice, a longer AGD means stronger singing behavior: males with a larger AGD have higher testosterone levels, and sing longer, more complex songs (Pasch et al., 2011). Similarly, a shorter AGD in females predicts greater feminization. Female AGD can be predicted by intrauterine proximity to male littermates. This is due to prenatal exposure to androgens – females with longer AGDs were likely positioned between male littermates in the womb, and became subject to atypically high androgen levels (vom Saal & Bronson, 1980). These females are found to be less attractive to male mice in adulthood and have irregular estrous cycles (vom Saal & Bronson, 1980). This study plans to use AGD as an index for hormone levels in tested females and stimulus males, to investigate whether AGD can mediate female sexual preference.

**Conclusions**

Female preference is largely important to the behavioral and evolutionary tendencies of most every species. Female singing mice have the potential to make decisions about mate quality based on male signaling in the form of unique, sophisticated singing behavior. The songs male singing mice perform in the presence of females vary in frequency, length, and amplitude (Miller and Engstrom, 2007; Hooper and Carleton 1970; Fernandez-Vargas, Tang-Martinez & Phelps, 2011) and songs that require more effort to produce are sung by males of higher physiological quality (Burkhard, Westwick & Phelps, 2018). Female lab mice have the ability to distinguish between male lab mouse ultrasonic vocalizations (Chabout, Sarkar, Dunson and Jarvis, 2015; Asaba et al., 2014), and singing mice have shown behavioral and song modulation responses to another mouse's song (Oboki et al, 2019), which provides further reason to think that female singing mice may be distinguishing between songs to choose a superior mate. High effort songs may also be indicative of superior genetic quality, per the "good genes" hypothesis, which

suggests that males more favored by females produce offspring of higher viability (Hamilton and Zuk, 1982; Byers and Waits, 2006).

The question of whether females will select for higher quality males based on song remains to be elucidated. This study aims to use existing knowledge of female estrus and sexual behavior, AGD, and interpretation of male song characteristics to begin answering such questions. *Scotinomys Teguina*'s highly sophisticated turn-taking, social context-dependent system of communication provides a platform by which to investigate female preference, and could perhaps be predictive of some human patterns of communication in courtship behavior. Preference testing females based on male song effort may provide insight into male vocalization behavior, and the social cognition behind female sexual preference across species.

**Methods**

**Experimental Design**

This study aims to investigate female sexual preference in singing mice, contingent on male song quality. Young female singing mice underwent estrus induction by hormonal injection before being exposed to behavioral validation with a male and a two-choice test between high and low effort male songs. Control females were given injections of sesame oil following the same schedule. Song effort is determined by frequency, amplitude, and duration: high frequency, duration and amplitude all constitute a "high effort" song, and low profiles in each produce a "low effort" song. During the trial females were tested in a U-shaped two-choice apparatus. Songs played in alternation from speakers within the two sub-chambers, one male per speaker, and females were free to move through the chamber during playback. Preference was measured by recording total duration of time spent in each zone and total duration of time spent investigating the mesh in front of each speaker. The dependent variable was female preference, and the independent variable was varying song quality and thereby male condition. Female anogenital distance was measured as an additional variable reflecting hormone level to determine whether it moderates preference behavior. I hypothesized that females would display preference for high effort male song, as an indicator of male condition.

**Subjects**

Twenty sexually inexperienced female singing mice (*Scotynomys Teguina*) between 8 and 10 weeks old (10-16 grams) were used. Subjects were descendants of wild-caught animals from the mountainous cloud forests of Central America. Females were divided into control, estradiol benzoate, and estradiol benzoate plus progesterone treatment groups: control animals received no estrus induction, estrus treatment females were subject to a hormonal injection estrus

induction schedule. Both groups underwent the same preference testing and behavioral validation measures. Beginning on the first day of injections, females were housed in separate home cages until testing, with food and water *ad libitum*. They stayed in their normal housing room with a light cycle of 12 L : 12 D (lights on at 10am).

**Stimuli**

Stimulus songs and song analyses were previously used by Burkhard, Westwick and Phelps (2018). Songs were collected from 38 sexually experienced male singing mice of varying condition. Males were captured with Sherman live traps in San Gerardo de Dota, Costa Rica. Nine male songs were used in this experiment. Songs were categorized based on number of notes performed, song length, trill rate, note frequency bandwidth (minimum frequency subtracted from maximum frequency), frequency modulation, and amplitude. Frequency modulation and amplitude were broken down into four and three variables respectively, and every variable was measured in each note of the song. Each singing mouse note is characterized by one simple descending frequency sweep (Fig. 1A). Frequency modulation measurement was composed of starting frequency, note curvature, and frequency slope (Fig. 1B). Amplitude measurement was composed of inter-note interval (time between the end of a note and the beginning of the next), note duration, time to peak amplitude, and time to peak amplitude within the first quarter of the note (Fig. 1B). Weight, residual body mass, length, 5 measures of nutritional status (plasma levels of cholesterol, phospholipids, glucose, triglycerides and non-esterified fatty acids), and hormonal levels of adiponectin, insulin, and leptin were measured in each male. A greater weight and length is indicative of a larger, more robust mouse, likely able to produce higher effort songs. Residual body mass, adiponectin, insulin, and leptin are indicators of long standing

energy balance, and a composite Z-score of the four strongly predict song effort (Burkhard,

Westwick and Phelps, 2018). High levels of each in a male would lead to more elaborate songs.
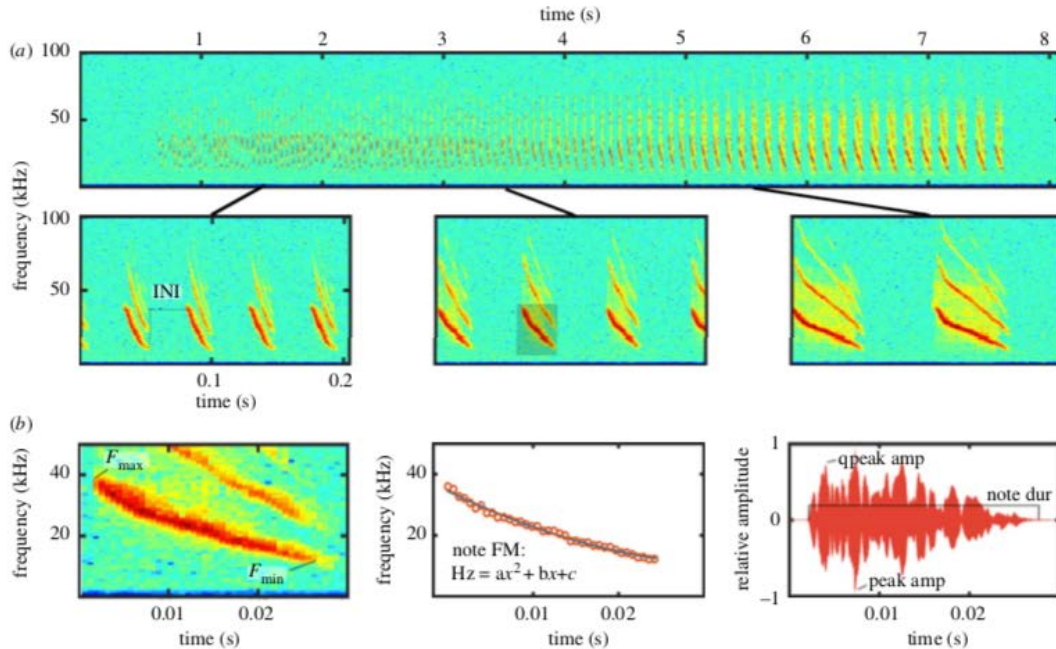


**Figure 1.** This figure is borrowed and modified from Burkhard, Westwick, and Phelps (2018). Structure of a singing mouse song. (*a*) Spectrogram of a typical male song: songs increase in amplitude and frequency as they progress. Subpanels show closeups of note groupings in 0.2 second intervals of the song. The first subpanel illustrates internote interval (INI), the time between the end of one note and the beginning of the next. The shaded portion in the second subpanel highlights a single note. (*b*) Amplitude and frequency modulation models. The left panel calculates note bandwidth by subtracting the minimum note frequency from the maximum note frequency ($F_{max} - F_{min}$). The central panel illustrates how frequency modulation was calculated by fitting the frequencies of each individual note to a quadratic curve. "a" is the curvature of the note, "b" is the slope, and "c" is the starting frequency. These are plugged into the quadratic equation to generate a frequency modulation value. The right panel depicts note amplitude in waveform, as given by peak amplitude, note duration, and quarter peak amplitude (peak amplitude reached within the first 25% of the note).

## Estrus Induction

Female singing mice were randomly divided into control (n=5), estradiol benzoate (n=7), and estradiol benzoate plus progesterone (n=8) treatment groups, which were counterbalanced for relation. Standard estrus induction protocols for lab mice were followed (Edwards, 1970),

and dosages were adjusted according to weight differences between lab mice and singing mice.

Females in the estradiol benzoate treatment group were injected subcutaneously with 1 ug

estradiol benzoate in 0.025 mL sesame oil once daily for three days preceding test day, and on

test day six hours preceding behavioral validation. Females in the estradiol benzoate plus

progesterone group were injected subcutaneously with 1 ug estradiol benzoate in 0.025 mL

sesame oil once daily for three days preceding test day, and on test day six hours preceding

behavioral validation. They were given an additional subcutaneous injection of 0.250 mg

Progesterone in 0.083 mL sesame oil on test day six hours preceding behavioral validation.

Control females were injected with 0.025 mL sesame oil once daily for three days preceding test

day, and on test day six hours preceding behavioral validation. 11 females from a previous estrus

testing cohort who received the same treatments (t-tests found no statistical differences between

the two cohorts behaviorally or morphologically) were included in estrus data analysis to

improve clarity, but they were not preference tested.

**Behavioral measures**

For behavioral validation of estrus, female receptivity was measured by total duration of

a number of behaviors that characterize female interest or lack thereof, such as "female

approaching," "female avoiding," "female rebuffing," "female sniffing male anogenital region,"

"female allowing male to investigate female anogenital region," and "lordosis." All measures are

well established in rodent behavioral evaluation (Becker et al., 2002; Beach, 1976; Page, Kuti, &

Sur, 2009; Wills et al., 1983; Blanchard and Blanchard, 2003; Asaba et al., 2015).

**Morphological Measures**

Estrus was further confirmed by examining morphological photo data. Before each

hormonal injection during the week, a photo was taken of every female's vaginal status. This

was accomplished by one researcher placing the female's forepaws on the lip of her home cage

and holding her shoulders in place while lifting her tail in the air to expose her anogenital region.

Another researcher took a close up picture. Photos were blindly scored according to the visual

method . Females in estrus have the following vaginal characteristics: a wide vaginal opening

that is slightly pink, slightly moist, and slightly swollen. If the photo taken on the day of testing

displayed these characteristics, the female was indisputably in estrus during preference testing.

### Preference Measures

For preference tests, female response was measured in terms of duration (s) spent

investigating each zone of the U-shaped apparatus and total duration of time spent investigating

the mesh in front of each speaker. All measures are well established in rodent behavioral

evaluation (Becker et al., 2002; Beach, 1976; Page, Kuti, & Sur, 2009; Wills et al., 1983;

Blanchard and Blanchard, 2003; Asaba et al., 2015).

### AGD Measures

AGD was measured in each female at the end of test day. One researcher held the mouse

on her back with her tail out of the way, and the other used a millimeter ruler to measure the

distance between her vaginal opening and anus in millimeters. Each female was then weighed for

AGD scaling (grams).

## Two Choice Test

### Apparatus

The testing space was a wood and wire caging rectangular apparatus with a U-shaped

interior (Fig. 2). It was 61 cm long, 30.5 cm wide, and 63 cm high in total. There was a 42 cm

long divider running lengthwise through the apparatus, creating two 13 cm wide, 42 cm long

"hallways." This left a neutral open space 30.5 cm wide and 21 cm long outside the "hallways."

The bottom 22 cm of the apparatus was wire caging, to allow free flowing sound, and the upper

41 cm was wood. Each hallway of the apparatus was visually (but not physically) subdivided by

a Zip Tie along the floor into two 21 cm by 13 cm zones, used to define subject proximity to the

speaker. The zone closest to the speaker was denoted as the sound zone, and the other as the

center zone (Fig. 2).

Two speakers (Pioneer TS-250) were attached to the back wire caging of the two

"hallways". The speakers were connected to RX6 audio processing hardware, which allowed for

clear and easy playback patterns through a software interface. A video camera was used to record

the trials. It was mounted several feet above the apparatus to capture preference behavior and any
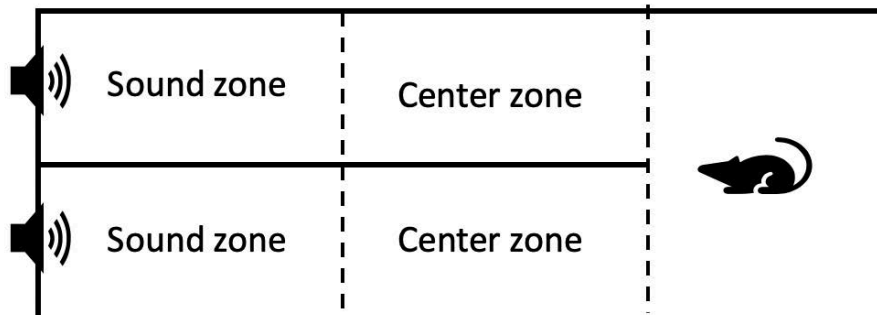
response songs.



**Figure 2.** Experimental apparatus: solid lines indicate walls and dashed
lines indicate zone parameters.

**Testing Procedure**

During the three days prior to testing, estrus was induced in the female using an injection

schedule of either estradiol benzoate or estradiol benzoate plus progesterone. The control group

was given sesame oil injections. A photograph of each female's vaginal status was taken before

each injection and after testing for morphological validation of estrus. Females were housed in

separate home cages beginning on the day of the first injection, and were given normal shelter, food, and water ad libitum.

On the test day, home cages containing females were placed in cabinet-style testing chambers for behavioral validation. Each chamber has a camera built into the ceiling, which is connected to a PC and footage can be viewed and recorded using Basler pylon camera software. Lights within the chamber are controlled on a smartphone by the Hue Philips app, and small fans to cycle out air are built into the side of each chamber. Each female was behaviorally validated using one of two males known to have previously produced litters. As six females were tested per week, three females were randomly assigned to each male, and were tested in a randomized order. The male was first presented to the female in a presentation chamber, which was a 12x12x25 cm plastic chamber with a 5x5 cm mesh panel on one side to allow sniffing. The presentation chamber containing the male was placed on one end of the female's home cage, and a "dummy" presentation chamber containing no male was placed on the other end. Female investigation was recorded for 10 minutes. After 10 minutes, both presentation chambers were removed and the male was released into the female's home cage. Behavioral testing proceeded for an additional 15 minutes.

After all females had been behaviorally tested, preference testing proceeded with the same randomized female testing order as used in behavioral validation. For each preference test, the female was removed from her home cages and with a transfer tube and habituated to the testing apparatus for 10 minutes prior to the first stimulus broadcast. Video recording began after this acclimation period. High and low-effort songs were then broadcasted antiphonally, three repetitions per condition with six trills in total, for three minutes of overall playback time. High and low-effort stimuli broadcasts were offset at 30 second intervals, 60 seconds between each

repetition of the same song. Order of the first song played (high or low effort) was varied across mice to ensure unbiased trials. Mice were free to investigate during playback. Female responses were recorded for an additional 5 minutes after playback was complete. Stimulus order and speaker side was randomized for each trial, preventing data from being confounded by bias. AGD (mm) and weight (g) were measured for each female immediately after testing. Females were then returned to their home cages and provided with mealworms.

**Statistical Analysis**

Effect of treatment on behavioral validation was analyzed with one-way analysis of variance and post-hoc tests. Effect of treatment on morphology between groups across days was analyzed with one-way analysis of variance and post-hoc tests. Within-treatment morphological progression from Day 1 to Day 4 (test day) was assessed with paired samples t-tests.

To determine whether there was an effect of song effort on female preference, data was assessed using a two-tailed paired-sample t-test, conducted between high and low effort song conditions. The null hypothesis predicts that there will be no evidence of preference in either direction: females is equally likely to approach and spend time around either stimulus. AGD was measured as a continuous variable and scaled with body weight (AGD (mm)/Body weight (g)), then plotted in a regression against duration of stay in the high effort sound zone and high effort speaker investigation.

# Results

## Behavioral Validation Results

One-way analysis of variance and post-hoc tests were used to assess differences in female sexual behavior during behavioral validation testing for estrus, with an alpha level of 0.05 for all statistical tests. No difference was found between treatment groups for generic contact between the male and female mice, and no difference was found between treatment groups for female approach behavior. There was also no significant difference between groups in female anxiety behavior, though control animals (M = 154.8) did display more than the estradiol benzoate group (M = 117.8) and far more than the estradiol benzoate plus progesterone group (M = 34), $F(2, 6) = 0.8$, $p = 0.48$. There was no significant difference between groups for "male sexual investigation of female" behavior, $F(2, 16) = 1.6$, $p = 0.23$, though both the estradiol benzoate (M = 60.33) and the estradiol benzoate plus progesterone groups (M = 42.8) showed more of this behavior than the control females (M = 17.8) (Fig. 3A). There was a significant effect of treatment group on "female sexual investigation
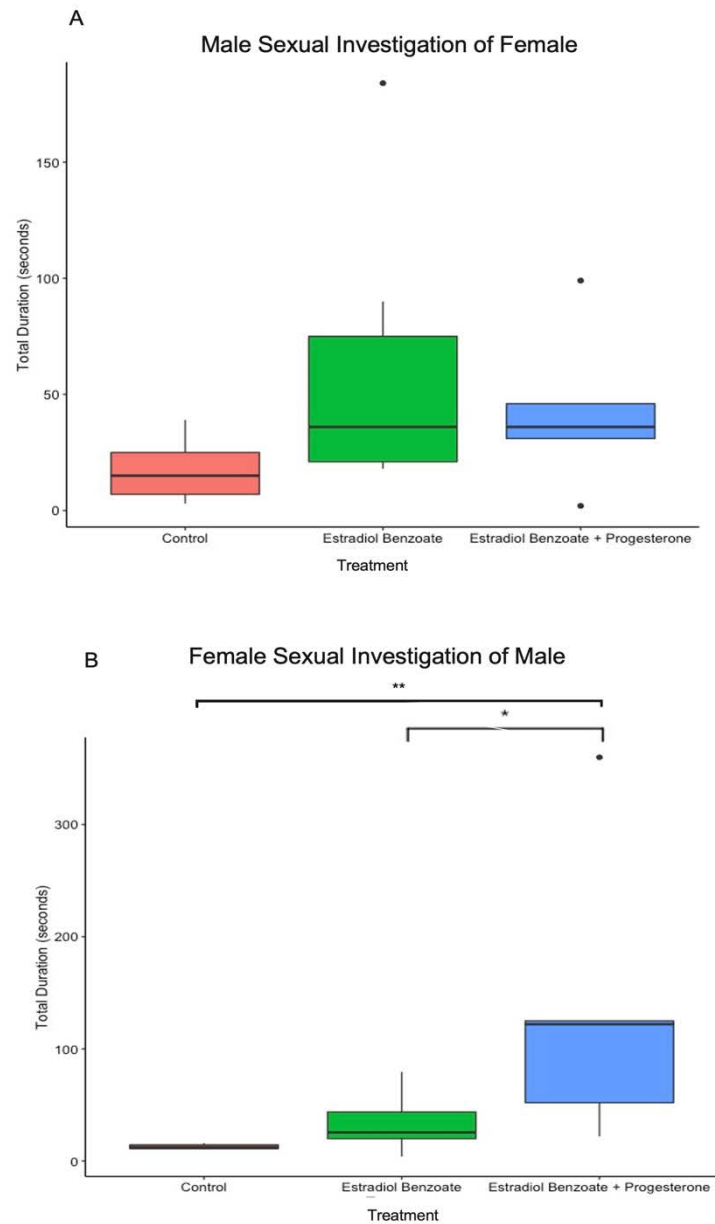


**Figure 3.** Behavioral validation responses across treatment groups. (A) "Male sexual investigation of female" behavior is insignificant across groups but both estrus treatments displayed more than control females. (B) "Female sexual investigation of male" behavior was significantly different across groups; estradiol benzoate plus progesterone displayed more than both other groups. Data are M ± SD.

of male" behavior, $F_{(2, 16)} = 4.7$, $p = 0.025$: estradiol benzoate plus progesterone females displayed more sexual proceptivity than both the estradiol benzoate group and the control group (Fig. 3B).

**Morphological Validation Results**

One-way analysis of variance and post-hoc tests with an alpha level of 0.05 found no significant difference in vaginal state between treatment groups on Day 1 of injections, $F_{(2, 26)} = 0.78$, $p = 0.55$ (fig. 4A). A significant difference was found in vaginal state between groups on Day 4 (test day): females in the estradiol benzoate treatment group had significantly greater vaginal perforation than the estradiol benzoate plus progesterone group and the control group, $F_{(2, 21)} = 10.6$, $p = 0.0007$) (fig. 4B). Estradiol benzoate plus progesterone (M = 0.94) females had greater vaginal perforation than the control females (M = 0.50) on Day 4, but not significantly so.
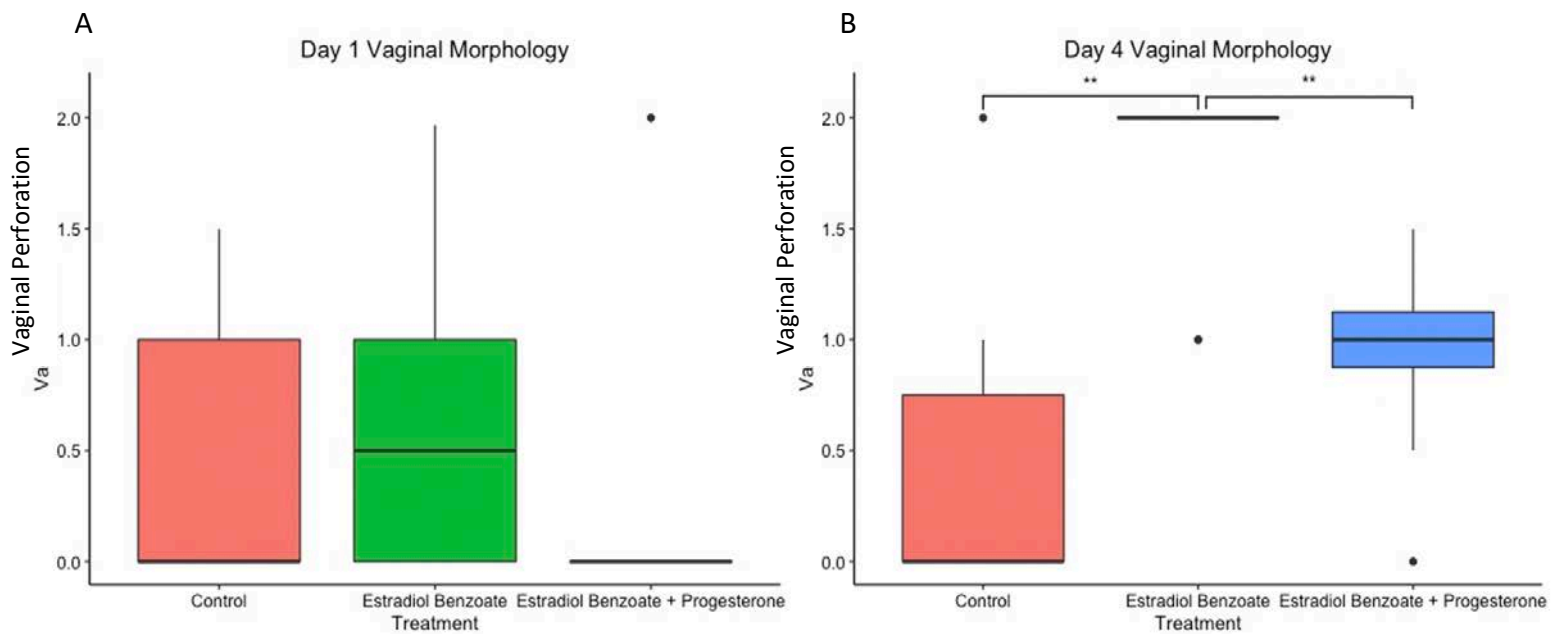


**Figure 4.** Vaginal morphology across treatment groups from Day 1 and Day 4 (test day). (A) Day 1 vaginal morphology showed no statistical differences between groups. (B) Day 4 vaginal morphology showed significant differences between groups: estradiol benzoate females were much more perforate than control and estradiol benzoate plus progesterone, and estradiol benzoate plus progesterone was more perforate than control. Data are M ± SD.

Paired sample t-tests found that the estradiol benzoate group had significantly greater vaginal perforation on test ay than on Day 1 of hormonal injections, $t(8) = -5.0$, $p = 0.001$ (Fig. 5B). There was also a marginally significant difference in vaginal perforation within the estradiol benzoate plus progesterone group between Day 1 and Day 4, $t(7) = -2.3$, $p = 0.05$ (Fig. 5B). No significant difference in vaginal perforation was found within the control group between Day 1 and test day, $t(6) = -0.7$, $p = 0.51$ (Fig. 5A).
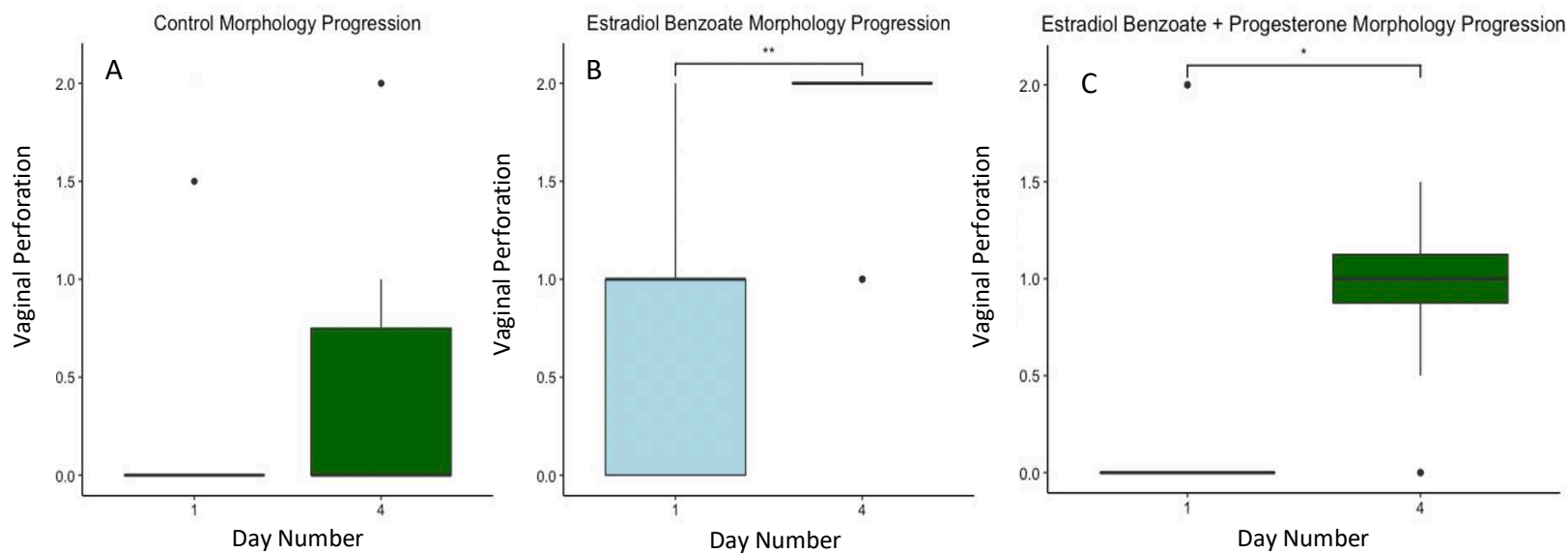


**Figure 5.** Vaginal morphology within treatments from Day 1 to Day 4 (test day) of hormonal injections. (A) Control females showed no significant difference in vaginal morphology from Day 1 to Day 4. (B) Estradiol benzoate females showed significantly greater vaginal perforation on Day 4 than Day 1 of injections. (C) Estradiol benzoate plus progesterone females showed significantly greater vaginal perforation on Day 4 than Day 1 of injections. Data are M ± SD. *$p<0.05$, **$p<0.01$.

**Female Preference Test Results**

Paired sample t-tests with an alpha level of 0.05 were used to examine female preference results. There was an overall significant effect of male song effort on duration of time spent in the sound zone, $t(18) = 2.5$, $p = 0.025$ (Fig. 6A). Females significantly preferred to spend time in

the high effort song side of the sound zone versus the low effort side. There was also an overall significant effect of male song effort on female preference for duration of speaker investigation, t(18) = 2.4, $p$ = 0.027 (Fig. 6A). Females spent significantly more time investigating (characterized by sniffing) the high effort speaker than the low effort speaker. There was no significant effect of song effort on female preference in the center zone, t(18) = -0.04, $p$ = 0.97; females displayed no preference for high or low effort songs (Fig. 6C). However, females did spend a slightly greater amount of time on the high effort side (M = 31.7) than the low effort side (M = 29.8).
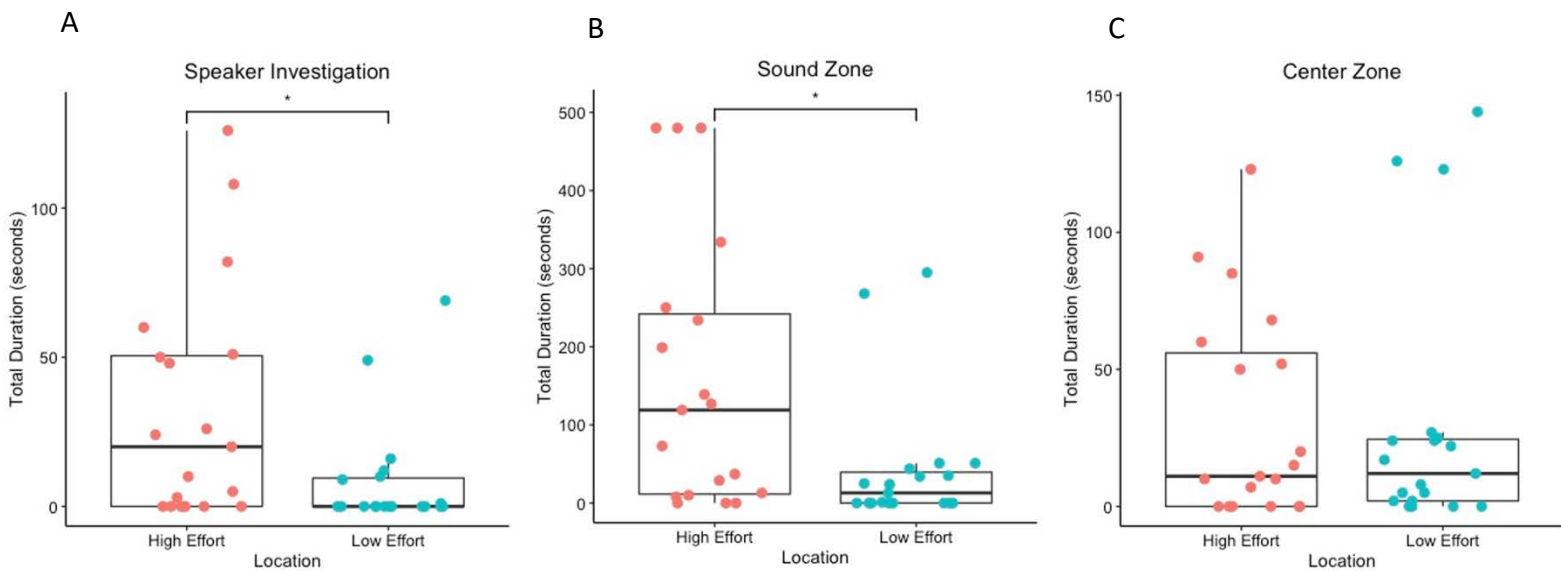


**Figure 6.** Female preference between high and low effort songs for each apparatus zone and speaker. (A) Females spent significantly more time investigating the high effort speaker than the low effort speaker. (B) Females spent significantly more time in the high effort sound zone. (C). There was no significant difference in duration spent on high and low effort song sides of the center zone. Data are M ± SD. *$p$<0.05.

One-way analysis of variance and post-hoc tests with an alpha level of 0.05 were used to assess the effect of estrus treatment on duration of time spent in each zone location. There was no significant effect of treatment on duration of time spent in the high effort sound zone, F(2,16) = 1.5, $p$ = 0.26, or on duration spent in the low effort sound zone, F(2,16) = 0.4, $p$ = 0.66 (Fig.

7B). There was also no significant effect of treatment on duration of time spent investigating the

high effort speaker, $F(2, 16) = 2.8$, $p = 0.09$, or on duration of time spent investigating the low

effort speaker, $F(2, 16) = 0.7$, $p = 0.52$ (Fig. 7A). Finally, no significant effect was found of

treatment on duration spent in the high effort center zone, $F(2,16) = 1.103$, $p = 0.36$, or the low

effort center zone $F(2, 16) = 1.9$, $p = 0.18$ (Fig. 7C).
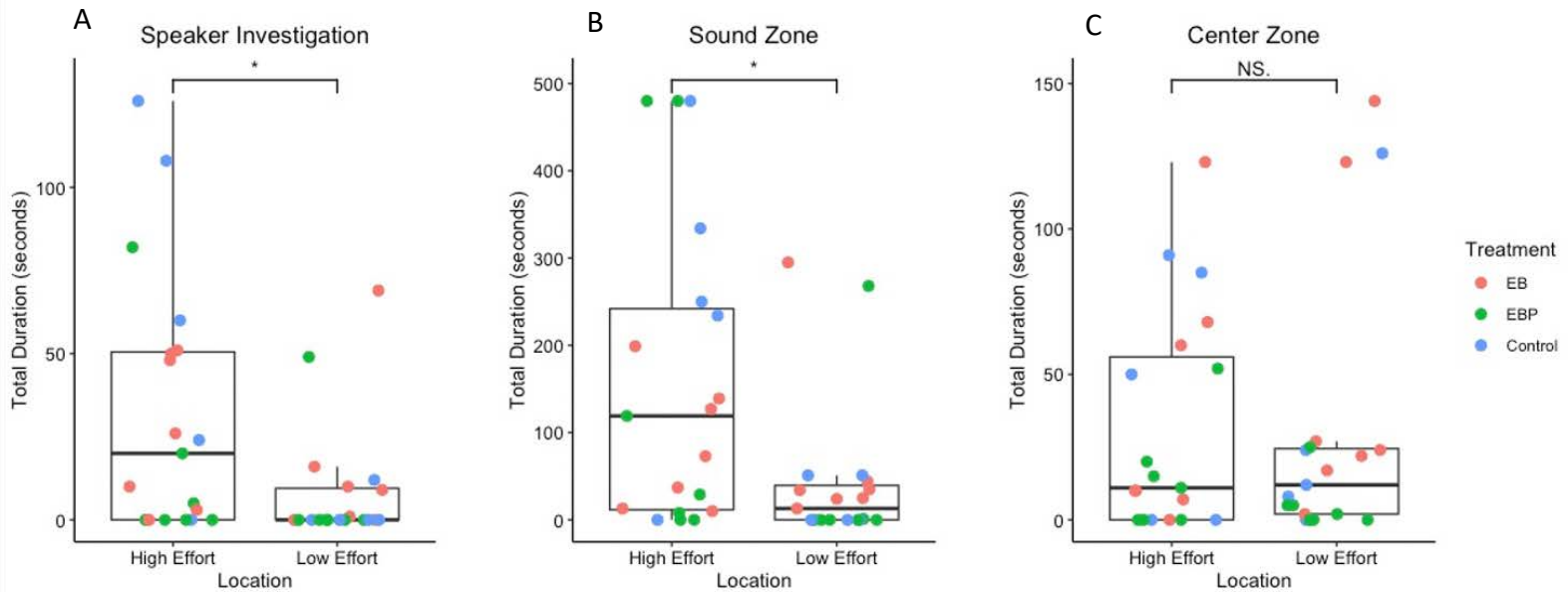


**Figure 7.** Female preference responses to high and low effort songs (see Fig. 6) colored by treatment. Significance bars reflect overall song effort preference, not treatment. (A) There was no significant effect of treatment on speaker investigation preference. (B) There was no significant effect of treatment on sound zone song effort preference. (C) There was no significant effect of treatment on central zone song effort preference. Data are M ± SD. *$p$<0.05.

**AGD Results**

Analyses using Pearson's correlation coefficient assessed the relationship between female

anogenital distance (AGD) and strength of preference for high effort stimuli. There was no

significant correlation between AGD and duration of time spent in the high effort sound zone

$r(17)= 0.09$, $p = 0.70$ (Fig. 8A). There was a visible negative correlation between AGD and high

effort speaker investigation, but it was not statistically significant, $r(17) = 0.34$, $p = 0.15$ (Fig
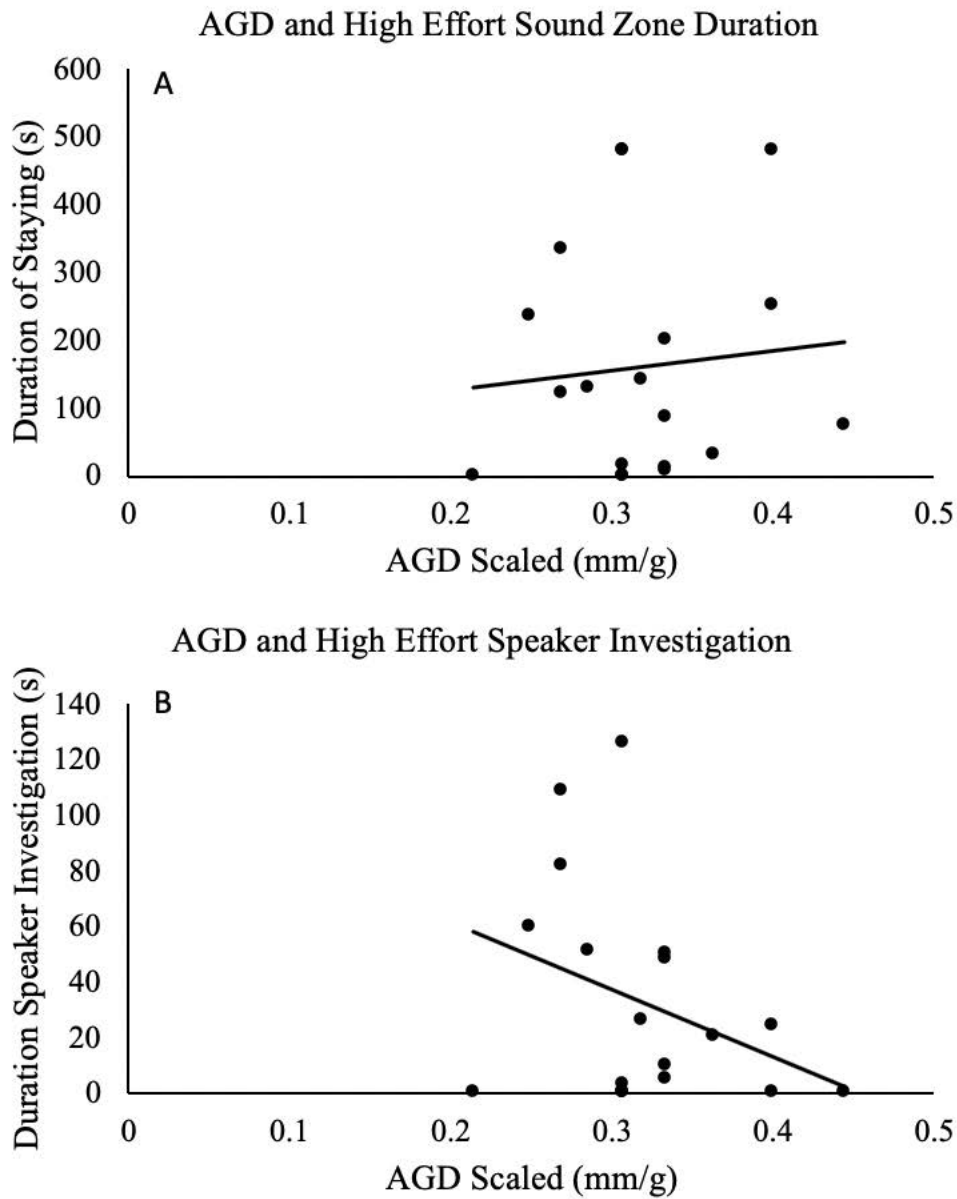
8B).

**Figure 8.** Correlation of female AGD with high effort preference strength. (A) No significant correlation was found between AGD and duration of time spent in the high effort song sound zone. (B) No significant correlation was found between AGD and high effort speaker investigation, but a negative trend is visible in the data.

**Discussion**

The behavioral validation results suggest a stronger effect of behavioral estrus in the two treatment groups than the control group, though not always significantly so. Control females showed more anxious behavior than both treatment groups, suggesting that females who were given estrus hormones were more interested in the male mice and comfortable in the sexual testing setting, whereas control females were highly anxious and often made attempts to escape the presence of the male mice. The far higher display of "male sexual investigation of female" behavior in the estradiol benzoate and estradiol benzoate plus progesterone groups than the control females suggests stronger female receptivity to male sexual interest. For the male to investigate her genital region, the female must stay still and invite or allow the sexual attention. Generally, the control mice would actively avoid this behavior. A significantly higher display of "female sexually investigating male" behavior from the estradiol plus progesterone mice indicates proceptivity in these females. The female actively approaching and sexually investigating the male suggests that the addition of progesterone may bring females to a behavioral estrus state, in which they are actively interested in pursuing a mate. It is difficult to confirm estrus based on these observations alone, but they are supplemented by the morphological data. There is no established method in the literature for estrus induction in female singing mice, and this as well as other previous attempts may serve as a stepping stone to future, more dependable estrus induction techniques.

Insignificant ANOVA test results between treatment groups on Day 1 of injections demonstrate that there was no morphological difference between groups prior to hormonal injections, and also that females were not yet naturally phasing through estrus (Fig. 4A). The differences between the treatment and control groups on Day 4 show that there was a significant

physiological effect of hormonal treatment on the treatment females, and none on the control. This is further exemplified by the within-treatment analyses, which showed that there was no physiological difference between Day 1 and Day 4 in the control females, but a significant difference between Day 1 and Day 4 in both treatment groups (Fig. 5). The strong significance in the morphological data provides evidence that both treatment groups, particularly the estradiol benzoate group, were in estrus during preference testing.

As hypothesized, preference test results suggest females can indeed differentiate between male song effort, and when given the choice display strong preference for high effort songs. Females spent significantly more time investigating the high effort speaker and the high effort sound zone (Fig. 6), implying more interest in locating the male producing this song than the low effort song. Given the positive correlation between song effort and male condition, such as larger BMI, higher leptin levels, and higher testosterone levels (Burkhard, Westwick, and Phelps, 2018; Pasch et al., 2011), these results suggest females may be using song effort to select higher quality mates.

Treatment did not affect female preference of high and low effort songs, which was unexpected. Females displayed varying preference for high effort stimuli regardless of estrus phase. One reason for this may be inconsistent estrus induction results. Presently, there is not a well-established way to induce estrus in female singing mice. Another explanation may be that female singing mice will seek out superior mates at all phases of the estrus cycle. Previous studies in lab mice have found that estrus phase significantly enhances response to male signals, but singing mice may deviate from this norm (Sanchez-Andrade et al., 2005; Hanson and Hurley, 2012).

Though statistically insignificant, the negative correlation between AGD and high effort speaker investigation has a reasonably strong Pearson's correlation coefficient. As the scaled AGD values increased, the duration spent investigating the high effort speaker decreased (Fig 8). This could be accounted for by the greater hormonal feminization correlated with a shorter AGD in females. Previous studies have shown that a female mouse with a shorter AGD has lower androgen levels and is more attractive to males (Vom Saal and Bronson, 1980). Though insignificant, these results may suggest that AGD can be used as a proxy for preference strength: the shorter the female's AGD, the longer she spends investigating a potential high-quality mate. A more precise mechanism of AGD measurement and further data collection may clarify any trends.

In agreement with the hypothesis, these results suggest the variation in male singing mouse song effort and complexity may predict female singing mouse mate choice. Variation in male song complexity is well established – some males sing songs that require higher effort and energy (Fernandez-Vargas, Tang-Martinez & Phelps, 2011; Miller & Engstrom, 2007). Male singing mice that are able to produce longer, louder, more elaborate songs are of superior physiological condition (Burkhard, Westwick, and Phelps, 2018; Pasch et al., 2011), and may additionally have superior genetic quality, such as higher fitness or parasitic immunity (Hamilton and Zuk, 1982; Byers and Waits, 2006). Female ability to use the sophisticated, reciprocal communication patterns of singing mouse song as a mate quality signal would provide an evolutionary edge.

**Limitations and Future Directions**

The COVID-19 pandemic prevented full data collection for preference testing. These results recommend further investigation into female mate preference based on song, considering

how complex a variable singing mouse song is. Establishment of a more consistent estrus-induction technique in singing mice may improve variability, and would generally benefit female choice research. It may also provide a more thorough explanation for the female sexual behaviors and preference behaviors observed in this study.

**References**

Anders Pape Moller and Rauno V. Alatalo, (1999). Good genes effects in sexual selection. *Proceedings: Biological Sciences*, Vol. 266, No. 1414

Asaba, Akari, et al., (2014). Developmental Social Environment Imprints Female Preference for Male Song in Mice. *PLoS ONE*, 9:2. doi:10.1371/journal.pone.0087186.

Asaba, A., Kato, M., Koshida, N., & Kikusui, T. (2015). Determining Ultrasonic Vocalization Preferences in Mice using a Two-choice Playback Test. *Journal of visualized experiments : JoVE*, (103), 53074. https://doi.org/10.3791/53074

Banerjee, Phelps, and Long. (2019). Quick guide: Singing mice. *Current Biology* 29:R190-191.

Barash, D. P. (1973). The social biology of the Olympic marmot. Anim. Behav. Morzg. 6, 173-245.

Beach, F. A. (1976). Sexual attractivity, proceptivity, and receptivity in female mammals. *Hormones and Behavior, 7*(1), 105-138. doi:10.1016/0018-506X(76)90008-8z

Becker, Jill B., et al., (2002). *Behavioral Endocrinology*. 2nd ed., Cambridge, MA: MIT Press.

Blanchard, D. C., & Blanchard, R. J. (2003). Bringing natural behaviors into the laboratory: A tribute to paul MacLean. *Physiology & Behavior, 79*(3), 515-524. doi:10.1016/S0031-9384(03)00157-4

Burkhard, Westwick and Phelps. (2018). Adiposity signals predict vocal effort in Alston's singing mice. Proceedings of the Royal Society B 285. doi: 10.1098/rspb.2018.0090

Byers, J. A., & Waits, L. (2006). Good genes sexual selection in nature. Proceedings of the National Academy of Sciences, 103(44), 16343–16345. doi: 10.1073/pnas.0608184103

Caligioni C. S. (2009). Assessing reproductive status/stages in mice. *Current protocols in neuroscience*, *Appendix 4*, Appendix–4I. doi:10.1002/0471142301.nsa04is48

Chabout J., Sarkar A., Dunson D.B. and Jarvis E.D. (2015) Male mice song syntax depends on social

    contexts and influences female preferences. *Front. Behav. Neurosci*. **9**:76. doi:

    10.3389/fnbeh.2015.00076

Cox, K. H., & Rissman, E. F. (2011). Sex differences in juvenile mouse social behavior are influenced

    by sex chromosomes and social context. *Genes, brain, and behavior*, *10*(4), 465–472.

    doi:10.1111/j.1601-183X.2011.00688.x

Crawley J.N., Chen T, Puri A, Washburn R, Sullivan TL, Hill JM, Young NB, Nadler JJ, Moy SS,

    Young LJ, Caldwell HK, Young WS (2007) Social approach behaviors in oxytocin knockout

    mice: Comparison of two independent lines tested in different laboratory environments.

    Neuropeptides 41:145–163.

Deborah N. Shelley, Elena Choleris, Martin Kavaliers, Donald W. Pfaff, (2006). Mechanisms

    underlying sexual and affiliative behaviors of mice: relation to generalized CNS arousal. *Social*

    *Cognitive and Affective Neuroscience*, Volume 1, Issue 3. https://doi.org/10.1093/scan/nsl032

Edwards, D. A. (1970). Induction of estrus in female mice: Estrogen-progesterone interactions.

    Hormones and Behavior, 1(4), 299–304. doi: 10.1016/0018-506x(70)90022-x

Ferguson J.N., Young LJ, Hearn EF, Matzuk MM, Insel TR, Winslow JT (2000) Social amnesia in mice

    lacking the oxytocin gene. Nat Genet 25:284– 288

Fernandez-Vargas, Tang-Martinez and Phelps. (2011). Singing, allogrooming, and allomarking

    behaviour during inter- and intra-sexual encounters in the Neotropical short-tailed singing mouse

    (Scotinomys teguina). Behavior 148:945-965.

Fisher R. A. (1915). The evolution of sexual preference. *The Eugenics review*, *7*(3), 184–192.

Hamilton, W., & Zuk, M. (1982). Heritable true fitness and bright birds: a role for parasites? Science,

    218(4570), 384–387. doi: 10.1126/science.7123238

Hanson, J. L., & Hurley, L. M. (2012). Female presence and estrous state influence mouse ultrasonic

courtship vocalizations. *PloS one*, *7*(7), e40782. doi:10.1371/journal.pone.0040782

Howard, R. D., Whiteman, H. H., & Schueller, T. I. (1994). Sexual Selection in American Toads: A Test

of a Good-Genes Hypothesis. *Evolution*, *48*(4), 1286. doi: 10.2307/2410386

Jaquiéry, J., Broquet, T., Aguilar, C., Evanno, G., & Perrin, N. (2010). Good Genes Drive Female

Choice for Mating Partners in the Lek-Breeding European Treefrog. *Evolution, 64*(1), 108-115.

Retrieved from http://www.jstor.org/stable/27743495

Kirkpatrick, M. (1982). Sexual Selection and the Evolution of Female Choice. *Evolution*, *36*(1), 1. doi:

10.2307/2407961

Lande, R. (1981). Models of speciation by sexual selection on polygenic traits. *Proceedings of the*

*National Academy of Sciences*, *78*(6), 3721–3725. doi: 10.1073/pnas.78.6.3721

Liu, Z., Jiang, N., Tao, X., Wang, X., Liu, X., Xiao, S., & Liu, Z. (2020). Assessment of Sexual

Behavior of Male Mice. *Journal of Visualized Experiments : JoVE*, (157).

https://doi.org/10.3791/60154

Miller, J. R., & Engstrom, M. D. (2007). Vocal Stereotypy and Singing Behavior in Baiomyine Mice.

Journal of Mammalogy, 88(6), 1447–1465. doi: 10.1644/06-mamm-a-386r.1

Merkwitz, C., Blaschuk, O., Eplinius, F., Winkler, J., Prömel, S., Schulz, A., & Ricken, A. (2016). A

simple method for inducing estrous cycle stage-specific morphological changes in the vaginal

epithelium of immature female mice. Laboratory Animals, 50(5), 344–353.

https://doi.org/10.1177/0023677215617387

Moller, A., & Alatalo, R. (1999). Good-Genes Effects in Sexual Selection. *Proceedings: Biological*

*Sciences, 266*(1414), 85-91. Retrieved from http://www.jstor.org/stable/51346

Moy S.S., Nadler JJ, Young NB, Perez A, Holloway P, Barbaro RP, Barbaro JR, West LM, Threadgill

DW, Lauder JM, Magnuson TR, Crawley JN (2007) Mouse behavioral tasks relevant to autism:

Phenotypes of ten inbred strains. Behav Brain Res 176:4–20.

O'Donald P. (1969). The selective coefficients that keep modifying genes in a

population. *Genetics*, *62*(2), 435–444.

Okobi, Banerjee, Matheson, Phelps, M.A. Long. (2019). Cortical control of vocal interaction in

Neotropical singing mice. Science 363:983-988.

Page, D. T., Kuti, O. J., & Sur, M. (2009). Computerized assessment of social approach behavior in

mouse. *Frontiers in Behavioral*

*Neuroscience,* doi:http://dx.doi.org.ezproxy.lib.utexas.edu/10.3389/neuro.08.048.2009

Pandit, R., et al., (2016). Role of Leptin in Energy Expenditure: the Hypothalamic

Perspective. *American Journal of Physiology-Regulatory, Integrative and Comparative*

*Physiology*, vol. 312, no. 6. doi:10.1152/ajpregu.00045.2016.

Pasch, B., George, A. S., Hamlin, H. J., Guillette, L. J., & Phelps, S. M. (2011). Androgens modulate

song effort and aggression in Neotropical singing mice. Hormones and Behavior, 59(1), 90-97.

https://doi.org/10.1016/j.yhbeh.2010.10.011

Saal, F. Vom, and F. Bronson. "Sexual Characteristics of Adult Female Mice Are Correlated with Their

Blood Testosterone Levels during Prenatal Development." Science, vol. 208, no. 4444, 1980, pp.

597–599., doi:10.1126/science.7367881.

Sanchez-Andrade G., James B.M., Kendrick K.M. (2005). Neural encoding of olfactory recognition

memory. The Journal of Reproduction and Development, vol. 51 (pg. 547-58)

Schwartz, C.L., Christiansen, S., Vinggaard, A.M. et al. Arch Toxicol (2019) 93: 253.

https://doi.org/10.1007/s00204-018-2350-5

Weinhold, L., & Ingersoll, D. (1988). Modulation of male mouse genital sniff, attack, and mount

behaviors by urogenital substances from estrous females. *Behavioral and Neural Biology*, *50*(2),

207–228. https://doi.org/10.1016/S0163-1047(88)90891-6

Williams, George C. (1966) Adaptation and Natural Selection. Princeton University Press.

Wills G. D., Wesley A. L., Moore F. R., Sisemore D. A. (1983). Social interactions among rodent

conspecifics: a review of experimental paradigms. Neurosci. Biobehav. Rev. 7, 315–

32310.1016/0149-7634(83)90035-0

Winslow J.T. (2003) Mouse social recognition and preference. In: Current protocols in neuroscience,

8.16.1–8.16.13. New York: Wiley

Zahavi, Amotz. (1975). Mate Selection-A Selection for a Handicap. *Journal of theoretical biology*, 53.

205-14. 10.1016/0022-5193(75)90111-3.