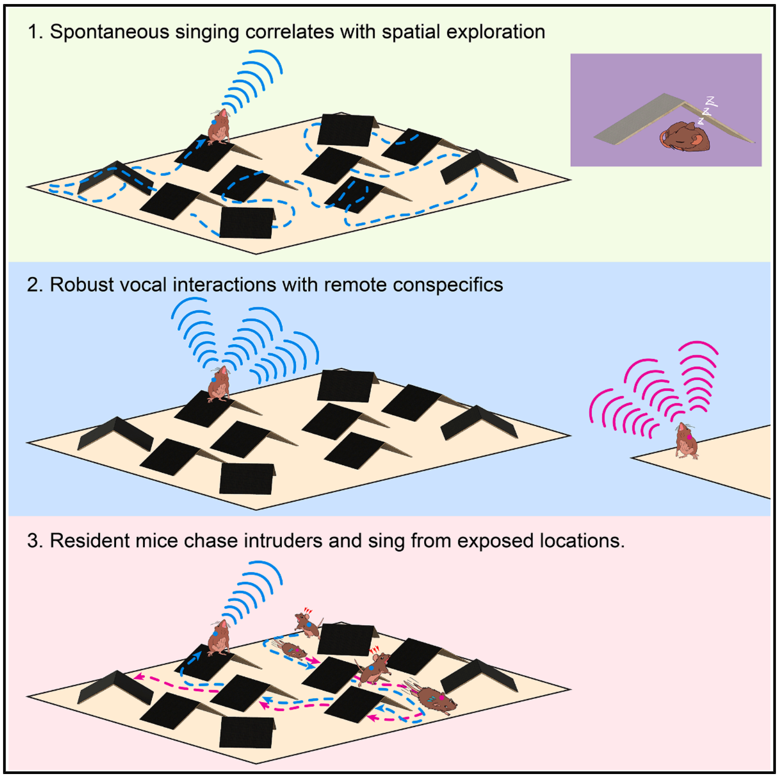
Current Biology

# Advertisement vocalizations support home-range defense in the singing mouse

## Graphical abstract



## Highlights

- Individual songs feature invariant acoustic properties and consistent response times
- Spontaneous singing is correlated with spatial exploration of home range
- Singing mice most reliably respond to acoustically distant conspecifics
- During interactions, aggressor mice produce “broadcast songs” from exposed locations

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## In brief

Fujishima and Long combine sound analysis with thermal imaging and position tracking in a semi-natural environment to demonstrate that singing mice (*S. teguina*) produce identifiable advertisement calls whose initiation is affected by the location of conspecifics and social status, suggesting a role in home-range defense.

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Report

# Advertisement vocalizations support home-range defense in the singing mouse

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## SUMMARY

Alston's singing mice (*Scotinomys teguina*) are highly vocal Central American rodents that produce structured "songs" (duration: 5–10 s),[1–3] often as part of dynamic vocal exchanges with response timing that resembles that observed in human conversation.[4–7] Although this behavior has been thought to function in both mate attraction[8–11] and male-male competition,[12–14] its precise ethological relevance remains elusive. To address this issue, we developed a semi-natural terrarium for tracking the activity of *S. teguina* using thermal cameras capable of monitoring the movement of individual mice, even when visually obscured under a shelter. Given the known sex differences in the behavior,[15,16] we focused on the behavior and interactions of males. We found that each mouse produced unique songs[16–18] with reliable counter-singing response latencies, allowing for a vocal signature that can be distinguished by conspecifics. Although individual mice sang spontaneously and with remote partners, we found that pairs of mice within the same terrarium rarely engaged in short-latency counter-singing. We used song playback to demonstrate that counter-singing can most robustly be elicited at distances of ∼2 m, demonstrating the tendency of this species to preferentially interact with others in nearby home ranges.[19] Finally, when an "intruder" mouse entered the environment during staged interactions, the resident mouse often responded aggressively by chasing the intruder and broadcasting songs from outside the safety of a shelter. Taken together, our findings indicate that the *S. teguina* song functions as an announcement of one's home range to nearby conspecifics.

## RESULTS

### Individual differences in song structure

Previous work has demonstrated that the *S. teguina* song,[1–3] as well as its underlying motor cortical activity,[4,20] is highly stereotyped across renditions, particularly when singing is performed in isolation[4] (i.e., without a partner). A recent study used a small subset of songs (2–14 per individual) to demonstrate that certain acoustic features are consistent within individual mice.[16] However, to act as an identifier, each mouse's song must also be distinguishable from those of conspecifics.[16–18] To test this idea, we analyzed 4,302 individual songs from 5 isolated male *S. teguina* (range: 402–1,497 songs/mouse; Figures 1A–1C, S1A, and S1B). We found that the acoustic features of individual notes (Figures 1B and 1D–1F) and the structure of the overall song (Figures 1C, 1G–1I, and S1B) exhibit unique components (STAR Methods; Figures S1C–S1G). Using a linear discriminant analysis model, we found that the identity of the singer can be predicted with high accuracy across renditions (92.9% ± 1.3%, chance: 20%; STAR Methods) (Figures 1J, 1K, S1C, and S1D). High prediction accuracy was retained even when restricting the analysis to features that are the most resilient to distance-related degradation—namely, number of notes, note density, and minimum frequency (76.6% ± 2.4%) (Figures S1E and S1F). Taken together, we find that the vocal displays of male *S. teguina* could reliably signify individual mice, with discrimination arising from a combination of both temporal and spectral features.

We next investigated whether the interactive timing of the song is specific to individual mice. We focused on the temporal latencies during counter-singing[3,4,21] (Figures 1L and 1M; STAR Methods). We reasoned that vocal response latency may systematically differ as a function of the individuals engaged in the vocal exchange. To test this hypothesis, we recorded the vocal behavior of 6 separately housed mice paired into 9 unique combinations for 24-h observation periods (Figure S1G). Across all pairs, we observed a total of 2,170 counter-songs (range: 50–473 counter-songs per interaction; STAR Methods). Response latencies varied widely; on average, some mice avoided overlap by subsecond margins (Figure 1L), whereas others often overlapped with their partners (Figure 1M). To probe whether response times of the same responder are more similar than those of different individuals, we defined a similarity index (STAR Methods) between possible pairs of response time distributions (Figures 1N–1Q) and found that the response time distribution is significantly more similar when comparing a single responder with different initiators, rather than the opposite configuration (Wilcoxon rank-sum test, $p = 0.00022$). Therefore, in addition to invariant acoustic features during spontaneous singing, individual mice also have preferential response latencies during social episodes.

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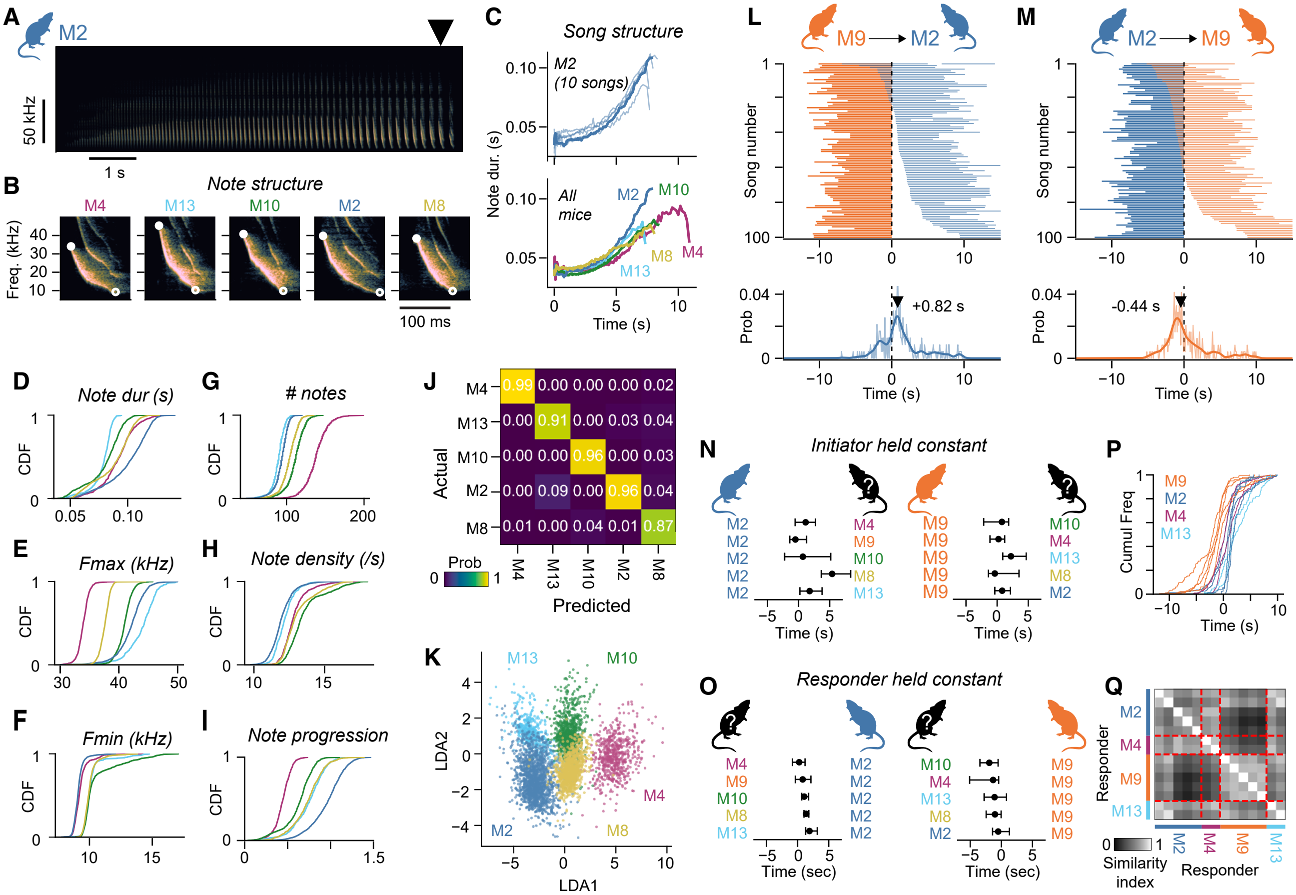

**Figure 1. Individual differences in *S. teguina* song structure and response latency**

(A–C) Spectrogram of an entire representative song from M2 (A). Longest notes for all mice (B) with M2 indicated by black arrow in (A). White circles mark the maximum (closed) and minimum (open) fundamental frequencies. Note durations are plotted as a function of their onset times (C); example from (A) indicated with a thick line. See also Figures S1A and S1B.

(D–I) Cumulative distribution functions of acoustic features: note duration (D), $F_{max}$ (E), $F_{min}$ (F), number of notes (G), note density (H), and quadratic coefficient of note progression (I).

(J) Confusion matrix of singer identity classification, averaged across 100 iterations.

(K) All data points ($n$ = 4,302 recorded songs across 5 mice) transformed into linear discriminant analysis space. Individual mice indicated by color. See also Figures S1C–S1F.

(L and M) Randomly selected examples in which M9 (L) or M2 (M) initiates a counter-song. Time is aligned to the offset of songs from the vocally initiating mouse. Trials are sorted by response latency. At bottom, the estimated probability density functions of response latency for examples shown above. Bin size = 0.1 s (thin line). Smoothed (Gaussian filter: sigma = 0.4 s) indicated with thick line. Arrow indicates median latency across trials. See also Figure S1G.

(N and O) Median ± IQR response latency when vocal initiator (N) or responder (O) is held constant.

(P) Cumulative density plots of response latencies of individual sessions in which the responder was held constant.

(Q) Similarity matrix of response latencies of individual sessions. Red lines indicate the separation of vocal responder identities.

## Singing behavior in a simulated natural environment

We next developed a more complex environment to better understand the behavioral relevance of both spontaneous and social songs. To accomplish this, we placed individual singing mice into a 0.91 (depth) by 1.22 m (width) terrarium (Custom Cages) filled with moss bedding and woodchips (Figures 2A and 2B; STAR Methods). Field recordings had revealed that singing mice (a diurnal species) often nest under fallen leaves or rotten logs in the forest.[1] To provide a similar set of burrows, we created "A-frame" mouse shelters (19.1 × 13.3 × 5.7 cm) constructed with black cheesecloth and plastic mesh (Figures 2A and 2B) that provided sufficient coverage to encourage normal singing but maintain the capability to monitor behavior with a thermal camera (Figures 2A and 2C; Boson 640; horizontal field of view, 95°; focal length, 4.9 mm). Using this arena, we could easily measure singing (Figure 2C) and animal movement (Figure 2D) with machine-learning-based behavioral tracking methods[22] trained on points of interest from the thermal images (STAR Methods), ultimately allowing us to continuously monitor the behavioral repertoires associated with song production in *S. teguina*. We first examined the activity of 5 isolated adult males across 8.3 ± 2.2 days (range: 4.9–10.7 days). Individual *S. teguina* exhibited two distinct modes: intense movement and relative immobility (Figure 2E). The amount of singing was strongly correlated with these high movement states (Figures 2E and 2F), with 97.0% ± 2.4% of songs occurring

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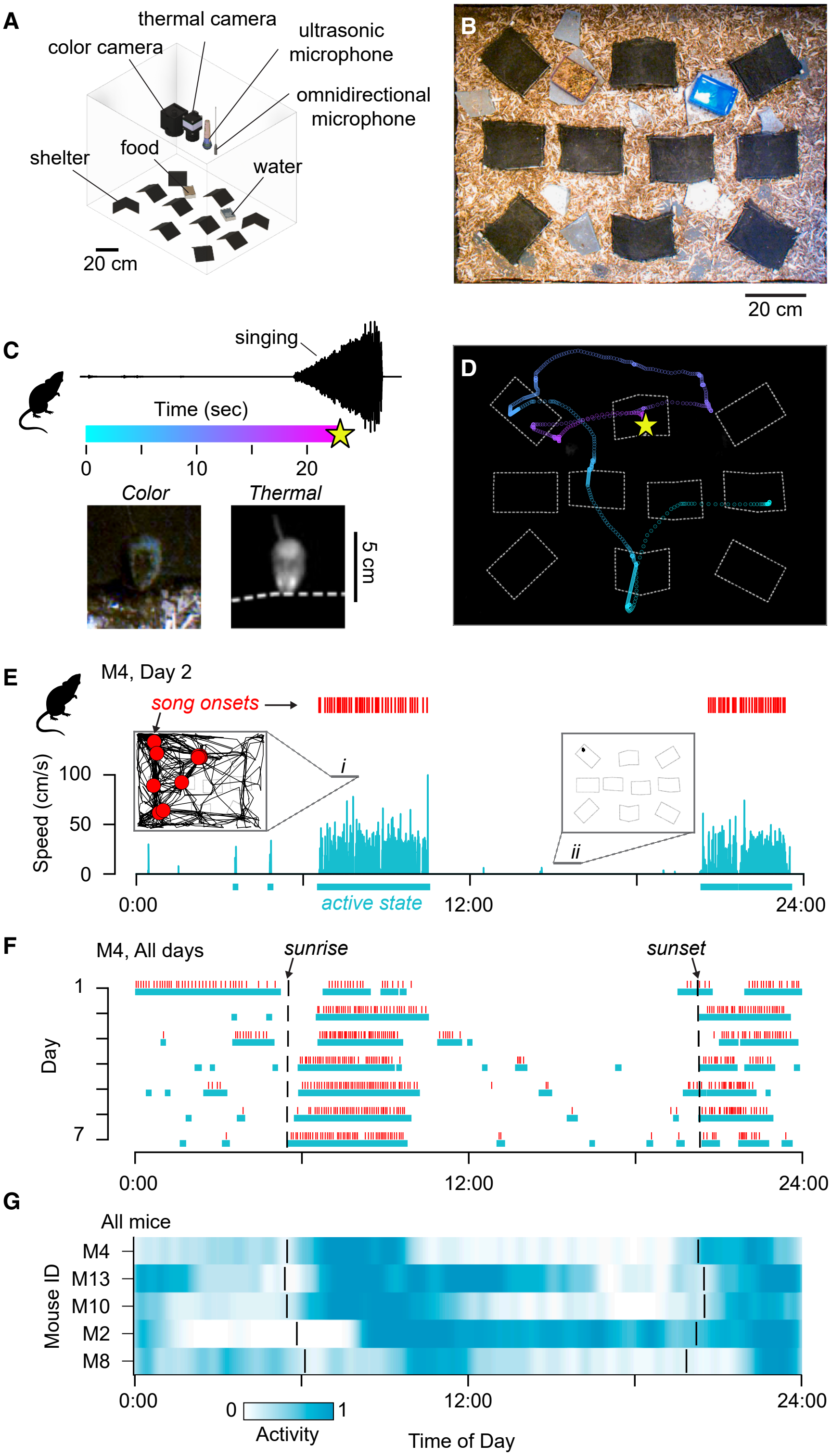

**Figure 2. A custom terrarium for quantifying song-related movements**

(A) Schematic of the terrarium highlighting two cameras (thermal and color), two microphones (omnidirectional and ultrasonic), and 10 thermally transparent shelters.

(B) Photograph of the terrarium (top view).

(C) Example song recorded in terrarium shown at top. Simultaneous color and thermal images of mouse during singing provided at bottom, corresponding to the time point indicated by the star.

(D) Using a thermal camera to track the movement of a single mouse before and during song production. Elapsed time indicated by color, corresponding to timescale in (C).

(E) Animal speed and song timing of an example mouse (M4). Ticks indicate the onset of each song, and blue lines show distance traveled within 1-s time windows. Horizontal blue bars indicate times of significant movement (STAR Methods). Two 1-h example epochs are highlighted in which the mouse is in an active state (07:00–08:00) and a period of relative immobility (15:00–16:00). Red dots indicate the position where the song was produced.

(F) Ethogram showing consistent active states (blue lines) and song timing (red ticks) of an example mouse (M4) across 7 days. Black vertical bars indicate the sunrise and sunset times for individual days, respectively. See also Figure S2.

(G) Probabilities of being in an active state reveal unique activity patterns for each recorded mouse ($n$ = 5). Black vertical bars indicate the median sunrise and sunset times across days.

during spatial exploration bouts. These active states accounted for 46.9% ± 12.3% of each day, and mice displayed robust patterns of movement across the observation period (Figure 2F), and these singing patterns appeared to differ across individuals (Figures 2G and S2). Taken together, our customized monitoring system enabled us to track the behaviors associated with spontaneous *S. teguina* song production.

## Distance dependence of vocal responses

To investigate counter-singing, we introduced 7 pairs of male mice into the terrarium and observed their behavior for 2-h sessions. The number of songs produced was significantly lower in the terrarium (11.1 ± 5.8 songs/h) compared with the remote condition (68.8 ± 28.3 songs/h, Wilcoxon rank-sum test, $p$ = 0.00086) (Figure S3A, left), and the proportion of counter-singing responses were also highly suppressed (terrarium, 0.03 ± 0.05; remote, 0.28 ± 0.18; Wilcoxon rank-sum test, $p$ = 0.0026) (Figure S3A, right). Taken together, the total instances of counter-singing were nearly abolished in this shared social environment (0.50 ± 0.76 counter-songs/h; Figures 3A and 3B) compared with sessions in which mice were recorded in different cages (STAR Methods; $n$ = 9 pairs; Figures 3C and S3A; 22.2 ± 19.7 counter-songs/h, Wilcoxon

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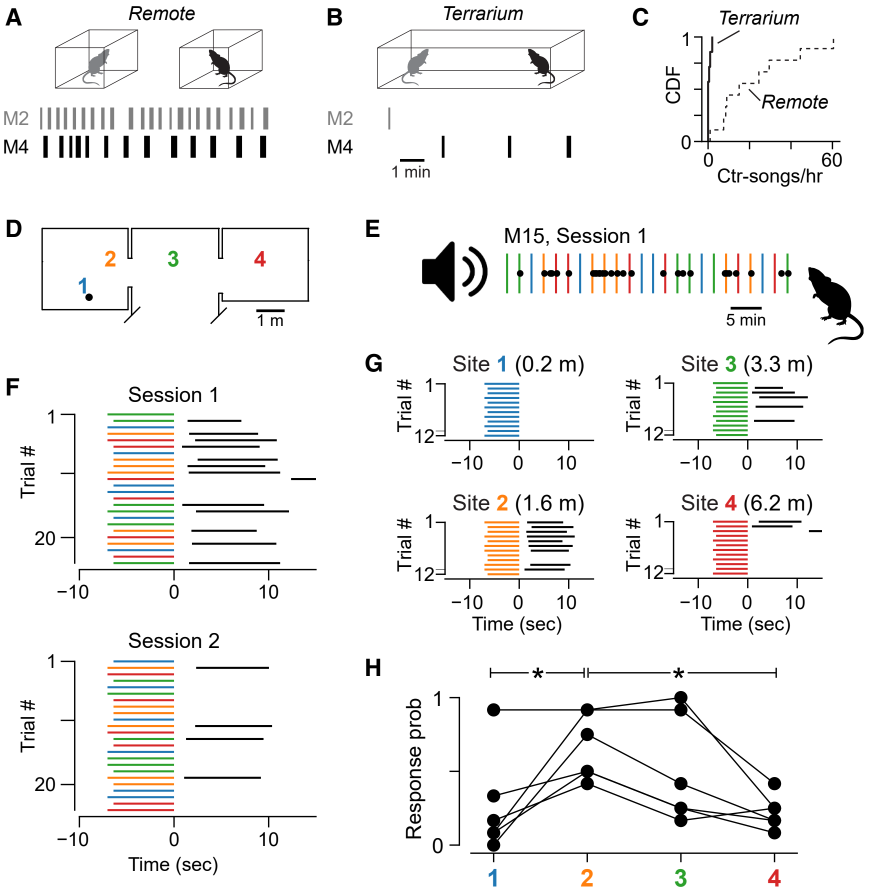

**Figure 3. Distance dependence of vocal responsiveness in *S. teguina***

(A and B) Remote (A) and terrarium (B) sessions of an example pair (M2 and M4). Schematic of the experimental setup at top; song times shown at bottom.

(C) Counter-song amounts of all pairs in separate enclosures ($n = 9$) and all pairs in a shared terrarium ($n = 7$) (Wilcoxon rank-sum test, $p = 0.0018$). See also Figure S3A.

(D) Schematic of the experiment room. Numbers indicate the locations of the playback speaker, and the black dot shows the position of the mouse. See also Figure S3B.

(E) Song playback times (vertical lines) from different locations, as indicated by color (which corresponds to locations in A). Black dots represent onset times of songs from M15 in a single session.

(F and G) Response times of an example mouse (M15) across two sessions shown in chronological order (F) and sorted by the speaker locations (G). Only songs initiated after playback onset are shown. See also Figure S3C.

(H) Population ($n = 6$ mice) response probabilities for all 4 locations tested (*$p < 0.05$).

rank-sum test, $p = 0.0018$). These observations are consistent with the notion that counter-singing is primarily used for long-distance social interactions.

To explicitly test this hypothesis, we played back *S. teguina* songs from various locations (0.2, 1.6, 3.3, and 6.2 m) with respect to the home cage (Figure 3D). This manipulation led to large differences in sound intensity and frequency profiles at the level of the receiver (Figure S3B). Sessions consisted of 24 total playbacks, with 2 different conspecific song motifs repeated 3 times at each location in pseudorandom order (Figures 3E and 3F). When we sorted responses by location, we found a strong relationship between distance and the probability of producing a counter-singing response (Figures 3G, 3H, and S3C) (Friedman test, $p = 0.023$). *S. teguina* were more likely to counter-sing to songs played from 1.6 m compared with more proximal ($p = 0.04$) or distal locations ($p = 0.04$) (Dunn's test, Benjamini-Hochberg adjusted $p$ values). These playback experiments further reinforce the notion that counter-singing is used by *S. teguina* as a long-distance communication signal.

## Territorial effects on singing behavior

If the *S. teguina* song can convey identity to long-range targets, then what is the nature of the singing behavior that occurs when two mice are placed in the same space? We hypothesized that song use in this species may be related to the establishment of their core home range. To investigate this, we conducted staged home-range disruptions in which a "resident" who had been living in the arena for at least 5 days interacted with a newly introduced intruder mouse. Consistent with the observation in the "neutral" setting (Figures 3B and 3C), counter-singing was rare (0.09 ± 0.19 counter-songs/h). In addition, this arrangement led to frequent "chase" events (Figures 4A, 4B, and S4A; STAR Methods), often preceded or followed by additional aggressive behaviors (e.g., biting), and was more likely to occur with the resident pursuing the intruder (Figure 4C; 22.3 ± 38.7 episodes/session) compared with the opposite configuration (Figure 4C; 1.76 ± 3.7 episodes/session, linear mixed model, $\chi^2(1) = 4.77$, $p = 0.029$). Despite the near absence of counter-singing of *S. teguina* pairs in the terrarium in this resident-intruder paradigm, 23 out of 34 mouse-sessions produced songs, with an average of 13.0 ± 10.5 songs (range: 1–40) throughout the 2-h session, although the number of songs produced by residents and intruders did not significantly differ (Figure 4D; linear mixed model, $\chi^2(1) = 3.07$, $p = 0.080$).

Upon closer inspection of the behavioral tracking data, we noticed a change in the context in which songs were produced. In certain cases, mice broadcast their songs widely (i.e., not hidden from view), most often by climbing on top of a shelter (Figure 4E). We found that residents made significantly more exposed "broadcast" songs compared with intruders (Figures 4E–4H; linear mixed model, $\chi^2(1) = 10.37$, $p = 0.0013$), a posture that was also observed when mice were alone in the terrarium during active periods (Figures S4B–S4D). The tendency of the resident mouse to favor broadcast songs was observed even in "within-animal" cases in which former resident mice were reintroduced as intruders (Figures 4F–4H), consistent with the notion

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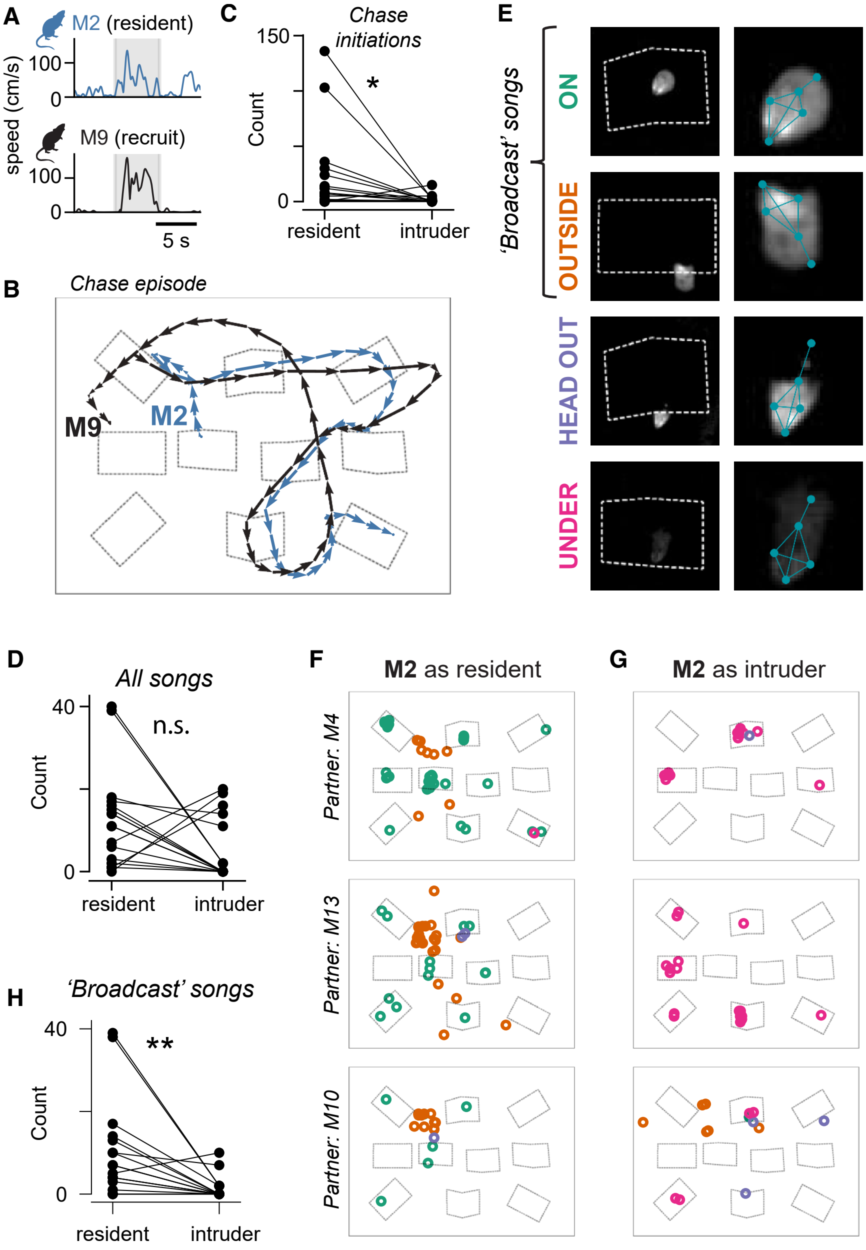

**Figure 4. Territorial effects on song production**

(A and B) A chase episode is associated with an increase in movement speed (A) and a clear directional pursuit (B). See also Figure S4A.

(C) Chases were more often observed in residents pursuing an intruder rather than vice versa (linear mixed model, $\chi^2(1) = 4.77$, $p = 0.029$).

(D) The number of songs produced by the resident and the intruder did not significantly differ (linear mixed model, $\chi^2(1) = 3.07$, $p = 0.080$).

(E) Four behavioral categories defined by the mouse's position relative to the shelter. At right, a close-up thermal camera view of examples from each song posture. The categories "on" and "outside" were defined as "broadcast" songs.

(F and G) The song positions of a single mouse (M2) are affected based on territorial context (resident at left and intruder at right). Colors correspond to categories in (E). See also Figures S4B–S4D.

(H) Residents sang more broadcast songs than intruders (linear mixed model, $\chi^2(1) = 10.37$, $p = 0.0013$).

For (C), (D), and (H), each pair of connected symbols represents the activity of a recorded pair of singing mice in a session.

that this behavior is related to social context rather than individual differences across mice. Furthermore, this set of experiments supports the notion that residents are actively seeking sites that increase the active space of their song, emphasizing the role of the song as an advertisement display.

## DISCUSSION

In this behavioral study, we investigated the structure and function of songs of Alston's singing mice. We found acoustic features, which have been shown to be consistent in individual mice,[16] are easily distinguishable from conspecifics and that these vocalizations could act as a marker for space. By playing back recorded conspecific songs from different locations, we discovered that male singing mice have a propensity to respond to conspecific songs signaled from moderate distances (~2 m), suggesting that the target of these vocalizations is likely in adjoining home ranges. When a home-range dispute was simulated by placing an intruder mouse within the same terrarium, the resident often broadcast their songs from atop the shelters, with intruders typically hiding below. Taken together, these results reveal the behavioral significance of *S. teguina* counter-singing, an interchange that may be central for territorial defense in male-male social contexts.

This study advances our current understanding of the ethological relevance of *S. teguina* singing behavior.[8,10] Previous field research[19] (see also for related species in the same genus, *S. xerampelinus*[23]) has used radio telemetric tracking of singing mouse locations (without audio) and found that home ranges of individual male mice in their natural habitat are in the order of 255–1,620 m$^2$, with extensive overlaps between males and across sexes. Our findings further allow us to speculate that the prolific spontaneous production of the songs during exploration and counter-singing may contribute to the maintenance of exclusive regions within these home ranges. The spatial exploration pattern during the "active state" of individual singing mice resembles the patrolling behavior observed in other territorial rodents, including the laboratory mouse (*Mus musculus*),[24,25] in which animals

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regularly move through their territory while scent-marking. Singing mice may have evolved to vocally reinforce their territories during such behaviors, perhaps in response to environmental factors (e.g., rich vegetation of the cloud forest).

When two male mice were placed in the terrarium, the resident frequently acted aggressively toward the intruder and produced their songs from a vantage point that enabled the greatest vocal spread (e.g., from on top of the shelter), suggesting that the songs are used to convey local defense, balanced against predatory and retaliation risks[14] as well as metabolic costs.[18] Longitudinal behavioral observations in either a larger laboratory environment or in the field are needed to determine how the home-range disputes evolve over time with repeated exposures and the extent to which the borders of the home range can be modified.[13] Additionally, because both male and female *S. teguina* are capable of counter-singing,[4,21] the dynamics of social vocalizations may be even more complex.[26]

Our findings are consistent with the notion that *S. teguina* use counter-singing for long-distance communication. Although the results from the playback experiment indicate that audition is sufficient to influence the decision to counter-sing, the impact of multimodal sensory information (i.e., vision and olfaction) on vocal interactions remains to be investigated. For instance, such signals may have contributed to the attenuation of counter-singing during direct interactions in the terrarium. Future work can explicitly test which sensory modality or combination thereof is primarily responsible for counter-singing suppression. Additionally, recent observations that pairs of singing mice produce ultrasonic vocalization (USVs) at close range[27] raises the intriguing possibility that the communication modality must be actively selected based on the location of an intended receiver. Nonetheless, the observation supports the notion of counter-singing as a strategy for long-distance communication.

The characterization of behavior in the current study opens opportunities to investigate underlying biological mechanisms. Although our results suggest that conspecific vocalizations can lead to a rapid response, how the brain processes the sensory information to give rise to the perception of conspecifics is largely unknown.[28–31] Using the vocal response as a readout, further experiments with neural recordings will be able to investigate the computations underlying the integration of acoustic parameters that convey conspecific identity[31,32] and spatial information.[28–30,33] Furthermore, recent studies in *S. teguina*[4,20] reported that the orofacial motor cortex is required to produce the temporally precise response and the song length modulation observed in vocal interactions, but other processes that enable the recognition of certain conspecifics, the decision to respond, and the diurnal patterns of spontaneous singing remain to be explored. Taken together, our behavioral experiments provide strong support for the function of songs as signals to adjoining territories that are central to male-male interactions. This work establishes the *S. teguina* song as an ethologically relevant behavior that appears to integrate information related to identity and home territory and can enable future studies to uncover fundamental mechanisms of sensorimotor processing in the service of social interactions.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Michael Long (mlong@med.nyu.edu).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

Data and code used to generate figures in this study have been uploaded to Zenodo (https://doi.org/10.5281/zenodo.14963288).

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## AUTHOR CONTRIBUTIONS

Y.F. and M.A.L. conceived the study and designed the experiments; Y.F. conducted the research; Y.F. and M.A.L. performed data analysis; Y.F. and M.A.L. created the figures; Y.F. and M.A.L. wrote the initial draft of the manuscript; Y.F. and M.A.L. edited and reviewed the final manuscript; Y.F. and M.A.L. acquired funding; and M.A.L. supervised the project.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Deposited data | | |
| Behavioral data | This paper | Zenodo: https://doi.org/10.5281/zenodo.14963288 |
| Experimental models: Organisms/strains | | |
| *Scotinomys teguina* | Laboratory colony | N/A |
| Software and algorithms | | |
| MATLAB | MathWorks | https://www.mathworks.com/products/matlab.html |
| Python 3.12.5 | Python Software Foundation | https://www.python.org/ |
| R 4.4.3 | R Foundation | https://www.r-project.org/ |
| Ultrasonic audio recording software | Avisoft Bioacoustics | Avisoft-RECORDER https://avisoft.com/downloads/ |
| Camera acquisition software | Teledyne FLIR | SpinView https://www.teledynevisionsolutions.com/ |
| Pose-tracking software | SLEAP developers | SLEAP https://sleap.ai/ |
| Other | | |
| Thermal camera | Teledyne FLIR | Boson 640, 95° (HFOV) 4.9 mm |
| Color vision camera | Teledyne FLIR | Blackfly USB3 - Model: BFLY-U3-23S6C-C: 2.3 MP, 41 FPS, Sony IMX249, Color |
| Color vision lens | Edmund Optics | 3.5mm C Series Fixed Focal Length Lens |
| Omnidirectional microphone | Audio-Technica | AT803 |
| Ultrasonic microphone | Avisoft Bioacoustics | CM16/CMPA |
| Ultrasonic digitizer | Avisoft Bioacoustics | UltraSoundGate 116Hb |
| Ultrasonic playback interface | Avisoft Bioacoustics | UltraSoundGate Player 116H |
| Ultrasonic speaker | Avisoft Bioacoustics | Vifa |
| Audio amplifier | ART | ART TPS II 2-channel Tube Microphone Preamp |
| Analog terminal block | National Instrument | BNC-2090A |
| Acquisition board | National Instrument | PCI-6221 |
| Pulse stimulator | AMPI | Master-8 |
| Digital sound level meter | Nady | DSM-1 |
| Terrarium | Custom Cages | 36''H x 48''L x 36''D H3 Small Animal Cage |
| Moss beddings | Zilla | Jungle Mix |
| Woodchips | Zoo Med | Forest Floor Bedding |
| Recording chamber | Med Associate | CT-SAC-322024 |
| Sound absorption foam | Soundcoat | Soundfoam ML HY ULb |
| Paint Marker | Mitsubishi Pencil | Uni-Paint PX-20 Oil-Based Paint Marker, Medium Point, Metallic Silver (63614) |

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals used in this study were adult (> 3 months) male *Scotinomys teguina*. All animals were laboratory-reared offspring of wild-captured *S. teguina* from La Carpintera and San Gerardo de Dota, Costa Rica. The animals were maintained in a temperature- and humidity-controlled environment. The light cycle was on a 12/12 hr light/dark schedule in enclosed recording chambers, while our terrarium was illuminated with natural sunlight from a nearby window. All animal maintenance and experimental procedures were performed according to the guidelines established by the Institutional Animal Care and Use Committee at the New York University Langone Medical Center.

## METHOD DETAILS

### Terrarium design

A terrarium was constructed using an arena (0.91 m × 1.22 m) (Custom Cages, Neenah, WI). The floor was filled with moss bedding (Zilla, Franklin, WI), woodchips (Zoo Med, San Luis Obispo, CA), and several slate stones. The inner walls were lined with wallpaper

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depicting a forest scene. Ten ‘A-Frame’ mouse shelters (19.1 × 13.3 × 5.7 cm) were built using bamboo sticks for the frame and covered with black cheesecloth and plastic mesh. These shelters were distributed across the terrarium, with their corners secured to the base using double-sided adhesive strips. The terrarium was equipped with an ultrasonic condenser microphone (Avisoft Bioacoustics, CM16/CMPA) and a standard omnidirectional condenser microphone (Audio-Technica, AT803). For visual monitoring, a long-wave infrared thermal camera (Teledyne FLIR, Boson 640, 95° (HFOV) 4.9 mm) and a color vision camera (Teledyne FLIR, BFLY-U3-23S6C-C) provided a top-down view of the terrarium.

### Sound-isolated recording chamber design

Sound attenuating cubicles (81.3 × 50.8 × 61.0 cm) (Med Associate, St. Albans, VT) were further sound-isolated by lining the inner walls with sound absorption foam (Soundcoat, Deer Park, NY). Each chamber was equipped with an omnidirectional microphone and an ultrasonic microphone.

### Audio recording

Signals from omnidirectional microphones were amplified using an audio preamplifier (ART, TPS II) and then sampled at 10 kHz via a data acquisition board (National Instruments, PCI-6221), ensuring shared timestamps for audio signals of different mice. Signals from ultrasonic microphones were sampled independently using an ultrasonic recording interface (Avisoft Bioacoustics, UltraSoundGate 116Hb) at 250 kHz and were later synchronized (see ‘audio signal synchronization’). By recording with both ominidirectional and ultrasonic microphones, we were able to align timestamps across modalities (i.e. audio from another recording chamber and camera streams) while retaining spectral resolution needed to capture the frequency range of *S. teguina* vocalization. For longitudinal recordings of individual mice, the ultrasonic microphone was used with a trigger mode, and timestamps were provided by commercially available acquisition software (Avisoft Bioacoustics, Avisoft-RECORDER).

### Image acquisition

During multi-animal sessions in the terrarium, thermal frames were captured at 60 fps in free-run mode, and were saved as 640 × 512, 16-bit images. Color images (1280 × 1024, 8-bit) were acquired by the color vision camera driven with a pulse stimulator at 10 Hz (AMPI, Master-8), and these commands were also sent to the data acquisition board as a temporal reference of image collection. To synchronize thermal and color image streams, we used visual cues at the beginning and end of recording sessions and provided timestamps for the thermal images by interpolation based on the color images’ timestamps. We used the OpenCV Python package to control the thermal camera and a commercially available software (Teledyne FLIR, Spinview) to interact with the color vision camera. During long-term monitoring of individual mice over multiple days, thermal frames were acquired at 1 fps.

### Remote interaction experiment

Two mice, housed in individual home cages, were placed in separate sound-isolated recording chambers (described above) that were adjacent to each other. Although the linear distance between the location of the home cages was ~1 m, the doors of the recording chambers were slightly opened, providing limited acoustic access to one another without any visual cues. Each recording session lasted 24 h.

### Terrarium interaction experiment

We observed interactions between two freely moving male mice in the terrarium for 2 hours. Before introducing the mice to the terrarium, we painted a dot on the trunk of one of the mice with a silver paint marker (Mitsubishi Pencil, Tokyo, Japan) for additional confirmation of animal identity (see animal Tracking). For the ‘neutral social interaction’ paradigm (i.e., Figures 3A–3C), we introduced both mice to the terrarium at the same time. For the ‘resident-intruder’ paradigm (i.e., Figure 4), we introduced an ‘intruder’ mouse to the terrarium, where the ‘resident’ mouse had been living for at least 5 days.

### Playback experiment

During the playback experiment, the responding mouse was kept in a recording chamber, while the position of the playback speaker was changed to one of 4 locations across trials. To avoid the potential influence of acoustic features other than those that correlate with behavior (e.g., partner identity), our stimulus set included audio files of 2 spontaneous songs recorded from a male *S. teguina* (not included as a subject). For each location, each song of the 2 audio files was played 3 times, totaling 24 songs per session. Songs were played on average every 2 minutes (interval: 119.62 ± 11.15 s) in a pseudorandomized order such that 8 consecutive playbacks had all conditions (4 locations x 2 audio files) to avoid the potential effects of adaptation within a session. Songs were broadcast using an ultrasonic dynamic speaker (Avisoft Bioacoustics, Vifa) via an ultrasound playback interface (Avisoft Bioacoustics, UltraSoundGate Player 116H). The analog signal was split from the converter to the National Instruments data acquisition board for synchronization. Sessions for each mouse were spaced at least 6 days apart. For analysis, we calculated the response ratio for each mouse and location as the proportion of playback trials that elicited a counter-song. Volume levels for playback were

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adjusted using a portable digital sound level meter (Nady, DSM-1) so that the maximum sound pressure level of the songs was at 75 dB SPL at 15 cm.

## Data analysis

### Preprocessing of thermal images

Flat-field correction was applied to 16-bit thermal frames to address variability across pixels. To enhance the visibility of the mice, we retained only the upper 5% of pixel values (corresponding to the warmest regions) and set all other pixel values to zero. Contrast in the mouse's body structures was further improved using Contrast Limited Adaptive Histogram Equalization (via skimage.exposure.equalize_adapthist). The images were then converted to 8-bit format. Finally, to address distortions introduced by the wide-angle lens, a fish-eye correction was applied using a MATLAB function (undistortFisheyeImage).

### Animal tracking

Pose tracking was performed on the preprocessed thermal images of mice in the terrarium using SLEAP.[22] A total of 101 frames were manually labeled for training, focusing on five body parts: snout, right ear, left ear, middle, and tail base (e.g., Figure 4E). The multi-mice top-down algorithm was used for training, with the following parameters: anchor_part = middle, sigma for centroids = 2.5, and sigma for nodes = 2.5. All other parameters were set to default. The trained model was then used to infer body parts on newly acquired images. Mouse identities were tracked across frames with the following configuration: tracker = simple, track_window = 10, similarity = centroid, match = greedy, min_new_track_points = 1, min_match_points = 1, and clean_instance_count = 0. The tracking results were visually inspected using the SLEAP GUI. Frames where body parts were incorrectly predicted were removed, and missing positions were linearly interpolated in each dimension (x, y) using scipy.interpolate.interp1d. Mislabeling of mouse identities was corrected manually on the GUI. True identities were determined based on a small, silver, highly reflective paint mark on the trunk of one of the mice, which appeared as a bright ('warm') point in the thermal images. Further behavioral analyses (e.g., position, speed) were computed based on the 'middle' body part.

### Animal speed

For single-animal video tracking (1 fps), the mouse's speed was calculated as the distance traveled between consecutive frames. Speed values smaller than 2 cm/sec were set to 0 to eliminate noise introduced by pose-tracking estimation errors. For multi-animal recordings (60 fps), the mouse's speed was determined as the vector norm of the first derivative of animal position, smoothed using a Savitzky-Golay filter (scipy.signal.savgol_filter) with a window length of 1 second. All distances were converted from pixels to centimeters using a scaling factor of 0.21.

### Audio signal synchronization

To align timestamps for the ultrasonic audio signals (250 kHz) across mice, we first up-sampled the audio signal of the omnidirectional microphone (10 kHz) to 250 kHz, matching the ultrasonic microphone signal. Then, we used cross-correlation (via scipy.signal.correlate) to calculate the time lag between the ultrasonic signals containing a song and the corresponding audio signals recorded by the omnidirectional microphone (10 kHz).

### Song and note detection

To detect singing mice songs in continuous audio signals, we first screened for signal blocks exceeding 10 dB above the background noise, which were then stored as potential songs. Each of these sound blocks was visually inspected in spectrograms to confirm the presence of a song. To identify individual notes within a song, we followed a strategy described in previous studies from our laboratory.[4,20] The sound waveform was smoothed using a 4-ms sliding Hanning window. Signals with a relative amplitude exceeding 10 dB above the background noise were considered notes. Note onsets and offsets were determined as the first and last crossings of 1% (20 dB quieter) of each note's maximum relative amplitude. For notes with a maximum relative amplitude of less than 20 dB, a threshold of 1 dB was used. The start and end of a song were defined as the onset of the first note and the offset of the last note, respectively. We visually inspected the detected notes and removed false positives (e.g., noise). We excluded vocalizations shorter than 2 seconds.

### Feature extraction

For the singer identity classification, 6 features were extracted: (1) maximum note duration, (2) minimum dominant frequency, (3) maximum dominant frequency, (4) number of notes, (5) note density, (6) note progression. Features (1) – (3) depicted characteristics of the note of the maximum duration in a song ('note features'), whereas features (4) through (6) were acoustic patterns of an entire song ('song features'). (1) Maximum note duration was the time difference between detected onset and offset of the longest note. (2) Maximum dominant frequency and (3) minimum dominant frequency were taken from detected pitch on the note of maximum duration. The pitch was computed using librosa.piptrack (parameters: fmin = 5000, fmax = 50000, hop_length = 1, win_length = 512) on a corresponding segment of high resolution (250 kHz) audio signal. (4) Number of notes were the count of detected notes in each song. (5) Note density was computed as the number of notes divided by the length of the song. (6) Quadratic coefficient of note duration trajectory is the value of the quadratic coefficient ($Q$) of a quadratic function ($y = Qx^2 + \beta$) fitted to the note duration trajectory, where y is duration (in seconds) of notes and x is the onset time (in seconds) of each note.

Based on the prior knowledge that a *S.teguina* song starts with short, quiet notes and the notes become louder and longer towards the end of the song, the notes occurring in the latter half of each song were considered for the longest note. We limited our analysis to songs whose length was greater than 4 seconds (> 98% of cases). After the feature extraction process, we removed 71 songs from further analysis if the song met one or more of the following criteria: (1) $Q < 0$ or $Q > 0.0015$, (2)

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librosa.piptrack algorithm failed to determine dominant frequencies, (3) maximum dominant frequency exceeded 55 kHz or fell below 30 kHz.

### *Singer identity classification*

Using the linear discriminant analysis (LDA) module from the scikit-learn library (sklearn.discriminant_analysis.LinearDiscriminantAnalysis), we trained a classifier on six extracted features from individual songs. Absolute values of Pearson's correlation coefficients between all pairs of features were $< 0.8$. Cross-validation was performed by repeating the following process 100 times: in each iteration, since the number of songs varied among individual mice (402–1497), we randomly selected 200 samples from each of the five mice. To ensure a conservative approach, we split the selected data into a training set (70%, 140 songs) and a test set (30%, 60 songs). We then fit a linear discriminant model to the training set and predicted the singer identity in the test set. The confusion matrix represents the average cross-validation results, showing the probability of correctly identifying each singer. Mean LDA coefficients were computed as the absolute values for individual features, averaged across singer identities for 100 iterations. Empirical feature importance was computed with sklearn.inspection.permutation_importance (n_repeats = 10) for test data sets across iterations, and the average importance of each feature was reported. This analysis quantifies how much the prediction accuracy deviates when each feature is permuted.

### *Response latency distribution similarity*

To compare response latencies of counter-songs, we defined a similarity index as:

$$S_{KS} = 1 - D_{KS}$$

$$D_{KS} = \sup_{x} |F_a(x) - F_b(x)|$$

where $D_{KS}$ is the Kolmogorov-Smirnov statistic between two response latency distributions, and $F_a(x), F_b(x)$ are empirical cumulative distribution functions of response latency distributions. $S_{KS} = 1$ indicates that two response latency distributions are identical, while $S_{KS} = 0$ represents the maximum possible difference between the two distributions. We obtained a similarity matrix by computing a similarity index between all combinations of response latency distributions of cases in which the mouse produced more than 40 counter-songs. Then, we performed a Wilcoxon rank-sum test between the similarity indices of distribution combinations of the same responder and those of different responders.

## Quantification of movement periods

Movement periods were defined as time windows during which the mouse's speed exceeded 4 cm/s. If consecutive windows were separated by less than 5 minutes, they were merged into a single window. Additionally, windows lasting less than 1 minute were excluded from analysis. To capture potential pre- and post-movement singing activity, a 5 min buffer was added to both sides of each valid movement window. To visualize whether each mouse exhibits a conserved pattern in the occurrence of movement periods across days, we computed the probability of movement periods by averaging the occurrence of movement periods (binary) in 1 s bins across full-day recordings (i.e., excluding day 0 and the last day).

## Singing location extraction and singer identity labeling

To determine the singing location in relation to a shelter, we visually inspected the frames surrounding detected songs and manually labeled the singer's location as 'on', 'outside', 'headout', or 'under' in relation to the shelters. When two mice were in the terrarium, the singer was identified by their characteristic upright posture.[3,9,34] In 3 out of 456 total songs from the multi-animal terrarium sessions, identifying the singer was difficult, and these three events were discarded from the analysis.

## Counter-song occurrence rate comparison

To compare the occurrence of counter-songs during interactions with different social conditions, we calculated the counter-song occurrence rate below. For remote vocal interaction, we computed the counter-song occurrence rate as the total number of counter-songs made (by either mouse) in the first 2 h of recording sessions, divided by 2. For terrarium sessions, we considered all counter-songs detected in individual sessions (2 h).

## Chase episodes

We first detected time windows in which the speed of at least one mouse exceeded 100 cm/s as potential chase episodes. The start of a candidate episode was defined as the moment one mouse began moving (> 4 cm/s), and the end was marked when both mice stopped moving (< 4 cm/s). Candidate episodes shorter than 1 second were merged. To identify the chaser, we leveraged the fact that *S. teguina* often moved by hopping between shelters. We defined 10 'zones' surrounding each of the 10 shelters (Figure S4D), and for each of the two mice, we quantified events ('chase episode') in which the mouse enters the zone in which the opponent is present, and the opponent leaves that zone. For a given candidate episode, the mouse with the higher count of chaser moments was determined to be the chaser of the episode. Candidate episodes with no chase moment (e.g., mice that were never in the same zone, or cases in which a mouse entered the opponent's zone and left without the opponent evacuating) were discarded.

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## QUANTIFICATION AND STATISTICAL ANALYSIS

We used Python packages including SciPy and scikit-posthocs for non-parametric comparisons (Wilcoxon rank-sum test, Friedman test, and Dunn's test) throughout the manuscript unless mentioned otherwise. The linear mixed model analysis was conducted using lme4 package in R (v4.4.3) as follows: we fitted linear mixed-effects models for each behavioral outcome (e.g., chase count) with role (i.e., resident or intruder) as a fixed effect and included mouse identity and session ID as random intercepts. We calculated p-values using likelihood ratio tests, comparing each full model to a reduced model excluding the role term via one-way ANOVA.

Values are reported as mean ± SD unless indicated otherwise.