



Female preferences for high vocal effort in singing mice

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Abstract

Although vocalizations are essential to mammalian sociosexual behaviours, little is known about female preferences for male vocal displays in non-model mammalian species. Here, we characterized female preferences for the advertisement songs of male Alston's singing mice (*Scotinomys teguina*). We developed procedures for inducing oestrus, using vaginal perforation as a morphological indicator of oestrus. We then broadcasted 'high-effort' and 'low-effort' songs recorded from wild-caught males to virgin laboratory-reared females, using a two-choice experiment. Our results indicate that females spent more time investigating speakers playing high-effort songs; surprisingly, this phonotactic response is independent of oestrus status. In wild singing mice, acoustic characteristics of high-effort male songs positively correlate with body condition. Our data suggest that females could use acoustic cues to select mates in good condition, thus providing preliminary support for adaptive mate choice hypotheses. More generally, our results support the hypothesis that elaborate *Scotinomys* song may be shaped by female choice.

Keywords

mate choice, sexual selection, singing mice, advertisement song, honest signalling, vocalization, female preference, *Scotinomys*.

1. Introduction

Females choose mates by assessing behavioural, morphological, or other phenotypic components of male display, and they often tend to favour the most exaggerated traits (Ryan & Keddy-Hector, 1992; Ryan & Cummings, 2013). Many explanations for such preferences have been proposed

(reviewed in Kirkpatrick & Ryan, 1991). Attractive traits may excite female sensory or cognitive systems (Ryan et al., 1990; Ryan & Cummings, 2013), reduce search costs (Lindström & Lehtonen, 2013) or inbreeding (Blouin & Blouin, 1988), predict parental quality (Norris, 1990), or become genetically entangled with the female preferences themselves (Wilkinson & Reillo, 1994). Other explanations posit that female choice may be adaptive by selecting for mates better suited for survival. Evidence from empirical studies across taxa supports this hypothesis, demonstrating that elaborate male displays can be correlated with a variety of important male traits, including body mass, immune status, stress, parasite load, nutritional state, and genetic-survivorship quality (Borgia & Collins, 1989; Hill & Montgomerie, 1994; Kotiaho et al., 1998; Welch et al., 1998).

While female preferences and their targets exist in all modalities, auditory preferences have been particularly well studied because acoustic signals are easily manipulated and presented in two-choice playback experiments. Many such experiments have focused on anurans and songbirds, which produce audible vocalizations that can be played and recorded with common audio equipment, facilitating their study. Findings from these studies reveal that females prefer exaggerated or elaborate acoustic characteristics — such as long duration (Neubauer, 1999), increased complexity (Searcy, 1992; Mountjoy & Lemon, 1996; Ryan et al., 2019), and high calling rate (Gerhardt, 1991; Gerhardt & Huber, 2002) — that provide indicators of male condition or survivorship. In zebra finches, for example, females prefer elevated male song rates that positively correlate with male body condition (amount of fat reserves; Birkhead et al., 1998).

By contrast, much less is known about female acoustic preferences in mammalian species. Mammals can be challenging to study for a variety of reasons (reviewed Charlton, 2008). Female preferences in some mammalian species may be revealed only during periods of sexual receptivity (Zinck & Lima, 2013). Furthermore, the use of ultrasonic frequencies (>20 kHz) is widespread among mammalian clades, including in bats, muroid rodents, primates, and marine mammals (Masterton et al., 1969; Fitch, 2006). Ultrasonic vocalizations are inaudible to the human ear, and require specialized equipment to record and broadcast, posing a barrier to studying these mammalian sounds (Pierce & Griffin, 1938; Anderson, 1954; Sales & Pye, 1974). Finally, unlike most songbirds, the majority of mammalian species are nocturnal (69%; Bennie et al., 2014), making their observation and capture more difficult.

A small but growing body of evidence suggests that mammalian female acoustic preferences are analogous to those found in birds and anurans. Females prefer high call rate in laboratory mice (Nomoto et al., 2018) and red deer (McComb, 1991), more complex vocalizations in geladas (Gustison & Bergman, 2016) and bats (Davidson & Wilkinson, 2004), and low-pitched vocalizations in koalas (Charlton et al., 2012) and red deer (Charlton et al., 2007). These acoustic characteristics are associated with larger body sizes or greater resource holding potential in some species (McComb, 1991; Charlton et al., 2007; Charlton et al., 2012), but the link between male condition, male vocalization, and female preference is even less often explored. As a result, our understanding of female acoustic preferences in mammalian species remains limited, particularly when compared to other vertebrate taxa.

In this study, we examined female preferences for advertisement vocalization in wild Alston's singing mice (*Scotinomys teguina*), an insectivorous and diurnal species that inhabits high-elevation cloud forests throughout Central America and Mexico. Singing mice are named for their distinctive advertisement trills, which are long (averaging 7–10 s) and span a wide range of human-audible and ultrasonic frequencies (Hooper & Carleton, 1976; Miller & Engstrom, 2007). These acoustic characteristics mark a sharp evolutionary departure from the relatively soft, short, and entirely ultrasonic vocalizations produced by both close and distant relatives of singing mice, including *Baiomys* pygmy mice, *Peromyscus* deer mice, and *Mus* (Miller & Engstrom, 2007, 2012; Von Merten et al., 2014; Kalcounis-Rueppell et al., 2018; though see *Onychomys* grasshopper mice as a notable exception, Green et al., 2020). Advertisement song likely plays an important role in mate attraction (Hooper & Carleton, 1976; Fernández-Vargas et al., 2011; Pasch et al., 2013; George, 2014). In laboratory settings, for example, both female presence and removal are sufficient to incite male song (Hooper & Carleton, 1976; Fernández-Vargas et al., 2011).

Interestingly, song may function as an 'honest' signal of male condition, providing a possible mechanism for adaptive female mate choice. Recently, we demonstrated that in wild-caught singing mice, songs produced by male mice differ in vocal effort (Burkhard et al., 2018). 'High-effort' songs are longer, have elongated first notes, are quicker to reach peak amplitude, and have shorter inter-note intervals than 'low-effort' songs. This acoustic variation is predicted by physiological and morphological measures of male body condition, especially adiposity hormones that predict long-term energy

resources (Burkhard et al., 2018). Experimental work in lab-reared mice corroborates this relationship: leptin-injected mice sing at higher rates than saline-injected controls (Giglio & Phelps, 2020).

The covariation of male song and body condition provides a putative mechanism for adaptive female choice, and earlier work by the lab has presented preliminary evidence that female singing mice may use male song to assess potential mates (Pasch et al., 2011). Pasch et al. found that female singing mice preferred supernormally high-performance songs (i.e., songs artificially sped up) indicative of high levels of testosterone and high body condition over natural songs (Pasch et al., 2011). However, whether females attend to natural variation in male song — and specifically, to natural acoustic variation signalling male condition — is unknown.

In this study, we assessed whether females prefer high-effort male songs that predict good body condition. We provided laboratory-reared female singing mice choices between naturally varying songs recorded from wild-caught male mice, then evaluated female preferences. Since female preferences emerge only during oestrus in many mammalian species (Clutton-Brock & McAuliffe, 2009; Charlton, 2013), we also tested the effects of oestrus induction on female responses. We predicted that females would prefer high-effort songs, which serve as phenotypic cues for good male condition. We further predicted that oestrus-induced females would have a stronger response to male signals than control group females.

2. Methods

All research was performed with approval from the University of Texas Institutional Animal Care and Use Committee (IACUC) and following the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

2.1. Focal animals

All animals in this study were descendants of wild-caught singing mice from San Gerardo de Dota, Costa Rica kept in an outbred laboratory colony at the University of Texas at Austin. All mice in this colony were provided with cat chow and water ad libitum and were kept on a light:dark cycle of 12:12 at 20°C. We weaned mice at 28 days old, then housed them in same-sex pairs until study recruitment.

We moved individual focal females into $42.5 \times 26.6 \times 18.5 \text{ cm}^3$ cages containing nesting cotton and moss, a PVC tube, Sani-Chip wood bedding (PJ Murphy, Montville, NJ, USA), and a running wheel (BioServ, Flemington, NJ, USA). Focal females were virgin female mice between 8 and 10 weeks old. In total, we recruited 31 females from 16 separate breeding pairs, and our study included both full-sister, same-litter relationships and full-sister, separate-litter relationships (see the Supplementary material at 10.6084/m9.figshare.21904173). We mitigated the effect of relatedness by assigning sisters from the same litters to different treatment groups. However, one set of littermates was assigned to EBP, and two sets of littermates were assigned to EB (see the Supplementary material at 10.6084/m9.figshare.21904173). All 31 focal females were included in the oestrus induction experiment, but due to logistical issues (i.e., the pandemic-related lockdown of the University in 2020) only 19 females were tested in the phonotaxis experiment.

2.2. Oestrus induction and evaluation

Many female rodents demonstrate sexual interest only when in oestrus, a period of sexual receptivity caused by physiological changes (Becker et al., 1992). We provided hormonal injections following standard oestrus-induction protocols to induce oestrus in our focal female mice, with dosages adjusted to accommodate the smaller body mass of young adult singing mice (9–13 g). Because there are no established methods for inducing oestrus in singing mice, we tested oestrus-induction protocols for both voles (Cushing & Hite, 1996; Fowler et al., 2005) and laboratory rodents (Edwards, 1970; Ajayi & Akhigbe, 2020) against a negative control. We randomly assigned female singing mice to receive oestradiol benzoate ('EB'; $N = 14$), as used in vole oestrus induction; oestradiol benzoate and progesterone ('EBP'; $N = 7$), as used in lab mouse oestrus induction; or a control, sesame oil ('CON'; $N = 10$). Hormones were delivered in sesame oil and injected subcutaneously. Control injections were also given subcutaneously.

Females in the EB group received $1 \mu\text{g}$ of oestradiol benzoate once daily for four consecutive days (Day 1–Day 4). Females in the EBP group received $1 \mu\text{g}$ of oestradiol benzoate once daily for three consecutive days, then were injected with $1 \mu\text{g}$ of oestradiol benzoate and 0.25 mg of progesterone on the fourth day. Control females were provided with sesame oil injections once daily for four consecutive days. In all cases, the first three injections

were made between 1600 and 1700 h, and the fourth injection was provided between 0800 and 0900 h. Females were returned to home cages after receiving injections and were monitored for 15 min post-injection. We looked for behavioural and physical signs of adverse responses to injection (e.g., shock, hunched posture, lethargy) and watched for signs of normal behaviour and activities (e.g., alertness, wheel running, eating, burrowing). Females in this study were also consistently monitored by the University of Texas Animal Resources Center (ARC) staff, who performed daily wellness checks on all animals in the colony and were made aware of the experimental proceedings. All focal females in this study returned to normal activities within 15 min of being released into home cages.

Female mice in oestrus have swollen and perforate vaginal openings, which can be identified via visual inspection (Byers et al., 2012). We photographed each female's vaginal opening on Day 1, before giving the first injection (pre-treatment), and four hours after the final injection was provided on Day 4, between 12h00 and 13h00 (post-treatment). The photo timing on Day 4 fell within the range of expected physical signs of oestrus in induced voles and mice (Edwards, 1970; Cushing & Hite, 1996). We used the same location and lighting for all photos. One researcher placed the female's front paws on the lid of her home cage and held her shoulders in place while lifting her tail in the air to expose her anogenital region. A second researcher took a close-up photo using a smartphone camera (iPhone 11, Apple). We then scored these photos as follows: 0 (imperforate), 1 (semi-perforate), or 2 (fully perforate) (Byers et al., 2012). To maintain consistency, only one researcher (E.R.S.) scored all photos; to reduce bias, she scored these photos blind to individual identity and pre- or post-treatment status. Each photo was scored twice, then averaged to calculate a vaginal openness score. This resulted in two scores per female, one pre-treatment and one post-treatment, that were used in downstream analyses. After photographs were taken, we returned females back to their home cages for two hours, where they had access to food and water ad libitum.

2.3. Female phonotaxis

2.3.1. Playback stimuli

To evaluate female phonotaxis, we tested focal females using a two-choice playback paradigm. During tests, we presented females with paired sets of 'high-effort' and 'low-effort' male singing mouse songs. To create high-effort/low-effort pairs of stimuli, we selected exemplar songs from a library

recorded from wild male mice caught in San Gerardo de Dota, Costa Rica between 2014 and 2016 (Burkhard et al., 2018). Exemplar songs were recorded at 32-bit, 195.3 kHz resolution with ACO Pacific microphones on Tucker-Davis RX6 hardware (Tucker-Davis Technologies (Alachua, FL, USA)). Previously, we described acoustic variation in these songs by using principal components analysis, finding that PC1 explained differences in song effort: high-effort songs scored strongly positively on PC1 while low-effort songs scored strongly negatively on PC1 (Burkhard et al., 2018). Songs that were considered high in effort are longer, comprise more notes, reach peak amplitude faster, and have shorter inter-note intervals. We selected three males whose songs ranked as high effort ($PC1 > 4$) and three males with songs ranked as low effort ($PC1 < -7$). Previously, we showed that long-term energetic reserves, which we assessed by measuring adiposity hormones and residual body mass (RBM) strongly and positively correlate with PC1 effort (Burkhard et al., 2018). While hormonal information was not available for all six stimulus males, RBM was available for each. RBM was positively correlated with PC1 effort for the six stimulus males (adjusted $R^2 = 0.51$, $P = 0.07$; Figure A1 in the Supplementary material at 10.6084/m9.figshare.21904173).

We then randomly selected three song files from each stimulus male's repertoire and appended them to produce one file of three appended songs for each selected male, creating three high-effort stimulus files and three low-effort stimulus files, each comprising three songs from an individual male. For example, 'high-effort file 1' comprised three different songs each produced by high-effort Male A; similarly, 'low-effort file 1' comprised three different songs each produced by Male B. During a playback experiment, only one 'high-effort' file and one 'low-effort' file were played. In all files, amplitude was normalized (peak amplitude = 1 V).

2.3.2. Playback apparatus

We tested females in a $61 \times 30.5 \times 63$ cm³ expanded PVC board and wire arena (Figure 1). The bottom 22 cm of each outer wall of the apparatus was constructed from PVC-coated wire mesh, which mitigated the reflection and scattering of sounds. The remaining 41 cm of each outer wall was constructed from expanded PVC board, which discouraged mice from climbing or escaping the apparatus. Following Asaba et al., the apparatus was bisected by a $42 \times 2 \times 63$ cm³ expanded PVC inner wall, creating two runways and one open space, the 'starting chamber' (Asaba et al., 2015). This

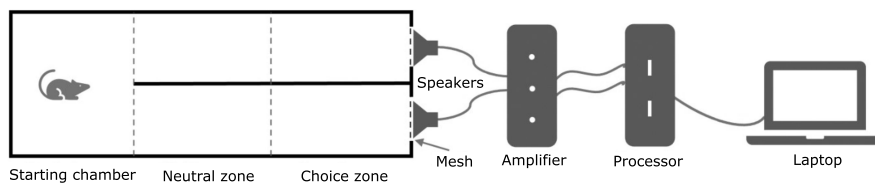


Figure 1. Schematic of two-choice experimental arena and playback devices. The test chamber comprises two runways connected to an open chamber. Solid lines indicate walls; dashed lines indicate zone parameters. Females began in the starting chamber and were free to explore runways and speakers during playback. The two-choice chamber and speakers were placed on the floor of a small testing room, and the trials were recorded from above. Song stimuli were broadcasted antiphonally from speakers located at the end of each runway.

wall was made entirely of expanded PVC. A Vifa XT25BG60-04 speaker was mounted at the end of each runway (flat on-axis frequency response to >40 kHz, Viborg, Denmark). Each runway was subdivided visually, but not physically, into two 21×13 cm² zones, allowing us to describe female proximity to the speaker. Following Asaba *et al.*, we designated the runway section closest to the speaker as the ‘choice zone’ and the runway section farther away from the speaker as the ‘neutral zone’ (Asaba *et al.*, 2015).

2.3.3. Playback tests

We tested females six hours following the final injection and two hours following handling for photographs (1400–1600 h). This timing is consistent with methods developed by the Gore lab in the College of Pharmacy, UT Austin (A. Gore, personal communication, February 3, 2020) and falls within the range of expected behavioural and physical oestrus in induced voles and mice (Edwards, 1970; Cushing & Hite, 1996). To mitigate handling stress, we transferred females from their home cages to the starting chamber of the choice arena via a PVC transfer tube. Once in the starting chamber, females were given 10 min to acclimate before acoustic playback.

We broadcast high-effort and low-effort song antiphonally, offset by 30 s. Stimulus order and speaker side were counterbalanced across females. One song within a stimulus file was presented once every 60 s for 3 min. For example, songs A, B, and C from high-effort file 1 were broadcast at minute 0, minute 1, and minute 2 while songs a, b, and c from low-effort file 1 were broadcast offset by 30 s, at minute 0.5, minute 1.5, and minute 2.5. We allowed mice to investigate the entire arena freely during playback and for an additional 5 min and 30 s after playing the final song. Thus, playback tests

lasted 480 s (8 min), not including the 10 min of acclimation pre-test. Trills were broadcast to each speaker using a Tucker-Davis MA3 amplifier and RX6 microprocessor with a sampling rate of 192 kHz and a peak amplitude of 50 dB SPL at 1 m, within the range of naturally produced trills in the field (Pasch et al., 2011).

We videotaped all behavioural trials using a Nikon handheld camcorder. All videos were scored blindly by one observer (E.R.S.). Female response was measured by analysing the following measures for each 480 s test (excluding the acclimation period): the total number of entries in each zone per trial; the duration in seconds spent in each neutral zone during the trial; the duration in seconds spent in each choice zone during the trial; and the duration in seconds spent investigating each speaker during the trial.

Investigation of the speaker broadcasting the stimulus (e.g., sniffing of the speaker) is a sign of preference used in many studies of mouse preference, including in singing mice (Pasch et al., 2011; Asaba et al., 2015). Close proximity, such as being in the choice zone (Asaba et al., 2015) or within 30 cm of the speaker (Pasch et al., 2011) is also interpreted as being a sign of preference. Conversely, time spent in the starting chamber or the neutral zone of either runway is not considered to be showing preference.

2.4. Statistical analyses

All statistical analyses were performed in R version 4.0.4 (2021-02-15, R Core Team, 2021).

2.4.1. Oestrus induction

To determine the effect of oestrus-induction treatments, we used paired t-tests to compare vaginal perforation pre-treatment (Day 1) and post-treatment (Day 4), and we used one-way analysis of variation (ANOVA) to compare post-treatment vaginal perforation scores among treatment groups. We used Tukey's HSD tests for post-hoc analysis. Three EB-treated females were fully perforate on Day 1 and were discarded from analysis of oestrus-induction treatments. The remaining EB-treated females were bimodally distributed in vaginal perforation pre-treatment, so we examined these females to see if family relationships could explain the bimodal pattern. We found that it did not: of the two sets of sisters in the EB treatment group, one pair scored differently pre-treatment, and while the sisters in the second pair both scored 0 pre-treatment, they had different scores post-treatment.

2.4.2. Female phonotaxis

To analyse female phonotaxis, we analysed the number of entries in high-effort and low-effort runways, the duration of stay in high-effort and low-effort neutral zones, the duration spent in high-effort and low-effort choice zones, and the duration spent investigating high-effort and low-effort speakers. One control female and three EBP females did not move from the starting chamber and were excluded from statistical analyses; thus, we included $N = 15$ females in subsequent analyses. We compared these responses using paired t -tests with a significance level of 0.05. We then checked to see if patterns of behavioural response were driven by family relationships. We verified the parentage of all outliers from the phonotaxis dataset; in no case of similar and extreme responses were females from the same litter or full sisters from different litters.

We fitted generalized linear mixed models (GLMM) with the `glmmTMB` package in R (v1.0.2.1, Brooks et al., 2017) to assess the influences of oestrus-induction treatment, vaginal score, and stimulus type on female response. Our final sample size was small ($N = 15$), limiting our power and amplifying the risk of overparameterization. To reduce parameterization of our models, we first investigated the amount of variance explained by Family and Individual ID. Our results indicated that both random effects explained little to no variance in our response variables, so we excluded random effects in our models. We then used the `buildmer` package in R to perform backwards stepwise elimination based on the change in AIC (v1.6) (Voeten, 2022), starting with a full model that included Stimulus type, Treatment, and Vaginal status as covariates and their interactions. Because both duration and number of entries were left-skewed, positive, zero-inflated, and integer response variables, we specified negative binomial family distributions in all models. We report all values from final models in Table 1.

3. Results

3.1. Oestrus induction

Prior to oestrus induction treatments, most female mice were imperforate (closed vaginal openings), and no mouse was fully perforate and swollen (mean vaginal score \pm SD = 0.19 ± 0.40). Oestradiol-treated (EB) females had significantly more perforate vaginal openings post-treatment than pre-treatment (paired t -test: $p < 0.001$; mean pre-treatment score = 0.36, mean

Table 1.
Outcome of final models.

Response variable	Estimate	SE	Z	$Pr(> z)$
Entries				
(Intercept)	2.7160	0.5080	5.347	8.95E-08***
Stimulus_type:low-effort	-0.6241	0.4412	-1.415	0.157
TreatmentEB	0.9136	0.5755	1.587	0.112
TreatmentEBP	0.3063	0.6121	0.5	0.617
Vaginal_score	-0.1927	0.3734	-0.516	0.606
neutral zone				
(Intercept)	3.9832	0.4866	8.185	2.72E-16***
Stimulus_type:low-effort	-0.1120	0.4290	-0.261	0.794
TreatmentEB	-0.0734	0.6365	-0.115	0.9082
TreatmentEBP	-1.1073	0.5786	-1.914	0.0556
Vaginal_score	-0.0574	0.3391	-0.169	0.8655
Choice zone				
(Intercept)	4.4328	0.5919	7.49	6.90E-14***
Stimulus_type:low-effort	-0.9762	0.6778	-1.44	0.149806
TreatmentEB	-0.2441	1.6293	-0.15	0.880891
TreatmentEBP	0.6243	1.0307	0.606	0.544708
Vaginal_score	0.32817	0.5740	0.572	0.567498
TreatmentEB:Vaginal_score	-0.0823	1.0829	-0.076	0.939428
TreatmentEBP:Vaginal_score	-4.5683	1.3169	-3.469	0.000522***
Speaker				
(Intercept)	3.6078	0.6956	5.187	2.14E-07***
Stimulus_type:low-effort	-1.4552	0.7188	-2.025	0.0429*
Vaginal_score	-0.5393	0.6818	-0.791	0.429
TreatmentEB	-0.1291	1.8651	-0.069	0.9448
TreatmentEBP	0.7363	1.1108	0.663	0.5074
Vaginal_score:TreatmentEB	0.7327	1.2180	0.602	0.5475
Vaginal_score:TreatmentEBP	-3.3654	1.4667	-2.294	0.0218*
Speaker (excluding F13)				
(Intercept)	3.7044	0.7235	5.12	3.06E-07***
Stimulus_type:low-effort	-1.8142	0.7988	-2.271	0.0231*
Vaginal_score	-0.5774	0.6923	-0.834	0.4043
TreatmentEB	-0.1129	1.8870	-0.06	0.9523
TreatmentEBP	-1.4789	1.3919	-1.063	0.288
Vaginal_score:TreatmentEB	0.8561	1.2365	0.692	0.4887
Vaginal_score:TreatmentEBP	-1.1484	1.7050	-0.674	0.5006

* $p \leq 0.05$; *** $p \leq 0.001$.

post-treatment score = 1.4, Figure 2). Oestradiol benzoate and progesterone treated females (EBP) also had significantly more perforate vaginal openings post- than pre-treatment ($p = 0.02$; mean pre-treatment score = 0.0, mean post-treatment score = 0.71, Figure 2), though less strikingly so. Control females did not differ in pre-treatment vaginal openness and post-treatment vaginal openness ($p = 0.1$; mean pre-treatment score = 0.10, mean post-treatment score = 0.45, Figure 2). There were no differences in pre-treatment vaginal openness among females in EB, EBP, and control treatment groups (ANOVA: $F_{2,28} = 2.47$, $p = 0.1$). Conversely, at post-treatment, female vaginal scores differed significantly among treatments (ANOVA: $F_{2,28} = 7.70$, $p = 0.002$). Specifically, EB females scored significantly higher in vaginal openness than either EBP females (Tukey's HSD: $p = 0.05$) or control females ($p = 0.002$), but EBP females and control females did not differ in vaginal score ($P = 0.73$).

3.2. Female phonotaxis

Fifteen of the 19 female mice we tested responded to playbacks, i.e., moved from the starting chamber (EB: $N = 7$, EBP: $N = 4$, control: $N = 4$). Female mice did not differ in the number of times they entered high-effort or low-effort runways (paired t -test: $p = 0.32$, Figure 3a). Further, there were no significant effects of treatment, vaginal score, or stimulus type (low vs high effort) on the number of entries in high- or low-effort runways (GLMM, Treatment_EB: estimate = 0.91 ± 0.58 , $Z = 1.59$, $p = 0.11$; Treatment_EBP: estimate = 0.31 ± 0.61 , $Z = 0.50$, $p = 0.62$; Vaginal_score: estimate = -0.19 ± 0.37 , $Z = -0.52$, $p = 0.61$; Stimulus_low-effort: estimate = -0.62 ± 0.44 , $Z = -1.42$, $p = 0.16$; see the Supplementary material at 10.6084/m9.figshare.21904173).

Females did not differ in the duration spent in neutral zones or choice zones (Figure 3b,c; Table 1). Paired t -tests revealed no difference in duration spent in low-effort and high-effort neutral zones (mean \pm SD, high-effort neutral zone: 36.9 ± 38.1 s, low-effort neutral zone: 37.7 ± 49.2 s; $p = 0.96$, Figure 3b). Mixed models indicated that neither stimulus type, treatment, nor vaginal score had a significant effect on the duration spent in the neutral zone (Stimulus_low-effort: estimate = -0.11 ± 0.43 , $Z = -0.26$, $p = 0.79$; Treatment_EB: estimate = -0.07 ± 0.64 , $Z = -0.12$, $p = 0.91$; Treatment_EBP: estimate = -1.11 ± 0.58 , $Z = -1.91$, $p = 0.06$; Vaginal_score: estimate = -0.06 ± 0.34 , $Z = -0.17$, $p = 0.87$). Similarly,

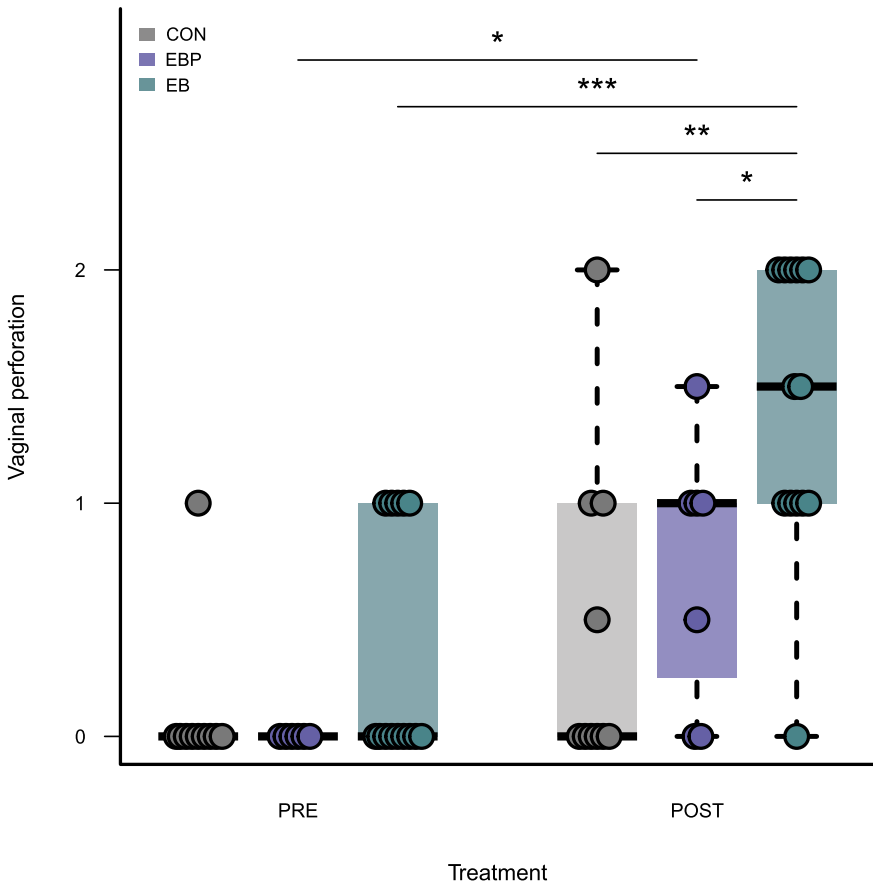


Figure 2. Vaginal perforation pre- and post-oestrus induction treatment. Boxplots show medians and interquartile ranges (IQR) of values, and whiskers extend to the most extreme data within $\pm 1.5 \times \text{IQR}$. Control females (CON) in grey; oestradiol benzoate and progesterone females (EBP) in purple; and oestradiol benzoate females (EB) in turquoise. Significant pairwise contrasts ($p \leq 0.05$) are marked with asterisks.

neither paired *t*-tests nor mixed models found a difference in the amount of time females spent in high-effort choice zones versus low-effort choice zones (mean \pm SD, high-effort choice zone: 79.3 ± 85.7 s, low-effort choice zone: 42.5 ± 73.8 s; paired *t*-test: $p = 0.2$; GLMM: Stimulus_low-effort: estimate = -0.98 ± 0.68 , $Z = -1.44$, $p = 0.15$, Figure 3c). However, there was a significant interaction between treatment and vaginal perforation (Treatment_EBP \times Vaginal_score, estimate = -4.57 ± 1.32 , $Z = -3.47$, $p = 0.0005$), such that EBP females with more perforate vaginas spent more

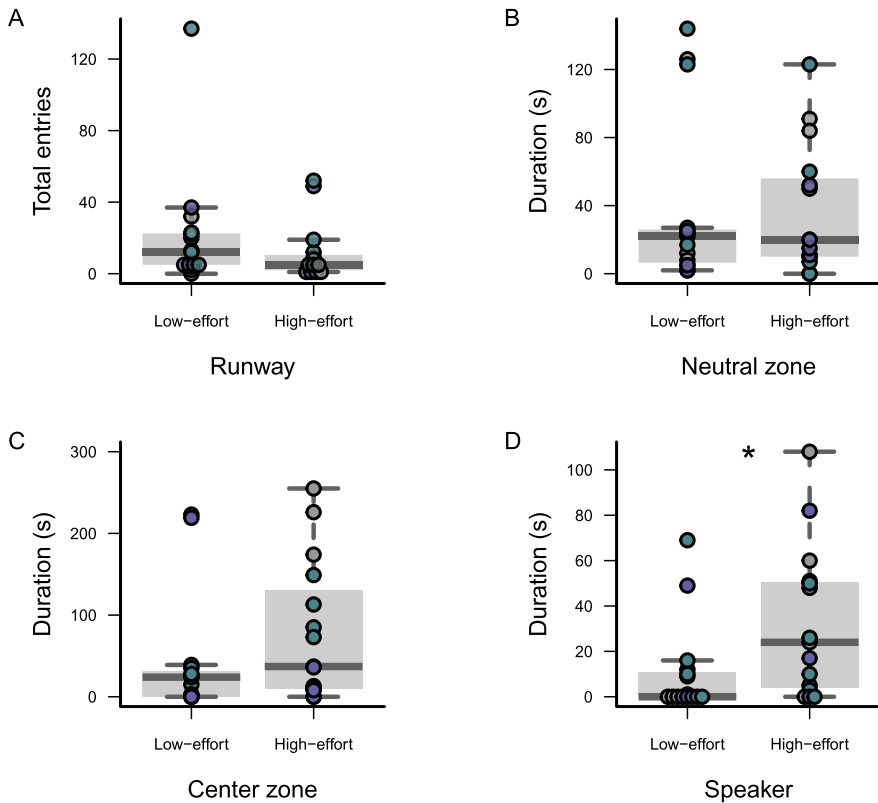


Figure 3. (A) Number of entries in high-effort and low-effort runways. (B) Time spent in neutral zone. (C) Time spent in choice zone. (D) Time spent at high vs low effort speakers. Boxplots show medians and interquartile ranges (IQR) of values, and whiskers extend to the most extreme data within ± 1.5 IQR. CON females in grey; EBP females in purple; and EB females in turquoise. Significant pairwise contrasts ($p \leq 0.05$) are marked with asterisks.

time in low-effort than in high-effort choice zones. This relationship seems driven by one EBP female (F13) who spent >200 s in the low-effort choice zone and not indicative of a greater trend, however (Fig. 3c).

By contrast, female mice spent more time on average investigating high-effort over low-effort speakers (mean \pm SD; high-effort speaker: 32.3 ± 33.2 s, low-effort speaker: 11.1 ± 20.5 s; paired t -test: $p = 0.05$; Figure 3d, Table 1). Results from generalized linear mixed models including oestrus-induction treatment and vaginal score corroborated this result, indicating that female mice spent more time investigating high-effort speakers than low-effort speakers (GLMM, Stimulus_low-effort: estimate = $-1.46 \pm$

0.72, $Z = -2.10$, $p = 0.04$). There was once again a significant interaction between treatment and vaginal openness, indicating that EBP females spent more time investigating low-effort speakers than high-effort speakers (Treatment_EBP \times Vaginal_score: estimate = -3.37 ± 1.47 , $Z = -2.30$, $p = 0.02$). However, just as before, this pattern was driven by one EBP female (F13). Unsurprisingly, removal of this female strengthened the finding that females spent more time in high-effort choice zones than in low-effort choice zones (GLMM, Stimulus_low-effort: estimate = -1.81 ± 0.80 , $Z = -2.27$, $p = 0.02$) and clarified that neither treatment nor vaginal openness influenced female response (Treatment_EB: estimate = -0.11 ± 1.89 , $Z = -0.06$, $p = 0.95$; Treatment_EBP: estimate = -1.48 ± 1.39 , $Z = -1.06$, $p = 0.29$; Vaginal_score: estimate = -0.58 ± 0.69 , $Z = -0.83$, $p = 0.40$).

4. Discussion

Male singing mice produce long and elaborate advertisement trills that function in mate attraction and male-male competition (Hooper & Carleton, 1976; Fernández-Vargas et al., 2011). These songs vary in many temporal, spectral, and amplitude-related characteristics (Campbell et al., 2010; Burkhard et al., 2018). Previously, we described acoustic variation in advertisement song from wild Costa Rican mice using principal components analysis (PCA), which revealed a component describing individual differences in temporal and amplitude-related characteristics (Burkhard et al., 2018). This we interpreted as variation in display effort, where ‘high-effort’ songs are longer, have elongated first notes, are quicker to reach peak amplitude, and have shorter inter-note intervals than ‘low-effort’ songs (Burkhard et al., 2018). In the present study, we presented lab-reared female singing mice with exemplar high-effort and low-effort songs recorded from wild-caught male mice. Female singing mice spent more time investigating speakers playing high-effort songs than speakers playing low-effort songs (Figure 3d), indicating a stronger interest in high-effort advertisement trills. This phonotactic response was independent of oestrus.

Our high/low stimulus pairs were designed to provide salient contrasts for the two-choice tests: we picked exemplars that scored strongly and oppositely on PC1 song effort (high-effort songs scored $PC1 > 4$; low-effort songs scored $PC1 < -7$). Given the complexity of interpreting principal components, however, we are unable to distinguish which song effort trait — note

duration, internote interval, or song length, for example — might contribute most to mate choice. Studies of mammals, birds, and anurans provide hints to which traits may be most important, demonstrating evidence of female preferences for long duration (Ryan & Keddy-Hector, 1992; Neubauer, 1999), quick rise to max amplitude (Lyu et al., 2016), increased rate of display (McComb, 1991), and rapid trill rate (Ballentine, 2009; Searcy et al., 2010). Work in wild and laboratory rodents also indicates female preferences for longer vocalizations (Hanson & Hurley, 2012), increased call rate (Nomoto et al., 2018), and faster trill rate (Pasch et al., 2011). Experiments presenting more exhaustive binary choice tests to females (e.g., in bats, Hemingway et al., 2019; and frogs, Kime et al., 1998) would help us parse out the traits playing the biggest role in mate choice. Of course, multiple traits may also be used in female mate choice (Candolin, 2003), including traits from considerably different modalities (Zhao et al., 2022). Regardless, our results contribute to taxonomically widespread evidence of female preferences for higher effort and more elaborate courtship vocalizations.

What explains these preferences? While this is beyond the scope of our study, we propose a few possible explanations. Some hypotheses in sexual selection predict that females use male traits to assess male quality, which may mean that males are better able to defend resources, are less likely to be parasitized, or may carry fewer deleterious mutations that could be passed on (Kirkpatrick & Ryan, 1991). In many species, acoustic characteristics like duration, trill rate, and complexity predict male motivation (Bernal et al., 2009; Demartsev et al., 2014), motor skills (Byers et al., 2010), immune function (Kubli & MacDougall-Shackleton, 2014) and body condition (Wyman et al., 2008; Vannoni & McElligott, 2009; Kagawa & Soma, 2013). These correlations of male vocalization and male condition support the hypothesis that female choice can select for healthier or more robust males. Likewise, in singing mice, males that produce high-effort songs have higher concentrations of adiposity hormones, which indicate consistent nourishment over long periods (Burkhard et al., 2018). We hypothesize that female acoustic preferences may serve to select higher quality mates in singing mice. A non-mutually exclusive alternative hypothesis is that preferences for high-effort songs help females locate mates more quickly. In the field, singing mice share montane grasslands and forests with many other noisy diurnal creatures. This complex soundscape ecology degrades signals and increases acoustic competition (Morton, 1975; Pijanowski et al., 2011),

and high-effort songs may be more noticeable in these conditions than low-effort songs (Wollerman & Wiley, 2002; Luther & Gentry, 2013; Wiley, 2017). If the cost of mate search is high, as demonstrated in other species (Alatalo et al., 1988; Vitousek, 2009), attending conspicuous advertisement songs could help female singing mice find mates quickly. Additional work is needed to determine whether female preferences accurately predict mating decisions in singing mice, and whether mating with preferred males provides benefits to females. Such insight will be important to understanding how sexual selection contributes to the evolution of elaborate mouse song.

We found that providing females with injections of oestradiol benzoate (EB) over four days effected swollen, perforate vaginal openings — anatomical indicators of oestrus (Byers et al., 2012) — by the final day of treatment. Cocktails of oestradiol benzoate and progesterone (EBP) also effected vaginal perforation in treated females but were less effective than EB. This difference in efficacy between the two methods is consistent with taxonomic differences in the mechanisms of oestrus induction. EB treatment is a well-established method for oestrus induction in voles (Cushing & Hite, 1996; Fowler et al., 2005), while EBP treatment is used in laboratory mice (*Mus musculus*) and rats (*Rattus rattus*) (Edwards, 1970; Ajayi & Akhigbe, 2020). Singing mice (subfamily Neotominae) and voles (subfamily Arvicolinae) are both members of the Cricetidae family of rodents, whereas laboratory mice and rats are of the Muridae family (Steppan et al., 2004; Steppan & Schenk, 2017). Singing mice and voles are thus more recently diverged than singing mice and laboratory rodents (earliest estimated split: singing mice and voles, 14 mya; singing mice and ancestor of *M. musculus* and *R. rattus*, 17.4 mya, Steppan & Schenk, 2017). The means of reliable oestrus induction in Cricetidae are known for few species, however, and whether such taxonomic patterns prove reliable await further work.

Hormonal and physiological changes affect female mate choice in a variety of vertebrates (Adkins-Regan, 1998; Lynch et al., 2006), and for many mammalian species, oestrus strongly influences female preferences (Clutton-Brock & McAuliffe, 2009; Charlton, 2013). Surprisingly, we found in this study that female phonotactic behaviour was independent of both hormonal treatment and vaginal openness score. Our results mirror those described in several studies of lab mice, however, which found that female mice are attracted to male ultrasonic vocalizations (USV) regardless of oestrus state (Hammerschmidt et al., 2009; Shepard & Liu, 2011). Hammerschmidt et

al. (2009) speculated that male USVs may function less to attract sexually receptive females than to assist mating by stimulating receptive behaviour in nearby females or by shepherding females into close range. Similarly, in *Peromyscus* deer mice, ultrasonic whistle vocalizations are used by both sexes to summon neighbours and mates (Briggs & Kalcounis-Rueppell, 2011; Kalcounis-Rueppell & Petric, 2013; Kalcounis-Rueppell et al., 2018). These functions have also been proposed for the audible calls of *Onychomys* grasshopper mice, which are detectable up to 50 m away (Hafner & Hafner, 1979; Pasch et al., 2017). In singing mice, male song can also carry over long distances in the field (detectable at 20 m, unpublished data). We propose that *Scotinomys* song may help bring females and males into contact. Field study or semi-natural field study will be necessary to understand how song is used in natural social contexts for singing mice, and for other muroid models for vocalization.

Overall, our study reveals female preferences for elaborate male song in a wild mammal species. Because *Scotinomys* advertisement song is condition dependent (Pasch et al., 2011; Burkhard et al., 2018; Giglio & Phelps, 2020), our findings highlight the potential role for female preferences in selecting higher quality mates. Our findings also support the hypothesis that female preferences for longer songs could drive the elaboration of song in *Scotinomys*. Additional work is necessary to provide greater insight into the mechanisms underlying female preferences and how such preferences may shape mammalian vocalizations.

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