SCALE DEPENDENT PROCESSING OF CONSPECIFIC SIGNALS IN THE GRAY TREEFROG *HYLA VERSICOLOR*

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Neuroscience
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2019

Urbana, Illinois

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ABSTRACT

Acoustic communication is both widespread and often essential for successful mate selection, territorial defense, and many social behaviors in many taxa. Laboratory studies often use ‘simplified’, artificial stimuli which have high signal-to-noise rations and are easy to manipulate, but often do not capture the true range and variation of sounds experienced by organisms in the field. In nature, acoustic signals from socially aggregating animals are often emitted together, clustered in space and time. As these signals come from conspecifics they tend to be self-similar in many parameters, providing a challenge for the auditory system to detect, localize, and discriminate between target signals amongst the background. Additionally, signal-environment interactions during transmission further increase the difficulty. In this dissertation, I explore the processing of natural signals in the eastern grey treefrog, *Hyla versicolor*. During the mating season, numerous males gather near water sources and form choruses, emitting a stereotyped advertisement calls, creating a challenging auditory environment. Females later approach the chorus from daytime resting sites, often up to several hundred meters distant. Females must first detect and orient to the chorus, and subsequently detect, localize, and discriminate among individual male calls embedded in the chorus background. In chapter 2, I quantify the attraction of the chorus using recordings made at increasing distances from a single calling male. In the field, females oriented to the chorus at distances up to 100 m. In laboratory playback experiments, females were only attracted to chorus recordings made up to 32 m from a male. Chapter 3 explores whether sound amplitude or temporal structure degradation,

dynamic acoustic features that changes with distance to a calling male, has a greater impact on chorus attractiveness and orientation. Through playback experiments we found that distance-dependent changes in call temporal structure had a larger effect on signal attractiveness than sound level, and that localization accuracy was proportional to sound level, signal attraction was not. This supported the necessity of perceptible fine temporal structure (pulse rise/fall time, pulse duration, etc.) in calls for female localization and discrimination, and that this information is probably not present at distances greater than 32 m. Finally, chapter 4 describes the representation of synthetic calls and natural chorus sounds in the central auditory system of *H. versicolor*. Single midbrain neurons had diverse response properties, including rapid, slow, and intermediate firing rate adaptation in response to stimuli, which resemble phasic, tonic, and phasi-tonic responses found in other anuran studies, respectively. The slowly-adapting cells had the least attenuation with recording distance from a calling male, and for an approaching female would likely be the first cells to respond to the chorus itself, or individual male calls embedded within. Rapidly-adapting cells, which demonstrate strong attenuation with distance and strong on/offset properties to call pulses are well positioned to aid in the detection of nearby individual male calls and the discrimination of temporal features essential for mate selection, which most likely take place at distances of no more than 4-8 m from a male calling in a dense chorus. Overall, we have just begun to explore auditory behavior and physiology with significant attention to the real-world context of the animal. The results of these studies demonstrate both the strict limits nature places on sensory systems, and their adaptability to the needs of the organism.

ACKNOWLEDGEMENTS

It has been a long, exciting, stressful, challenging, and fun road that brings me here, and I am blessed to have been given the chance. All things must have a beginning and that must be Dr. Albert Feng. His mentorship, guidance, advice, and years of experience in the fields of auditory neuroscience and neuroethology have proven invaluable, and I can only hope to carry them with me. It can truly be said that in the realm of studying such tiny, inconspicuous (though not acoustically!) organisms, Albert reigns as a true *giant*!

I would also like to thank my committee, Drs. Fuller, Gillette, Nelson, and Pandya for their patience, advise, expertise, and time. I would like to thank Rebecca for her advice and the opportunity to help explain the esoterica of my field to an 'outsider'. Mark was my first mentor here at Illinois, and never have I met a person more willing to help guide a budding scientist through the treacherous waters of graduate study. Regarding Rhanor....well what *would* life in the neuroscience program here be without you? Your experience, humor, and passion for all things spineless and squishy will warm my heart as I journey into the future. To Pritesh, your knowledge and advice has also proven of the highest sort, and I believe that you will certainly be an irreplaceable member of the cadre of neurophysiologists here on the prairie.

I would like to thank my collaborators away from the metropolis of Champaign-Urbana my friends at the University of Missouri - Columbia. I would especially like to thank Dr. Johannes Schul and Dr. Sarah Bush - you have been almost a second family away from Illinois – both academically and personally. I owe a great deal of my growth

as a student and scientist has been thanks to your wisdom and advice! To Dr. Carl Gerhardt, I thank you and feel honored to have been able to talk and meet with you. I would like to thank several graduate and postdoctoral colleagues at 'Mizzou': Dr. Oliver Beckers, Dr. Noah Gordon, Dr. Sarah Humfeld, and Dr. Allison Welch. Your hospitality and help are much appreciated. I would also like to thank my undergrad assistance: Paul Kimball, Stephanie Kleyman, and Leah Reynolds – thanks for staying up with me to wander out in tick-infested fields and leech-infested ponds at 1 AM!

To my colleagues in the Biology Department at the University of Iowa, I would like to thank Dr. Atulya Iyengar for aid with data analysis, Prof. Bernd Fritzsch for helping to critique my manuscripts and administrative help, and finally to my supervisor, Prof. Daniel Eberl, for editorial and moral support over the 'extended' gestation of the thesis.

To those lab-mates and colleagues in the program, I give my heartfelt thanks. It was a great experience working with Dr. Karla Melendez, Dr. Sungchil Yang, Dr. Vince Marshall, and (the newly-minted) Dr. Samit Shah. My special thanks to Dr. Wen-Yu Lin, our lab 'mother' – you are truly a master of the fine arts of amphibian surgery *and* making delicious dumplings! I want to thank Judy Whittington, our dearly departed (down-campus!) group secretary, life here in the second floor of Beckman will never be the same without you! I would like to thank Dr. Gene Robinson, in whose eternally-capable hands our program has been entrusted since before I arrived, and who I know can only deliver the program to even greater heights! Gene, you were always had a valuable word and ready ear for anyone in the program, and your personal guidance has been absolutely essential to my success here at Illinois. I would like to thank Dr.

Sam Beshers, who ‘knows all, sees all’ in regards to the Neuroscience Program. Your work and organization has made this program and this place truly special. I would like to thank all of my fellow graduate and postdoctoral colleagues, and extra thanks to Dr. Tsvi Achler, Dr. Seth Ament, Georgina Aldridge, Dr. Jason Ebaugh, Margaret Ferris, Dr. Sarah London, Claudia Lutz, and Dr. Ludmilia Yafremava.

I would also like to thank my friends around the country – to Kevin Crouse, you have been my friend through almost twenty years and three states, from grade school to grad school, from Sparta to St. Louis. To Michael Fernandez, Benjamin Cooper, Thomas Keeler, and Sean Gleason – I miss all of you and hope to visit D.C. as often as possible! Closer to home, I would like to thank my friend and roommate for many years, Eric Rozier, who has provided essential advice and asked many poignant questions – keep giving biologists hell for abusing statistics, Eric! I want to thank Chris Siefert, who preceded me in moving from Williamsburg to Champaign. To Dwight Divine, Ryan Lefever, Ken Keefe, and Sankalp Singh – thanks for driving me nuts and keeping me sane! I would also like to thank Dr. Richard Prosl and Dr. John Griffin – my advisors at the College of William and Mary – every journey begins with a single step, and you helped set me on that first step in science and research, which ultimately led me here.

Finally, I want to thank my family. To Mom and Dad – you were always there for me - without you, I am nothing, everything I have done was possible because of your love for me, and so everything I will do is dedicated to you. I want to thank and give my love to my sisters and my brother. You will always have all my love.

TABLE OF CONTENTS

CHAPTER 1: PROJECT BACKGROUND AND BRIEF OVERVIEW

1.1 PROJECT INTRODUCTION

The sensory systems of complex organisms have long been a subject of research – the nature of signal detection and processing, the structure and physiology of the sensory organs, etc. Historically, too little attention has been paid to the real-world contexts of sensory signals (Feng and Schul 2007). Signals used in the laboratory are often artificially simplified – easier to manipulate and study, but not always encapsulating the true range and variation experienced by organisms in the field. Laboratory studies commonly use repeated presentations of a single stimulus, while in nature, such signals are rarely encountered in isolation. Signals (regardless of modality) often have their origin in conspecifics, particularly in social organisms. As the physical distribution of social organisms is often uneven, signals can be emitted in groups or clusters, in space or time. As these signals tend to be self-similar in many parameters, the task of signal isolation and analysis can be a challenge for the sensory system in question. Examples include isolating a voice in a room with many ongoing conversations (auditory – the famous 'cocktail party problem'), searching for a familiar face in a crowd (visual), or discerning different flavor components of a well-cooked meal (gustatory and olfactory). Comparative work utilizing multiple model systems and sensory modalities can provide valuable information on the physiology and evolution of signal processing. Current artificial sensory and perceptual systems cannot yet rival the robustness or performance exhibited by biological sensory systems. The behavioral and

neural strategies used may also prove useful in fields such as computer vision, sound/voice recognition, artificial sensor development, and robotics.

The research projects described herein use the auditory system of anurans, including that of the gray treefrog, *Hyla versicolor*, which has been used as a model for auditory processing, neurophysiology, and behavior for decades (Gerhardt and Doherty 1988; Feng et al. 1990; Gerhardt and Watson 1995; Diekamp and Gerhardt 1995; Beckers and Schul 2004; Narins et al. 2007). During the mating season, male treefrogs gather nightly near ponds, lakes, and other permanent and temporary water sources. Each male in the chorus (which can number in the hundreds) emits a stereotyped, characteristic advertisement call. After the chorus assembles, females approach from their daytime resting and foraging grounds, often over distances of up to several hundred meters (Noah Gordon, personal communication). Females select a single calling male to mate with and fertilize her clutch of eggs, which requires the ability to successfully detect, localize, and discriminate individual calls among the continuous background of conspecific calls. In addition, much has been learned about the spectral and temporal characteristics preferred by females (Gerhardt and Doherty 1988; Gerhardt 1991, Diekamp and Gerhardt 1995; Gerhardt et. al. 2000; Gerhardt and Schul 1999). Combined with their readiness to behave in the laboratory, this makes *Hyla versicolor* an attractive model system in which to investigate the processing of clustered sensory signals.

I decided to explore the processing of clustered sensory signals – including signal detection, localization, and discrimination – in the context of mate finding during chorusing in the gray treefrog, *H. versicolor*. More specifically, I investigated the

behavioral and neurophysiological methods and strategies that gravid *H. versicolor* females use in the process of detecting an individual male's advertisement call from the chorus background, and how the background affects signal discrimination, leading to functional restrictions on mate choice in the field. Although successful mating requires the female to both detect and locate a potential mate's call, the task of call localization is fraught with many difficulties, and many details of how female treefrogs achieve these feats are unclear at best. As this is a relatively new area of study (Slabbekoorn and Bouton 2008), with much complexity in the signals and environments involved, the range of questions and potential experimental designs is vast. Therefore, in the current project I limited the investigation to the task of signal detection and orientation in a natural clustered sensory scene.

Three experimental projects/goals are described: (1) quantifying the attraction of a natural clustered auditory signal and how that attraction varies with distance, (2) quantifying the most salient alterations in signal properties of amplitude or temporal structure responsible for the above changes in attraction, and (3) exploring the representation of chorus noise and calls embedded in the chorus by the treefrog central auditory system. Previous work has shown that chorus noise has a notable effect on female perception of advertisement calls (Gerhardt and Klump 1988a; Gerhardt and Klump 1988b; Wollerman 1999; Schwarz et. al. 2001; Wollerman and Wiley 2002, Bee and Schwartz 2009; Vélez and Bee 2010; Kuczynski et al. 2010; Vélez et al. 2013), suggesting that call detection and representation in the nervous system may also be significantly altered, although to date few behavioral, and no neurophysiological experiments have used natural chorus sounds as stimuli.

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CHAPTER 2: PHONOTAXIS TO MALE'S CALLS EMBEDDED WITHIN A CHORUS BY FEMALE GRAY TREEFROGS, *HYLA VERSICOLOR*[1]

2.1 INTRODUCTION

Acoustic signals are widely used for communication in many taxa and serve numerous functions including sexual advertisement, mate choice, territorial defense, and other interactions. In socially aggregating animals, acoustic recognition, discrimination, and localization are made difficult due to the presence of competing background sound and changes in the signal features due to environmental interactions (Lohr et al. 2003; Feng and Schul 2007; Swanson et al. 2007). Noise from biotic (Gerhardt and Klump 1988a; Wollerman 1999; Wollerman and Wiley 2002) and abiotic (Bee and Swanson 2007) sources compromises signal detection and localization due to masking. Masking by signals of conspecifics is particularly problematic because they share many of the frequency and temporal characteristics with a signal of interest (Jouventin et al. 1999, Aubin and Jouventin 2002). Detection and localization of auditory signals are also hampered by environmental degradation, e.g., atmospheric attenuation, signal absorption and reflection from vegetation and the substrate (Forrest 1994; Luther and Wiley 2009). Signal-environmental interactions alter both the temporal and spectral characteristics of acoustic signals (Richards and Wiley 1980; Dabelsteen et al. 1993; Penna and Solis 1998).

[1] This chapter is published in its entirety in the Journal of Comparative Physiology A. This article is reprinted with the permission of the publisher and is available from https://link.springer.com/article/10.1007/s00359-010-0544-2 and using DOI: https://doi.org/10.1007/s00359-010-0544-2

During the reproductive season, male anurans aggregate and produce advertisement calls to attract mates, while females use these signals as a basis for mate selection (Narins et al. 2007). Most behavioral studies examining female mate choice use clean and well-defined acoustic signals with high signal-to-noise ratios (Gerhardt 1981; Gerhardt and Doherty 1988; Bush et al. 2002; Gerhardt 2005). Such studies have successfully elucidated, for a number of anuran species, the salient features in the male's call that attract female frogs and give rise to their behavioral preferences (Gerhardt 1981; Gerhardt and Doherty 1988; Gerhardt and Schul 1999; Gerhardt et al. 2000). However, we have limited understanding of the extent to which and the range from which a male's advertisement call within a chorus, or the chorus itself, is used to guide female's phonotaxis.

The use of acoustic cues for orientation in complex auditory environments has recently been investigated in a few taxa with mixed results (Slabbekoorn and Bouton 2008). Among anuran amphibians, there is evidence for positive phonotaxis to choruses in several species, including *Rana sylvatica* (Bee 2007), *Hyla gratiosa* (Gerhardt and Klump 1988b), and *Hyla chrysoscelis* (Swanson et al. 2007). These chorus attraction studies have focused on the attractive qualities of the chorus itself, without examining how distance to the chorus affects the spectral and temporal characteristics of individuals' calls. Males gray treefrogs (*Hyla versicolor*) also form dense choruses during the mating season from which they emit advertisement calls (Schwartz et al. 2004). The chorus sound can propagate over several hundred meters and remain at levels well above the measured hearing and behavioral thresholds for anurans (Gerhardt and Klump 1988b; Narins and Zelick 1988; Sinsch 1990a). Female *H.*

versicolor may travel hundreds of meters from daytime feeding and resting sites to breeding ponds (Johnson and Semlitch 2003). The sensory cues that females use to localize and orient towards the ponds remain unclear, however.

In the present study we examined the attraction of female *Hyla versicolor* to both natural chorus sounds as well as male calls embedded within a chorus. In the first part of the study, we studied females' responses to natural choruses in the field. In the second part, to assess the role of acoustic cues in directed orientation in the field we examined females' responses to male calls embedded within a chorus in a laboratory.

2.2 MATERIALS AND METHODS

2.2.1 SUBJECTS AND COLLECTION/STUDY SITES

We collected female gray treefrogs (*Hyla versicolor*) of the northwestern mitochondrial lineage (Ptacek et al. 1994) from breeding choruses in the Thomas Baskett Wildlife Area and Three Creeks State Park, near Ashland, Missouri. Gravid females in amplexus were caught by hand and placed in plastic containers on ice overnight to prevent oviposition. We carried out field and laboratory phonotaxis experiments on the following day. A total of 19 and 34 females were used for the field and laboratory phonotaxis studies, respectively. At the conclusion of each experiment, we released all animals back to their native ponds.

The study site for the chorus recording and field phonotaxis experiments was a large pond, approximately 60 m by 30 m, surrounded by woodland. The ground was level around most of the pond, but sloped on the north side, starting about 2-3 m from the pond's edge, and extending for ~3-4 m away and ~2 m down from the pond. The ground then gently sloped in a north-north-easterly direction. Vegetation consisted of

mixed hard and softwoods (with cedar and locust the most prevalent species) as well as woody shrubs < 1 m in height. Vegetation was most dense at the pond's edge, consisting of trees and shrubs. Beyond ~2 m, vegetation decreased in density and was mostly confined to tall (> 5 m) trees and short (< 1m) vegetation. The substrate was leaf litter laying on top of soil.

2.2.2 FIELD PHONOTAXIS STUDIES

All field phonotaxis studies took place at the Thomas Baskett Wildlife Area, between 22:00 pm and 1:00 am. Only gravid females originally collected from this site were used. Two transects were made from the edge of the main breeding pond out to a distance of 75-100 m. Care was taken to ensure that each transect led directly away from the main pond and not toward any other ponds which could contain choruses and act as potential stimuli. We constructed a series of 4 circular arenas along each transect at the following distances from the pond: 10 m, 25 m, 50 m, and 75 or 100 m (depending on local terrain). A series of 36 numbered marker flags was arraigned in a circle of 1 m radius around the center, in 10° arc intervals. In each arena, the majority of leaf litter, small plants, grasses, and shrubs was removed, leaving a substrate of soil with some leaves. The 10 m arena served as a control trial for the calculation of phonotaxis scores (see Statistics), and females were expected to orient directly to the pond at this distance. The other distances served as experimental trials.

Prior to each experimental session, we removed the females from ice and allowed them to warm to ambient temperature for 15 – 20 min. For each female we chose one of the two transects and tested the subject in each of the four arenas, in random order. At each distance, we placed the female in the center of the arena in an

acoustically transparent plastic release cage (random facing; ~12 cm diameter). Prior to this, the cage floor was dusted with an orange non-toxic UV fluorescent dye powder (DayGlo Color Corp.) – allowing tracking of the female (Windmiller 1996; Eggert 2002). All lights were switched off and the female was given >15-30 s to acclimate. Then the lid of the cage was remotely lifted by an observer standing outside of the arena border, and a timer started. The observer monitored the animal's locomotion with a night vision monocle (Night Patriot II, ATN Corp.). When the female left the arena boundary, the timer was stopped, and the time noted, along with the two flags through which the female passed to exit the arena. If the female did not leave the release cage after 10 minutes, the female was considered unresponsive, and data were not collected. Exit point determination and animal recovery were aided by visualizing the fluorescent powder the female deposited on the ground using a UV lamp (Blak-Ray ML-49, UVP). After the female was tested at all arenas, any residual powder was washed with pond water and the female was released.

2.2.3 CHORUS RECORDINGS

Recordings of frog choruses were made from ~22:00 pm to 1:00 am, when the chorus density and amplitude were at their peaks (personal observation). We measured the sound pressure level (SPL) of the chorus before and after recording of the chorus with a digital sound pressure level meter (Bruel and Kjær 2239, fast RMS, C-weighted, re: 20 µPa). C-weighting results in band-pass attenuation of frequencies < ~100 Hz and > ~6 kHz, with low-frequency roll-off less steep than standard A-weighting. The SPL was measured at four different positions (roughly north, south, east, and west of the breeding pond), each approximately 15 m from the edge of the breeding pond. Three

SPL values (10 s averaged L_{eq}) were taken at each of the four locations and used as a rough estimate of chorus intensity. The differences in SPL values between each location remained fairly constant throughout the duration of the experiment (data not shown).

We then selected an active calling male *H. versicolor* at the pond's edge (perching on a tree at a height of >20 cm from the ground) as a “focal” male. All focal males were at least 1 m distant from the closest neighboring males, to ensure that at the closest distances (1 – 4 m) the calls of the focal male are the most prominent. We recorded his vocal signals using a hypercardioid microphone (Seinnheiser model ME-66 passed through a Sound Devices MP-1 preamplifier) placed within 20 cm of the male. A reel of twine was run in a straight line away from the focal male and chorus, in a direction that avoided nearby ponds with potentially interfering choruses. The twine was used to mark various distances from the focal male: 1 m, 4 m, 8 m, 16 m, 32 m, 50 m, and 100 m. At each distance we set up a single omnidirectional microphone (Audio Technica model AT899 with AT8537 power module) at two elevations (~6 cm and ~1 m above the substrate) to record the chorus sounds. Output from both the hypercardioid and omnidirectional microphones were fed to a digital sound recorder (Nagra ARES-BB; 16-bit mode, 44.1 kHz sampling rate).

2.2.4 LABORATORY PHONOTAXIS STUDIES

We conducted laboratory phonotaxis experiments in a temperature-controlled and anechoic chamber at 20 ± 1 °C. The chamber measured 15 m long, 3 m wide, and 2.5 m high, with 10 cm acoustic foam (Technifoam, Circle Pines MN) on all surfaces, backed by 10 cm of sound absorbing material. Prior to the start of the experiment,

females were removed from the ice and placed in plastic containers in an incubator (kept at 20 °C) to acclimate to the temperature of the chamber. Within the chamber was a circular arena 2 m in diameter and 40 cm in height, composed of wire mesh covered in black fabric (to eliminate the potential use of visual cues). The circumference was divided into thirty-six 10° arc segments. Acoustic stimuli were presented via a 3" loudspeaker (Aura Sound NS3-194-8E in a custom-made casing; ± 4 dB frequency response from 200 Hz to16 kHz) placed on the floor outside the arena wall. For synthetic stimuli and the 1 m chorus stimulus, the loudspeaker was placed directly outside the arena wall, 1 m from the center of the arena. For all remaining chorus stimuli the loudspeaker was placed an additional 0.5 m from the arena wall (1.5 m distant from the center of the arena) to reduce or eliminate possible near-field effects not experienced by frogs exposed to distant chorus sounds. The loudspeaker was placed at different arc segments which were changed at random during the experiments to eliminate possible directional biases of females.

From the chorus recordings we selected exemplars on the basis of the chorus density, as approximated by the SPL measurements (unpublished data). Exemplars were taken from the three nights showing the highest average SPL values (5/19/2006, 5/23/2006, and 5/24/2006). Stimuli were chosen from recordings made from the ground, as females travel along the ground en-route to the chorus (personal observation). From the recordings made on each of the three nights mentioned above, we selected a 20 s segment of chorus sound when the focal male called uninterruptedly and not masked by calls of the nearest neighbors. This was looped to create a chorus stimulus with a total duration of 5 min. Chorus stimuli were played back at SPL levels approximating the

levels in the field at their respective distances, including fluctuations in SPL between 5-12 dB (Table 2.1), which matched well to values for this species encountered in the field (Gerhardt 1975).

Playback stimuli comprised chorus sounds and 3 synthetic stimuli: a synthetic conspecific call ('Hv') and a band-limited noise ('Noise'). The synthetic stimuli were created using Adobe Audition and presented at an inter-stimulus interval of 3.125 s – a period within the range observed from recordings in the field (unpublished data). The synthetic Hv call was a positive control stimulus, as it has been shown to be a highly attractive stimulus in previous phonotactic studies (Gerhardt and Doherty 1988; Bush et al. 2002; Beckers and Schul 2004) – the call consisted of 18 sound pulses of 25 ms duration with an inter-pulse interval of 25 ms, resulting in a total call duration of 875 ms (Fig 2.1a). Each sound pulse in the synthetic call consisted of 2 phase-locked sinusoids of 1.1 and 2.2 kHz (the dominant spectral components of natural *H. versicolor* calls), with the latter 6 dB more intense than the former, and had a rise/fall time of 80% and 20% of the pulse duration, respectively. To control for responses to acoustic energy in general, a band-limited noise stimulus with a flat spectrum over 0.1 – 22 kHz was also presented (Fig. 2.1b). All synthetic stimuli were played back at 85 dB SPL, measured at the center of the arena, equivalent to the amplitude of a calling male at ~1 m distance (Gerhardt 1975).

The general experimental protocol followed that of Bush et al. (2002) and Beckers and Schul (2004). For each female, we randomly selected a chorus exemplar; all chorus stimuli presented to the female were from the same recording date. An *experimental* trial comprised a single chorus exemplar or noise; presentation of

synthetic conspecific calls constituted a *control* trial. At the start of a trial, females were placed inside a wire-mesh release cage (~4” diameter) in the center of the arena with a random facing. After 15 s of stimulus playback, the cage was remotely lifted. The animal's movements were monitored and recorded by a video camera (Fig. 2.5). We recorded three measures of responsiveness: (i) the time for the female to touch the arena wall regardless of the location on the wall, (ii) the time for the female to touch the wall in the immediate vicinity (within 15 cm) of the loudspeaker, (iii) the location (arc segment) on the arena wall which the female contacted. A score of 'No response' was recorded if the female remained in the release cage for 3-min for synthetic stimuli, or 5-min for chorus stimuli. If a female took longer than 5-min to reach the arena wall for synthetic stimuli, or 10-min with chorus stimuli, a score of 'No response' was also recorded.

Each female began an experimental session with three control stimuli, with the first two functioning to acclimate the female with handling, while data was only recorded for the third. If on any subsequent *control* trial the female took longer to reach the loudspeaker than twice this value, the data for the previous two experimental trials was discarded and the experimental session was terminated for that female. After each control trial we presented the female with two experimental trials (stimuli chosen at random), followed by another control trial, then two more experimental trials, and so on. In between experimental trials females were returned to the incubator for a minimum of 5 minutes to maintain constant body temperature.

2.2.5 STATISTICS

For the field studies, the time it took a female to exit the arena boundary was used to calculate a *phonotaxis score,* as described by Bush et al. (2002). This measure of signal attractiveness was the ratio of the mean of the response times for the control (10 m) stimuli, to the response of the experimental trial (25, 50 and 75/100 m) itself ($t_{control}/t_{experimental}$). A score of 1 indicates that the female took the same amount of time to exit in response to an experimental stimulus as to the control stimulus. A score < 1 indicates reduced attractiveness (longer time to exit), while a score > 1 suggests a greater attractiveness to the stimulus. Data were pooled over transect for each distance in the field and compared with Kruskal-Wallis tests and Bonferroni comparisons (α = 0.05, Zar 1999). All scores are expressed as the mean ± standard error of the mean (SEM) for each chorus distance.

Orientation data from females exiting the arenas were pooled for each transect and were used to calculate a mean angle (± standard deviation) of orientation for each arena distance, an associated r-statistic (the length of the mean orientation vector), and 95% confidence intervals of the mean. Randomness of the distributions was tested using both Rayleigh and V-tests with a specified target angle of 0°, corresponding to a direct line to the chorus (α = 0.05, Batschelet 1981).

For the laboratory phonotaxis experiments, phonotaxis scores were determined in a similar manner to that described above. The phonotaxis score was the ratio of the mean of the response times for the control stimuli before and after an experimental trial, to the response of the experimental trial itself ($t_{control}/t_{experimental}$, where $t_{control} = (t_{hv\text{-}pre} + t_{Hv\text{-}}$

$_{post}$) / 2. 'No response' trials were given a score of 0. All scores are expressed as the mean ± standard error of the mean (SEM) for each acoustic stimulus type.

To justify pooling scores for wall- and speaker-contacts for chorus stimuli over all exemplar dates, we ran multiple Kolmogorov-Smirnov (K-S) tests (α = 0.05) on the cumulative distribution functions of the power spectra of the chorus samples used in stimulus creation. For each chorus distance, the power spectra of each exemplar was calculated with sound analysis software (Audacity; FFT: Hanning window, 512 samples), and the K-S tests was run using MATLAB (The Mathworks, Inc.). The null hypothesis of equal distributions could not be falsified, so the data was pooled over the exemplar dates. Wall- and speaker-contact derived phonotaxis scores were compared with Kruskal-Wallis one-way analysis of variance tests with post-hoc Bonferroni comparisons (Zar 1999). In addition, means ± 25% and 75% confidence limits were examined to see if they overlapped the value of 1.0, which would suggest that female responsiveness is similar to that of the synthetic control stimuli.

The wall-contact data in the laboratory were pooled by stimulus type over all 3 exemplar dates. From this we calculated mean angles (± standard deviation), the associated r-statistic, and 95% confidence intervals of the mean for each experimental distance. As in the field studies, we tested randomness using both Rayleigh and V-tests with a specified target angle of 0° (α = 0.05, Batschelet 1981). As females were tested multiple times with the control synthetic stimulus, a mean angle (± S.D.) was calculated for each female to avoid pseudoreplication (Hurlbert 1984). We averaged each female's responses to the control stimuli. The arena arc segment corresponding to the mean angle was then used as contact data for that female.

2.3 RESULTS

2.3.1 CHORUS RECORDINGS

At 1 m, the calls of the focal male were readily distinguishable from those of neighboring males (Fig. 2.1c, inset). Away from the focal male, his calls became less intense and the individual pulses became less distinct, while the calls of nearby conspecific males approached and could even exceed the intensity of that of the focal male (Fig. 2.1d,e, insets). At longer distances (> 16 m) the amplitude modulation of individual frog calls merged into the chorus background, the overall intensity dropped, and other biotic noise with energy in higher-frequency bands became more noticeable (Fig. 2.1f, inset,1e-h).

2.3.2 ORIENTATION IN THE FIELD

Females showed directed orientation to the chorus at all tested distances from the chorus, up to 100 m (Table 2.1, Fig. 2.2). Arena exit locations were non-random for all tested distances in the field (*V*-test, $p \leq 0.01$). The exit locations, reflected in the angles and lengths of the mean vectors (Fig. 2.3a-d), were directed toward the pond – the orientation degraded only slightly with increasing distance to the chorus. Their phonotaxis scores at any one distance, as well as the raw arena exit time, were not significantly different from the scores or times at any other distance (Kruskal-Wallis, $F_{2, 50} = 3.18$; $p \leq 0.05$). The standard error intervals for all three distances overlapped the control value of 1.0, suggesting that the mean phonotaxis scores do not differ from the values for the 10 m control.

2.3.3 PHONOTAXIS IN THE LABORATORY

The percentage of females reaching the arena wall in the laboratory ranged widely, from a low of 38% in response to noise to a high of 96% in response to conspecific control stimulus (Table 2.2). Chorus signals elicited increasing activity with decreasing chorus distance.

Phonotaxis scores were low for the noise stimuli, with values of 0.14 ± 0.4 and 0.02 ± 0.01, for wall- and speaker-contact, respectively (Table 2.2, Fig. 2.4). For the chorus sounds, responses were generally robust at short chorus distances (1 – 8 m) and became progressively weaker with increasing distance (Table 2.2 Fig. 2.4). Both wall- and speaker-derived scores showed significant differences in the responses to the various stimuli (Kruskal-Wallis one-way ANOVA: $F_{8,252} = 1.98$; $p \leq 0.05$; post-hoc pairwise comparisons). Scores from the 1 - 16 m stimuli (wall: 0.54 - 0.43; speaker: 0.47 - 0.27) indicate the quickest wall- and speaker-contacts. With stimuli recorded at increasing distances, phonotaxis scores dropped rapidly, with wall- and speaker-contact scores of 0.26 - 0.25 and 0.12 - 0.10, respectively, from 32 – 100 m. The phonotaxis scores at the furthest distances (50 and 100 m) were not significantly different from responses to noise. Only two stimuli, the 1 m and 8 m chorus distances, encompassed the theoretical normalized control value of 1.0 within their 25% and 75% confidence intervals of the mean scores – suggesting that the other stimuli were significantly less attractive.

Females' phonotactic trajectories for the various stimuli are shown in Figure 2.5. When presented with attractive stimuli, females moved in short bouts interspersed with pauses; females often re-oriented their trajectories in-between movement bouts, giving

rise to 'zig-zag' movement patterns described previously (Feng et al. 1976). In response to the conspecific control stimulus, females rapidly and accurately localized the loudspeaker (Fig. 2.5a). Accurate localization was also seen for the 1 m and 8 m chorus stimuli (Fig. 2.5c, d). With increasing distance (Fig. 2.5e-f), however, wall contacts became more random and less accurate.

When presented with noise stimulus, many females moved a short distance and stopped (Fig. 2.5b). Those females that moved for longer distances tended to wander with random orientations, and more females who left the cage failed to touch the arena wall compared to the other stimuli (Fig. 2.5b, unpublished data).

Analysis of the wall-contact data (Fig. 2.6a-f) showed a similar trend to the phonotaxis score data. Orienting responses to the synthetic conspecific calls were significantly non-random (*V*-test, $p \leq 0.01$), while those to noise were essentially random. Responses to the chorus stimuli (Table 2.2, Fig. 2.6c-f) were non-random for distances from 1 to 32 m, whereas at 50 m and 100 m, responses were random. Mean vector lengths (*r*) were similar at the closest chorus distances, e.g., 0.8 at 1 m and 0.77 at 8 m (Table 2.2) and decreased with increasing distance (Table 2.2).

2.4 DISCUSSION

Close to the breeding pond, the calls of the nearest males are distinct with clear temporal structure. Recordings made at further distances show a rapid reduction in the amplitude of male calls as well as the masking and degradation of the temporal structure as formerly distinguishable calls 'blend' into the chorus background. Female *H. versicolor* orient towards choruses in the field at distances up to 100m, but in

phonotaxis tests in the laboratory females show directed orientation only to recordings made at a distance of up to 32m.

A comparison of the field and laboratory orientation data shows that there is less variation in females' orienting responses in the field with increasing distance, on the basis of the arena-exit times (Table 2.1, Fig. 2.1) and the distribution of the arena exit angles (Fig. 2.2). A number of factors likely contribute to the discrepancies between the field and laboratory data. The sources of the acoustic stimuli differ in the two experiments. In the laboratory, the chorus is broadcast from a single source – the playback loudspeaker, whereas the chorus sound in the field is a distributed source with origins over a large geographical area. In theory, a stimulus originating from a point source can be localized more easily, considering the directional nature of the anuran external ear (Feng and Shofner 1981) and the absence of competing sources and interfering signals (Jørgensen and Gerhardt 1991; Schwartz and Gerhardt 1995). In the laboratory, the fact that distant chorus stimuli did not reliably elicit orientation, despite playback from a point source, suggests that *recognition*, rather than *localization*, is likely the primary problem, and in particular the temporal structure of the species vocal signal at long distances is compromised (see Fig. 2.1) making it difficult to recognize.

Another significant difference between the laboratory and field experimental conditions is the sensory cue(s) available to the frogs. In the laboratory, females have only the acoustic cues for orientation, whereas in the field females may use olfactory, visual, and/or magnetic cues in addition to auditory cues. Previous studies have shown that anuran amphibians utilize any or all of the aforementioned sensory cues to localize and orient to a breeding pond when manually displaced from it (Sinsch 1990a; Sinsch

1990b; Murphy 2008). The use of other modalities does not preclude the use of distant (> 32 m) chorus sound for orientation, which may act as an adjunct 'unreliable' signal (sensu Candolin 2003) that might aid orientation when presented in concert with other sensory cues, but by itself is not sufficient to localize distant choruses.

The fact that females oriented towards choruses with more challenging auditory characteristics (i.e. multi-dimensional vs. point) with superior accuracy suggests the important role of non-auditory cues. Future studies of the females' acoustically guided orientation to the chorus in the field should carefully control for the other sensory modalities.

Our results demonstrate that the attractiveness of male's calls embedded in a chorus varied with distance in a laboratory setting. Female *H. versicolor* reliably localize artificial conspecific calls and approach them within a short time (e.g. Diekamp and Gerhardt 1995; Bush et al. 2002; Beckers and Schul 2004). This was reflected in the high percentage of females that reached the playback loudspeaker (Table 2.2), the nonrandom distribution of contact angles (Fig. 2.6a), the statistics of the mean vector of orientation (Table 2.2), and the narrow distribution of movement trajectories in the arena (Fig 2.5a). Responses to band-limited noise were poor. Only 38% of females contacted the arena wall and contact angles were randomly distributed, resulting in the lowest phonotaxis scores for any tested stimuli (Table 2.2, Figs 2.4, 2.6b). We believe that this represents a baseline response to acoustic noise in general and indicates a non-salient stimulus.

Female *H. versicolor* were attracted to playback of male's calls embedded in a chorus recorded at short distances (1 – 32 m), but beyond which signal attractiveness

was drastically reduced (Fig. 2.6c-e). Results from other anuran species have had more mixed results. A laboratory study in American toads (*Bufo americanus*) and sibling gray treefrogs (*Hyla chrysoscelis*) also showed a reduction in attraction and orientation to chorus sounds recorded from 20 m to 80 m (Swanson et al. 2007). Barking treefrogs (*Hyla gratiosa*) were also attracted to chorus stimuli recorded at distances of up to 40 m (Gerhardt and Klump 1988b). *Rana sylvatica* showed attraction only at shorter distances <15 m (Bee 2007). Evolutionary and ecological factors may have a role in determining if a species responds to distant acoustic stimuli (Gerhardt and Klump 1988b; Bee 2007). Together, these results indicate that attenuation of the amplitude and/or degradation of temporal features of male advertisement calls at longer distances must reach a level that negatively impacts the female's ability to detect, discriminate, or locate the call (see Lang 2000).

The gray treefrog's phonotaxis scores to chorus sounds were lower than the scores to call models of individual males tested in a similar experimental setup (Bush et al. 2002; Beckers and Schul 2004). The mean speaker-derived phonotaxis score for the chorus sound at 1 m (that most closely resembles an isolated single male call), at a playback level of 83-86 dB SPL, was 0.42; Beckers and Schul (2004) could obtain the same score with a synthetic call at a playback level of just 43 dB SPL. The difference in experimental results is likely attributed to the difference in the sound stimuli; previous studies used synthetic *H. versicolor* calls with high signal-to-noise ratios (Fig. 2.3a) as opposed to calls embedded in a chorus in the present study (Fig. 2.3c-h).

In the laboratory phonotaxis studies, the response at 4 m measured by phonotaxis score and orientation show a marked decrease from the 1 m and 8 m

responses (Table 2.2, Fig. 2.4). This trend was consistent across animals and exemplar dates (data not shown). We believe these responses can be explained by local topography. The majority of chorus recordings were made roughly in an area with a downward slope extending from 3-6 m from the pond edge, which led away from any potentially interfering choruses. At the 4 m recording position, the microphone is shielded from the chorus by the substrate. This would act to both attenuate and filter propagating calls, which would alter the attractiveness of the call (Richards and Wiley 1980; Penna and Solis 1998). At further distances the slope no longer occluded a direct sound path to the microphone, and alterations in calls and chorus sounds would be significantly less, as shown by the increase in attraction of the 8 m calls (Fig 2.4).

There is a caveat with the laboratory phonotaxis experiments. When females respond to focal male's call in chorus sounds recorded at 'intermediate' distances (~4 – 16 m, which are distances at which the focal male's call is perceptible over the background chorus, but at which there are some other males calling), we cannot determine the particular calls (e.g., those made by the 'focal' male, or near neighbors, or all) to which the females are responding. To assess the relative contribution of individual calls to female responses, future studies should incorporate more advanced sound recording techniques (e.g., an array-microphone recording technique, such as in Jones and Ratnam 2009) whereby the calls of individual callers that have the highest signal-to-noise ratios can be observed along with female responses in real time.

The current study is the first to investigate the change in signal attractiveness as one moves away from a single calling male in a chorus. With distance, both temporal and spectral signal properties of the call are increasingly affected by the ambience (Fig.

2.3), resulting from substrate-signal interactions, reflection, spectral absorbance, thermal effects and the presence of competing biotic and abiotic sounds (Richards and Wiley 1980; Dabelsteen et al. 1993; Forrest 1994; Gerhardt and Klump 1988a; Holland et al. 1998, Wollerman 1999; Wollerman and Wiley 2002*;* Luther and Wiley 2009). If we are to understand how females process acoustic signals in real world listening conditions, it is imperative that we employ stimuli which resemble those the females encounter in the field – we feel that the current study is a valuable step in this direction. With increasing distance, the individual pulses of calling males become less distinct and have lower signal-to-noise ratios due to temporal 'blurring' of the pulses resulting from the presence of echoes and reverberations, other calling males and biotic sounds, and turbulent noises (Feng and Schul 2007). Further studies are being undertaken to study the independent effects of changes in sound attenuation and temporal degradation of male calls on female attraction and phonotaxis. In addition, neurophysiological studies of how chorus sounds are processed in the female auditory midbrain are underway.

Results of the present study suggest a dichotomy of female's attraction to chorus stimuli from two ranges: i) 1-8 m range for which the attractiveness is essentially the same, ii) 16-32 m range for which the phonotaxis score decreases progressively from 0.47 to 0.1 (Table 2.2). It would be of interest to investigate whether neurons in the frog central auditory system also show a dichotomy of physiological responses to chorus sounds recorded from different ranges.

2.5 ACKNOWLEDGEMENTS

We thank the Missouri Dept. of Conservation and the University of Missouri-Columbia for allowing studies and capture permits. Carl Gerhardt generously provided advice and material support. Vincent Marshall, Noah Gordon, Oliver Beckers, and Sarah Bush provided valuable advice and comments on the present study. Sarah Humfeld and Alison Welch aided in coordinating field activities and in providing material support. Eric Rozier provided many helpful comments and assistance with the statistical analysis. We would also like to thank the University of Missouri-Columbia undergraduate students who greatly aided in both animal collection and experimental execution: Paul Kimball, Stephanie Kleyman, and Leah Reynolds. This research was supported by a National Science Foundation Collaborative Research in Computational Neuroscience (CRCNS-0422073) grant to ASF and a National Science Foundation grant (IOB-0445286) to JS. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois.

2.6 TABLES

Distance (m)	10	25	50	75/100
SPL at arena center (dB SPL)	68 - 73	60 - 65	54 - 58	47 – 59*
n	19	16	19	18
Mean Angle *μ* (deg.)	357	341	6	13
S.D. (deg.)	38	56	44	62
Mean Vector length *r*	0.78	0.51	0.7	0.42
Phonotaxis score (Arena exit)	N/A	1.25	1.16	1.06
S.E.	N/A	0.79	0.89	0.66

Table 2.1 Data for the field phonotaxis experiment. Data taken from Figs. 2.5 and 2.6. The mean orientation angles are shown with the SD of the mean and associated mean vector length *r*, and phonotaxis scores are shown with SE of the mean. * - Greater variation due to different arena distances due to terrain

Stimulus	Hv	Noise	1m	4m	8m	16m	32m	50m	100m
Speaker Distance from arena center (m)	1.0	1.0	1.0	1.5	1.5	1.5	1.5	1.5	1.5
SPL at arena center (dB SPL)	85	85	83-86*	77-82*	72-78*	70-75*	65-69*	59-64*	47-53*
n	34	34	34	34	34	34	34	34	34
% Response (Wall touch)	96	38	85	76	85	76	65	71	62
Mean Angle *μ* (deg.)	356	147	352	31	4	31	16	102	30
S.D. (deg.)	7	64	36	61	39	52	61	68	70
Mean Vector length *r*	0.99	0.38	0.8	0.42	0.77	0.59	0.44	0.29	0.26
Phonotaxis score (Wall contact)	N/A	0.14	0.53	0.43	0.54	0.32	0.25	0.26	0.25
S.E.	N/A	0.04	0.07	0.07	0.06	0.05	0.06	0.04	0.05
Phonotaxis score (Speaker contact)	N/A	0.02	0.42	0.27	0.47	0.2	0.1	0.09	0.12
S.E.	N/A	0.01	0.07	0.08	0.07	0.04	0.03	0.02	0.03

Table 2.2 Parameters and data for the laboratory phonotaxis experiment. Data taken from Figs. 2 and 4. Percent response indicates the percentage of females contacting the arena wall during the presentation of the specified acoustic stimulus. Phonotaxis scores are shown using both wall contact and speaker contact (see Methods). * -

2.7 FIGURES

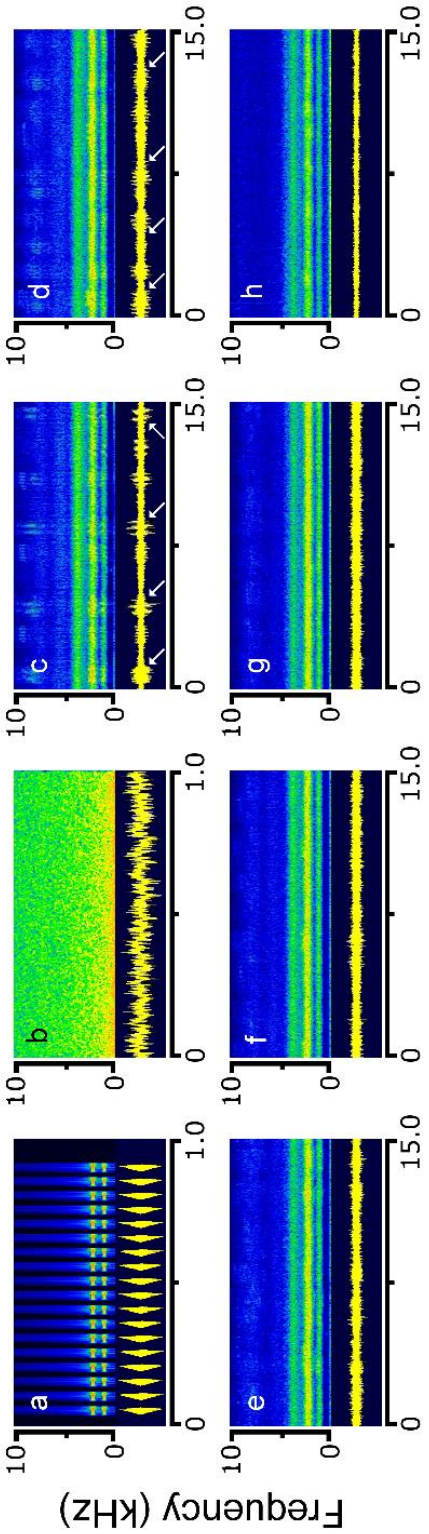

Fig. 2.1 Oscillograms (bottom) and spectrograms (top) of brief (400 ms) segments of control and chorus stimuli used in the laboratory phonotaxis experiments, taken at the middle of the focal male's call. (a) Synthetic Hv call, (b) Band-limited noise, (c) 1 m chorus, (d) 4 m chorus, (e) 8 m chorus, (f) 16 m chorus, (g) 32 m chorus, (h) 50 m chorus. Insets on c-f show oscillograms of chorus recordings (15 s duration) of chorus recordings during which the focal male called repeatedly. Arrows indicate calls emitted by the focal male

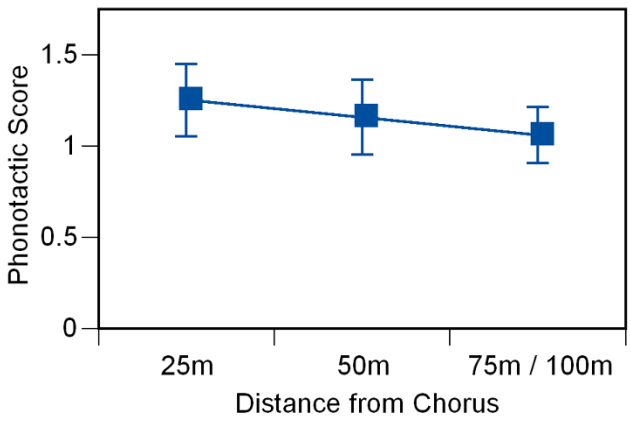

Fig. 2.2 Phonotaxis scores (mean ± SEM) from orienting responses to the chorus in the field; data were pooled from all transects. Scores were calculated as the ratio of the time to exit the arena at the 10 m distance to the time to exit the arena at the 25 m, 50 m, or 75/100 m distances. The 10 m scores are used as a reference

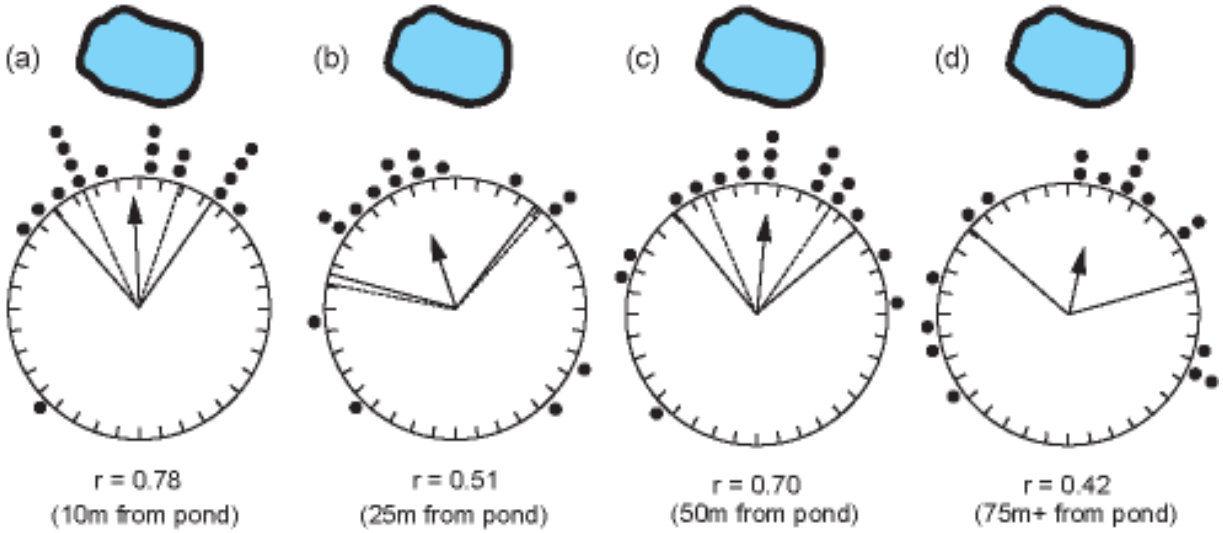

Fig. 2.3 Circular histograms indicating responses and mean directional vectors of females to choruses in the field, when released at (a) 10 m, (b) 20 m, (c) 50 m, (d) 75-100 m from the pond. Each dot represents a female exiting the arena between the flags staked at 10° arc segments, relative to a vector oriented directly at the chorus. All orientation distributions significantly differ from a random distribution (V-test, $p \leq 0.01$, Batschelet 1981)

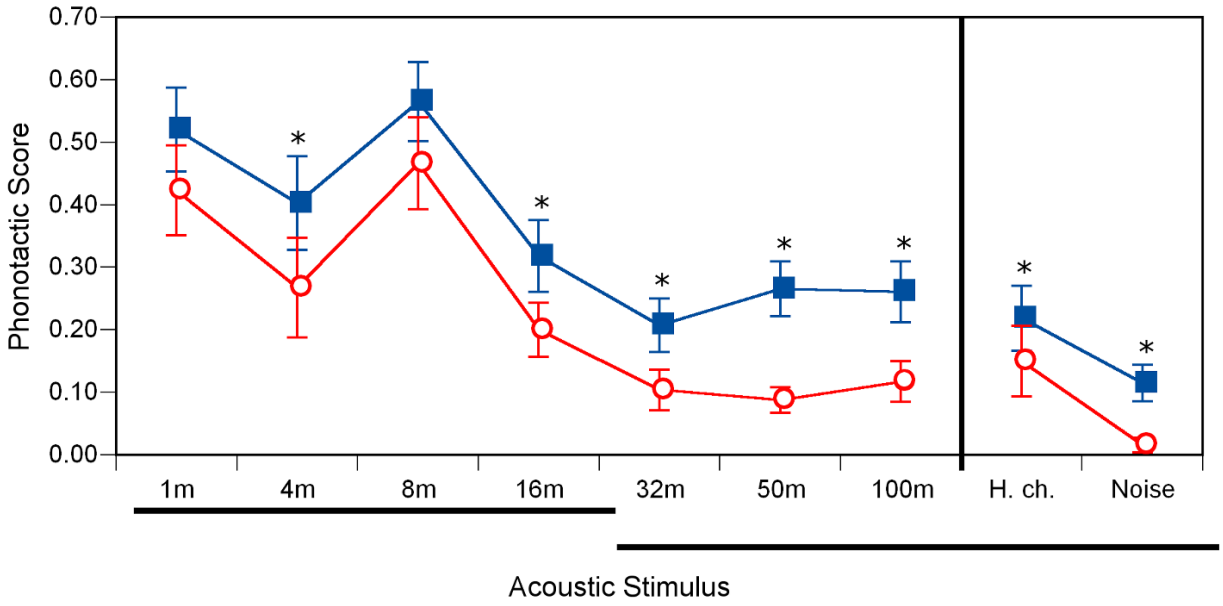

Fig. 2.4 Phonotaxis scores (mean ± SEM) for control and experimental acoustic stimuli from pooled data for all chorus exemplars. The circle and square symbols represent phonotaxis scores calculated using the time to reach the arena wall (irrespective of the location on the wall) and the time to reach the loudspeaker (within 10 cm), respectively. Horizontal bars indicate Bonferroni multiple comparison grouping ($p \leq 0.05$) after Kruskal-Wallis one-way ANOVA test. Speaker-derived scores whose 25% and 75% confidence intervals for the mean which did not include 1.0 (corresponding to the positive control stimuli) are denoted by asterisks (*)

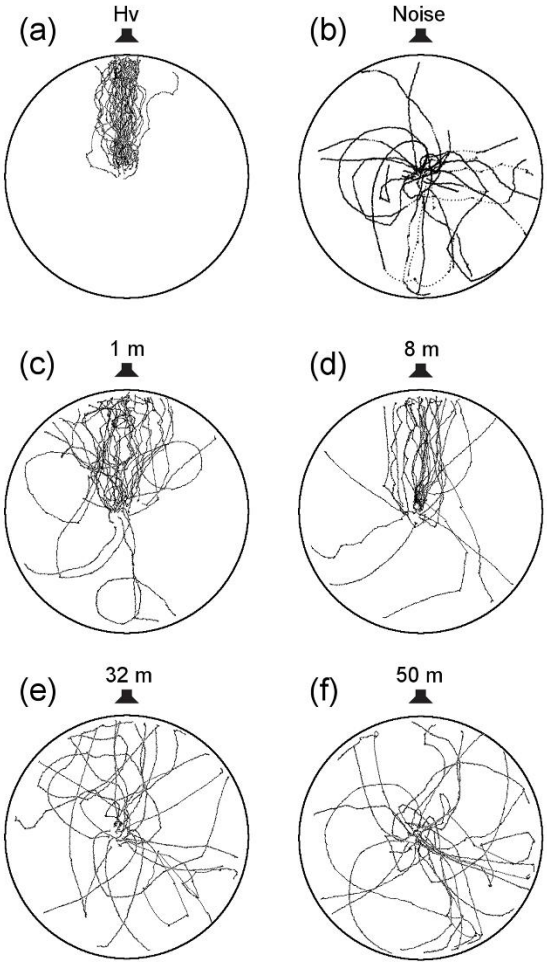

Fig. 2.5 Phonotaxis trajectories of female treefrogs for (a) synthetic Hv call (*n* = 35), (b) band-limited noise (*n* = *25*), (c) 1m chorus (*n* =31), (d) 8m chorus (*n* = 30), (e) 32m chorus (*n* = 23), (f) 50m chorus (*n* = 25). All traces have been aligned with respect to the acoustic stimulus presented from the top of each graph

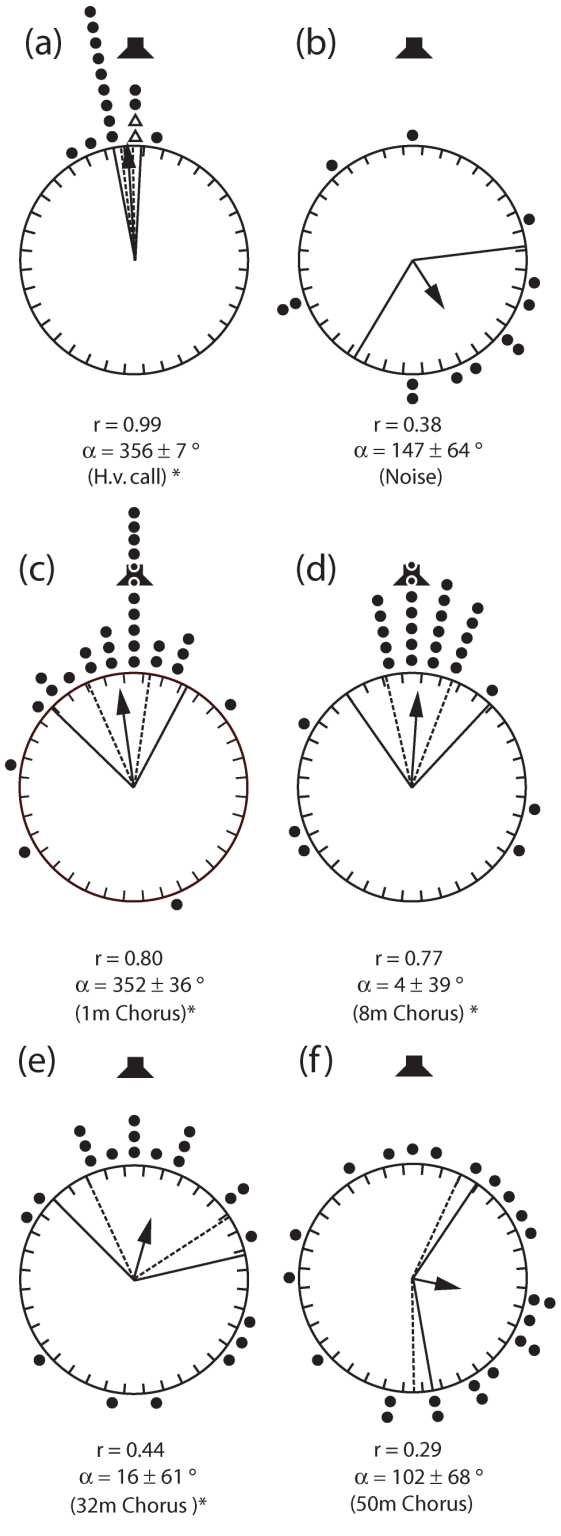

Fig. 2.6 Circular histograms showing the distributions of the locations of arena wall contacted by females (oriented with the direction of the stimulus at the top of each figure). Shown are results of phonotactic responses to (a) synthetic Hv call, (b) band-limited noise, (c) 1 m chorus, (d) 8 m chorus, (e) 32 m chorus, (f) 50 m chorus. Each dot represents a single wall contact in the corresponding 10° arena wall segment. Each open triangle represents 10 contacts. Arrows represent the mean directions of all contacts, with the length corresponding to the r statistic (shown below each histogram). Solid and dotted lines represent the S.D. and 95% confidence interval, respectively, for the mean angle. Asterisks denote contact distributions that significantly differ from random (V-test, $p \leq 0.01$, Batschelet 1981)

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CHAPTER 3: DIFFERENTIAL EFFECTS OF SOUND LEVEL AND TEMPORAL STRUCTURE OF CALLS ON PHONOTAXIS BY FEMALE GRAY TREEFROGS, *HYLA VERSICOLOR*[2]

3.1 INTRODUCTION

During the reproductive season, male gray treefrogs (*Hyla versicolor*) call nightly from choruses to attract females. Similar to other anuran species, the spectral and temporal structure of calls are essential for species recognition and mate selection (Narins et al. 2007). Male *H. versicolor* calls are trill-like and various behavioral studies based on female preference have shown that the duration and rise/fall times of each 'sound pulse', as well as pulse rates, numbers, and call durations represent salient features important for species recognition and call discrimination (Gerhardt and Doherty 1988; Diekamp and Gerhardt 1995; Gerhardt and Schul 1999; Schul and Bush 2002; Schwartz et al. 2010; Ward et al. 2013a,b; Schrode and Bee 2015). Most female preference studies were performed with 'clean' synthetic acoustic stimuli having high signal-to-noise ratios (SNRs), which may not accurately represent realistic signals females normally encounter in the field. Male choruses comprise the vocalizations of dozens to hundreds of calling males, distributed in space and time, making them a challenging auditory environment (Feng and Schul 2007; Luther and Gentry 2013; Vélez et al. 2013; Bee 2015). The overlapping spectral and temporal features of male calls

[2] This chapter is published in its entirety in the Journal of Comparative Physiology A. This article is reprinted with the permission of the publisher and is available from https://link.springer.com/article/10.1007/s00359-019-01325-5 and using DOI: https://doi.org/10.1007/s00359-019-01325-5

make detection, localization, and discrimination difficult tasks, analogous to the 'Cocktail Party Problem' (Bee and Micheyl 2008; Bee 2015; Lee et al. 2017; Wiley 2017).

As signals propagate in the forest environment, they are attenuated by absorption in the atmosphere and vegetation, and the fine temporal structures of sound pulses are degraded due to reverberations and interactions with the substrate and foliage (Richards and Wiley 1980; Brown and Handford 2000; Naguib 2003; Kuczynski et al. 2010; Lee et al. 2017). Various studies (Ryan and Sullivan 1989; Kime et al. 2000), including some in *H. versicolor* (Schwartz et al. 2001; Marshall et al. 2006; Schwartz and Marshall 2006), suggest that female selectivity on call traits is limited by these factors.

For several anuran species, females orient to chorus noise with degraded amplitude and temporal structures (Gerhardt and Klump 1988; Swanson et al. 2007; Christie et al. 2010, Kuczynski et al. 2010). We previously reported that female *H. versicolor* display phonotaxis in the laboratory to chorus sound recorded at 1 - 32 m (65-86 dB sound pressure level [SPL]) from a breeding pond, but not to choruses recorded beyond 32 m, which have a sound level of 47-64 dB SPL (Christie et al. 2010). As an increase in distance from a chorus reduces not only the sound level but also degrades the temporal features of a male's call (Chapter 2, Fig. 2.1b-e or Christie et al. 2010, Fig. 1b-e), it remains to be determined the relative contributions of these two factors to female phonotaxis, and whether they exert their effects independently of one another.

Female anurans are quite sensitive to sound amplitude, with just-meaningful-differences (JMDs) estimated at 2-4 dB SPLs in multiple species, including the sibling species *H. chrysoscelis* (reviewed in Fay and Simmons 1999; Bee et al. 2012).

Changes in signal amplitude also affect preferences for call temporal properties (Gerhardt and Schul 1999; Beckers and Schul 2004; Gerhardt 2008), and gradual changes in amplitude have a significant effect on the attractiveness of male *H. versicolor* calls (Beckers and Schul 2004). When given the choice between stimuli differing in SPL, female anurans prefer calls with higher intensity, presumably with higher stimulation of auditory receptors (reviewed in Ryan and Keddy-Hector 1992).

We investigated the relative influence of sound level and temporal fine structure by presenting female treefrogs with recorded calls made at increasing distances from an individual male embedded in a chorus at 85, 70, and 55 dB SPL. These intensities are consistent with recordings made at short (1 - 4 m), intermediate (16 – 32 m), and long (50 – 100 m) distances from breeding choruses, respectively (Christie et al. 2010). Both stimulus attractiveness and accuracy of sound localization were examined using a single-speaker no-choice paradigm (Busch et al. 2002, Beckers and Schul 2004, Christie et al. 2010). We hypothesized that 1) Female treefrogs show increased attraction and greater localization acuity towards a stimulus played at higher sound amplitudes, and 2) This improvement in attraction and orientation should be greater for signals recorded at closer distances to the calling male, as they retain more of the important temporal fine structure of the male calls.

Our study therefore differs from previous studies in *H. versicolor* and *H. chrysoscelis*, which employed synthetic calls and ‘chorus-shaped’ noise stimuli (Bee and Schwartz 2009; Kuczynski et al. 2010; Vélez and Bee 2010; Schwartz et al. 2013). While the use of synthetic stimuli allows for finer and more independent manipulation of call and chorus noise features, natural chorus recordings are a useful approximation of

the problem of call amplitude attenuation and temporal degradation in nature. While *H. chrysoscelis* females show weak to no attraction to 'chorus-shaped' noise (Swanson et al. 2007, Vélez and Bee 2010, Vélez and Bee 2011), they do show significant phonotaxis to playbacks of naturally recorded choruses (Swanson et al. 2007, Christie et al. 2010). This study augments previous work, using natural chorus noise to examine the effects of changing male call intensity and temporal structure on *H versicolor* female phonotaxis.

3.2 MATERIALS AND METHODS

3.2.1 SUBJECTS AND COLLECTION/STUDY SITES

Experimental subjects were female gray treefrogs (*Hyla versicolor*) of the northwestern mitochondrial lineage (Ptacek et al. 1994). Animals were collected from breeding choruses at the Thomas Baskett Wildlife Area and Three Creeks State Park, near Ashland Missouri. Females in amplexus were caught by hand and kept in plastic containers on ice overnight to prevent oviposition. All subjects (n = 94) were tested and released to their native ponds on the day following capture.

3.2.2 CHORUS RECORDINGS AND PHONOTAXIS STIMULUS PREPARATION

For detailed descriptions of chorus recordings and apparatus, see Christie et al. (2010). Briefly, we recorded choruses during their peak period (~22:00 - ~01:00). Before and after the recording session, we measured the chorus SPL with a digital SPL meter (Bruel & Kjær 2239, fast RMS, C-weighted, re: 20 µPa) at four different positions around the breeding pond. We took three measures at each position in order to get a rough

estimate of chorus intensity. Temperatures during recordings ranged from 19.4 – 25.6 °C, at 1 m above the forest substrate.

For each chorus recording session, a single 'focal' calling male *H. versicolor* was chosen at the pond's edge – each male was calling at a height >20 cm from the substrate and >1 m from the nearest conspecific male. Two microphones were used: 1) a hypercardioid microphone (Sennheiser model ME-66 passed through a Sound Devices MP-1 preamplifier) which was placed within 20 cm of the focal male and used to record his calls - termed the ‘focal’ microphone, 2) an omnidirectional microphone (Audio Technica model AT899 with AT8537 power module) which was used to record the chorus from various distances – termed the ‘distant’ microphone. We ran a twine reel in a straight line away from the chorus, and recorded the chorus at marked distances from the original male: 1, 4, 8, 16, 32, 50, and 100 m. At each distance the chorus was recorded at two elevations, ~6 cm and ~1 m above the forest substrate. All sounds from both microphones were recorded simultaneously by a digital sound recorder (Nagra ARES-BB; 16-bit mode, 44.1 kHz sampling rate) in stereo - twelve recording sessions were performed in total. Recordings for analysis and phonotaxis stimuli were created from selected exemplars of the chorus recordings made by the omnidirectional ‘distant’ microphone at ground level, to account for signal/substrate interactions that would affect the sounds heard by females traveling on the forest floor towards the pond (personal observation). Each of the exemplars was recorded on one of three nights (5/19/2006, 5/23/2006, and 5/24/2006) which had the highest chorus SPL values, and a stimulus – a 20 s segment of recording during which the focal male made uninterrupted calls - was created for each microphone distance.

3.2.3 CHORUS STIMULUS PREPARATION

The 20 s segments of recorded chorus were looped to create phonotaxis stimuli with a duration of 5 minutes, each of which was played continuously to female treefrogs during the experiment. Stimuli were broadcast at one of three sound levels (as measured at the center of the phonotaxis arena with a digital SPL meter): 85, 70, or 55 dB SPL.

In addition to the chorus sounds, females heard a synthetic conspecific call ('Hv'). This artificial stimulus was created using sound editing software (Adobe Audition) and had a playback interval of 3.125 s - similar to focal males' calling rate in the chorus (Supplementary Table 3.1). We used the Hv call as a positive control throughout the experiment to monitor females' behavioral motivation, as well as an experimental stimulus whose SPL was varied between females, to compare female behavior with chorus stimuli at the same SPL. The characteristics of the Hv call were comparable to those in previous studies (Gerhardt and Doherty 1988; Bush et al. 2002; Beckers and Schul 2004) and to call features of the 1 m recordings of the focal males used in phonotaxis stimulus generation (Supplementary Table 3.1; calls analyzed using Adobe Audition). Each call had 18 pulses, each 25 ms long, with an inter-pulse time of 25 ms, for a total duration of 875 ms (see Chapter 2, Fig 2.1a or Christie et al. 2010, Fig. 1a). Pulse properties were chosen as average values observed in the populations of the study area (Gerhardt and Doherty 1988). Each pulse consisted of 2 phase-locked sinusoids of 1.1 and 2.2 kHz, with the former -6 dB relative to the latter (Supplementary Fig. 3.1). The pulses had rise- and fall-times of 80% and 20% of the total pulse duration,

respectively. Hv stimuli used as controls were played at 85 dB SPL, while experimental Hv stimuli were played back at one of the above three SPLs.

3.2.4 CHORUS STIMULUS ANALYSIS

Individual calls from the 'focal' microphone were extracted from each 20 s chorus recording (*n* = 77). The simultaneous 'distant' microphone calls were temporally aligned with the 'focal' calls using the recording distance and speed of sound (343 m/s at 20 °C). Spectro-temporal analysis involved calculation of frequency spectra and spectrograms using the short-time Fourier Transform in MATLAB (The Mathworks, Inc; Temporal resolution = 20 ms; Frequency resolution = 172 Hz) as well as the frequency band with the most spectral power – termed the 'best frequency' (BF). The distributions of BFs with regard to exemplar date and recording distance were calculated in Prism (GraphPad Software Inc., 2013) and compared using one-way analysis of variance (ANOVA; $\alpha = 0.05$). We also calculated and averaged the number of pulses per call (P/C), call duration (CD), and inter-call interval (ICI) of each 'focal' call.

To quantify temporal degradation, we calculated a vector 'slice' from each call's 'focal'/'distant' spectrograms – the mean spectral power at the BF $\pm$ 500 Hz (see Supplementary Fig. 3.5b1-2). Calls recorded at closer distances to the focal male generally had lower degradation and a clear pulsatile structure, with lower spectral energy between successive call pulses (see Supplementary Figs. 3.5b1-2 and 3.5f1-2 for 1 m calls recorded by both 'focal' and 'distant' microphones). Calls recorded farther from the focal male showed more spectral energy between individual call pulses due to temporal 'smearing' and interference from other calling males, resulting in a loss of

pulse structure (see Supplementary Figs. 3.11b2 and 3.11f2 for 100 m calls recorded by the ‘distant’ microphone). The maximum cross-covariance between the ‘focal’ and ‘distant’ microphone vectors was calculated and averaged for each recording distance (MATLAB). Differences between median maximum covariances were compared with Kruskal-Wallis tests Dunn’s post-hoc tests (Prism; $\alpha = 0.05$). To validate this method, we devised a negative control – calculation of cross-covariance between signals with clear temporal structure – all 1 m ‘focal’ call recordings - and randomly generated white noise bursts which lack such structure, creating a low covariance ‘baseline’. For each ‘focal’ call, 100 random white noise bursts (1 s duration) were generated using MATLAB, and spectrogram ‘slice’ vectors calculated as described above. The cross-covariance between the noise and call signals was calculated and averaged. A grand median of noise-call covariance was determined for all ‘focal’ calls. Median peak covariance at each distance was compared to this value using the one-sample Wilcoxon signed-rank test ($\alpha = 0.05$).

All stimuli – synthetic and chorus stimuli (including 20 s recordings as well as time-aligned individual calls) are provided as .wav files (see Appendix A, Supplementary Files).

3.2.5 LABORATORY PHONOTAXIS

All phonotaxis experiments took place in an anechoic chamber at a temperature of 20 ± 1°C. The circular phonotaxis arena was 2 m in diameter and surrounded by a black-cloth covered wire mesh fence 40 cm in height, to prevent use of visual cues. The arena's circumference was divided into thirty-six 10° arc segments. Acoustic stimuli were presented via a loudspeaker (Aura Sound NS3-194-8E in a custom-made casing;

3” diameter, ± 4 dB frequency response from 0.2 kHz to16 kHz) placed on the floor outside of the arena wall. For stimuli played at 85 dB SPL, the loudspeaker was placed 1 m from the arena center, just outside of the arena wall. For chorus stimuli presented at 70 and 55 dB SPL, the loudspeaker was moved out to a distance of 0.5 m from the arena wall (1.5 m total distance from center) to reduce the effects of the acoustic near-field which would not be encountered by a frog responding to distant chorus sounds in the field. We moved the loudspeaker to different arc-segment positions throughout the course of an experiment to eliminate possible memory effects or directional biases by females. All female movements were monitored via video camera under IR-illumination and recorded on videotape for later analysis.

The experimental protocol was similar to that used in previous studies (Bush et al. 2002; Beckers and Schul 2004; Christie et al. 2010). Before each experiment, subjects were removed from ice and allowed to warm in plastic containers in an incubator at 20 °C. Each female was randomly assigned one of the three chorus exemplar dates and a stimulus SPL. All chorus stimuli presented to a female were from the same recording date/session. All stimuli (chorus or synthetic) – barring the control conspecific Hv call – were played at the chosen SPL (85, 70, or 55 dB SPL). An *experimental* trial consisted of a single chorus stimulus recorded at one distance or the synthetic conspecific call played back at the female's assigned SPL. A *control* trial was the synthetic call played back at 85 dB SPL.

For the experimental protocol, we initially tested each female with 2 control trials to acclimate them to handling and the testing procedure, and these data were not recorded. The female was then tested in a third control trial, whose data were used.

After a control trial, we tested the female with three experimental trials (with randomly chosen stimuli), then a control trial, then three experimental trials, and so on. If on any subsequent control trial, the female took more than twice the time to reach the arc segment corresponding to the loudspeaker than it took on the first recorded control trial, we ended the experiment for that female, and discarded the three previous experimental trial data.

At the start of each trial the female was removed from the incubator and placed in a ~10 cm diameter wire-mesh release cage in the center of the arena, with a random facing. The acoustic stimulus was played for 15 s, then the cage was lifted remotely. We recorded the time for the female to reach the arc segment corresponding to the loudspeaker, and the arc segment where the female first contacted the arena wall. If the female stayed in the release cage for 3 minutes during synthetic stimuli or 5 minutes for chorus stimuli, we assigned 'No response'. We also gave a score of 'No response' if the female took longer than 5 minutes or 10 minutes to reach the arena wall for synthetic and chorus stimuli, respectively. After each trial the female was placed back into the incubator for at least 5 minutes to reduce memory effects and maintain constant body temperature.

3.2.6 PHONOTAXIS STATISTICS

We calculated 'phonotaxis scores' (Bush et al. 2002, Christie et al. 2010) using the time it took the female to reach the loudspeaker location. Each score was the ratio of the mean of the response times for the *control* trials before and after each experimental trial, to the response time for a given *experimental* trial. A score of >1 indicated that the female took less time to reach the speaker in response to an

experimental stimulus compared to the control, and vice versa for a score of <1. 'No response' experimental trials were assigned a score of 0. Scores are given as the mean and median ±95% confidence intervals for each acoustic stimulus type/SPL combination (Supplementary Table 3.2, Fig. 3.1). For analysis, we used a between-subjects design, comparing scores for the same distances across SPLs. To justify pooling scores for a chorus stimulus type/SPL combination over all exemplar nights, we compared the cumulative distribution functions of the power spectra (FFT, Hanning window, 512 points) of the chorus samples used in stimulus construction using Kolmogorov-Smirnov (K-S) tests in MATLAB ($\alpha = 0.05$). We could not falsify the null hypothesis of equal distributions and continued the analysis pooling wall-contact and speaker-contact data for each stimulus type/SPL pair over all 3 exemplar nights. Phonotaxis score medians were tested and compared with Kruskal-Wallis tests and post-hoc comparisons with Dunn's test ($\alpha = 0.05$). We also compared the apparently linear relationship of phonotactic response/playback SPL seen in the synthetic call and those to natural stimuli with linear regression analysis. Changes in phonotaxis score moving from a distant to proximal stimulus distance were also calculated over all females for each playback SPL, and medians compared with Kruskal-Wallis tests and post-hoc comparisons with Dunn's test ($\alpha = 0.05$) and tested as significantly differing from 0 using Wilcoxon's signed rank test ($\alpha = 0.05$). All tests were performed using GraphPad Prism.

We pooled arena wall-contact data for each stimulus/SPL set as described above and calculated a mean angle (± standard deviation), the associated mean vector length (*r*), and 95% confidence intervals of the mean. We tested the randomness of the wall-

contact distributions using V-tests with a specified target angle of 0°, corresponding to the arc segment containing the loudspeaker (α = 0.05, Batschelet 1981).

3.3 RESULTS

3.3.1 CHORUS SPECTRO-TEMPORAL PROPERTIES

Excerpts of recorded chorus spectrograms (Fig. 3.1b-e; Supplementary Fig. 3.3) and averaged individual call spectra (Supplementary Fig. 3.2) showed a generally uniform distribution of acoustic energy extending past 10 kHz, with bands of increased power roughly centered around 1.1 and 2.2 kHz, with the latter more intense - characteristics typical of *H. versicolor* calls recorded in the field and used in behavioral experiments (Gayou 1984; Gerhardt and Doherty 1988; Gerhardt 1991). Spectra from both recording microphones were generally similar, especially at the 1 m recording distance (Supplementary Fig. 3.2a1-2). 'Focal' call BFs ranged from 1983 to 2371 Hz, with a grand average across exemplars of 2181 ± 28 Hz (Fig. 3.2a; Supplementary Table 3.1), which corresponds to the dominant second harmonic peak in the *H. versicolor* call. Comparing BFs between the three exemplar nights and both 'focal' and 'distant' microphones reveals some differences. Call BFs from the 'distant' microphone were higher than those from the 'focal' microphone, and this trend was consistent for all 3 nights. Regardless of recording source, calls from 5/19 had lower BFs – especially from the 'focal' microphone, and 'distant' calls showed increased BF variability. Nevertheless, the distribution of BFs for the 'focal' and 'distant' microphones were both within limits seen in natural *H. versicolor* choruses recorded at multiple times and geographic locations, as well as those which show female attraction, and have been used in

behavioral choice experiments (Gayou 1984; Gerhardt and Doherty 1988; Gerhardt 1991; Gerhardt et al. 1996; Gerhardt et al. 2000).

With increasing distance from the focal male, call spectra remained quite stable (Fig. 3.2b; Supplementary Fig. 3.2), with the main effects being an increase in frequency power below ~500 Hz (compare Supplementary Figs. 3.2a2 and 3.2d2) and a reduction in variance at frequencies >5 kHz. Peak power and low variability about the 1st and 2nd call harmonics are maintained at all distances. While average call BFs showed a slight (~78 Hz) increase beyond 4 m (Fig. 3.2b) which remained somewhat steady out to the 100 m, there were no significant differences between the focal and distant recordings at each recording distance. While BF variance was reduced in the 'distant' microphone recordings, both the mean and range of BFs remained similar to those reported previously at all recording distances. In distant stimuli spectrograms (Supplementary Fig. 3.3) a dispersion of power in the band centered about the call 1st harmonic (black triangles, ~1.1 kHz) was noticeable as the distance increased from 1 – 16 m, especially when compared with the 2nd harmonic (white triangles, ~2.2 kHz). This effect was more evident at the individual call pulse level (Supplementary Fig. 3.4). Note that even as pulse amplitudes are highly attenuated with distance, 2nd harmonic power remained less affected.

Call temporal properties were also examined: the number of pulses per call (PPC), call duration (CD), and inter-call-interval (ICI). Similar to call BFs, these parameters (Supplementary Table 3.1) are well within the range calculated from natural *H. versicolor* choruses (Gayou 1984; Gerhardt et al. 1996; Gerhardt et al. 2000). Mean PPC, and call duration were similar across exemplar nights, with grand means of 20.58

± 0.63 pulses and 939 ± 34.3 ms, respectively. Average inter-call-interval between the three exemplar males differed by ~400-800 ms, with a grand mean of 3624 ± 281 ms. The means and range of all three parameters fell within those determined from males in the field recorded at similar temperatures (Gayou 1984; Gerhardt et al. 1996).

Unlike the frequency spectra, the temporal structure of the call stimuli changed with recording distance (Fig. 3.2c, Supplementary Figs. 3.3, 3.5-3.11). From 4 – 100 m, inter-pulse 'gaps' were gradually filled-in with acoustic energy, visible both in oscillo- and spectrograms as a loss of distinct pulse structure (Supplementary Figs. 3.5a, b-3.11a, b). This is noticeable even at comparatively short distances (Supplementary Fig. 3.3b, c).

To quantify temporal degradation, we took a vector 'slice' through each call's spectrogram, centered at the BF (see Methods for details). Vectors from focal and relatively close distant calls (1 – 4 m, Supplementary Figs. 3.5c-3.6c) retained a clear quasi-sinusoidal structure, which was strongly attenuated at increasing distances (8 – 100 m, Supplementary Figs. 3.7c-3.11c), as acoustic energy increases between peaks. Using the maximum peak of the cross-correlation between simultaneous focal and distant call recordings as a measure of a call's pulse structure, a clear degradation was seen the farther a male's call propagates in the chorus environment (Fig. 3.2c, Supplementary Table 3.2). Increasing the recording distance from a calling male from 1 to 4 m resulted in a 59% reduction in peak cross-covariance. Moving from 4 m to 50 m resulted in diminishing decreases at each distance, ranging from 37-18% reductions. Stimuli recorded at 100 m showed a slight 26% increase in peak covariance. Median

maximum covariance at the 1 m distance was significantly larger than those from 16 – 32 m, while 4 m values were larger than at 32 and 50 m (Kruskal-Wallis one-way ANOVA and Dunn's post-hoc pair-wise comparisons; $H_{7,77} = 53.53$, $p < 0.0001$). Regardless of recording distance, median peak covariances were significantly larger than our negative minimally-covariant controls (one-sample Wilcoxon's signed-rank test, $p < 0.05$).

3.3.2 PHONOTAXIS SCORES

Results of phonotaxis experiments are shown in Figures 3.3 and 3.4, and Supplementary tables 3.3 and 3.4. For the experimental Hv calls, phonotaxis scores at 85 dB SPL were significantly higher than the scores at 70 and 55 dB SPL (Kruskal-Wallis one-way ANOVA and Dunn's post-hoc pair-wise comparisons; $H_{3,93} = 24.45$ $p < 0.0001$). Reducing the SPL resulted in a linear reduction in signal attractiveness (Fig. 3.3a, Linear-regression on mean scores; $r^2 = 0.2$, $p < 0.0001$). Even at the lowest sound level tested, females still oriented and approached the speaker rapidly, with a median score of 0.42 (mean 0.5) - a value greater than all chorus sounds except for the 1 m stimulus (Fig. 3.3b, Supplementary Table 3.3). Scores for the closest natural chorus stimulus did not show a linear increase with sound level ($r^2 < 0.0001$, $p = 0.99$), and although both median and mean scores showed a slight peak at 70 dB SPL, there were no significant differences between responses at any SPL ($H_{3,89} = 5.38$ $p = 0.07$).

Similar to the 1 m chorus stimulus and unlike the experimental Hv call, phonotactic responses to the other chorus stimuli were not linearly related to the sound level (all $r^2 \leq 0.03$, $p \geq 0.11$). Mean and median responses for 4 - 16 m had a slight inverted 'V' shape (Fig 3.4, Supplementary Tables 3.3, 3.4). Phonotaxis scores for

chorus stimuli presented at 70 dB SPL were significantly greater than those for at 55 dB SPL for the 4 m (Fig. 3.4a; $H_{3,90}$ = 6.67 p = 0.04), 8 m (Fig. 3.4b; $H_{3,91}$ = 7.03 p = 0.03), and 16 m (Fig 3.3c; $H_{3,89}$ = 12.11 p < 0.01) distances. Phonotaxis scores for the 4 m stimuli were less than those for the 1 m and 8 m stimuli at equivalent SPLs, although these differences were not significant. Overall, the responses to the most distant (32 - 100 m) chorus stimuli (Fig. 3.4d-f, Supplementary Table 3.3) were similar to, but slightly less than responses to the proximal chorus sounds (Fig. 3.4a-c, Supplementary Table 3.3, 3.4). Consistent with previous results (Chapter 2, or Christie et al. 2010), the overall attraction to distal stimuli was weaker. Phonotaxis scores were generally low, with no median or mean score exceeding 0.3, and there were no significant differences between any of the stimuli (32 m: $H_{3,89}$ = 1.36 p = 0.51; 50 m: $H_{3,89}$ = 4.14 p = 0.13; 100 m: $H_{3,88}$ = 1.75 p = 0.42).

Comparing the changes in phonotaxis score from distant to proximal stimuli generally reinforced the raw phonotaxis results, namely, showing larger changes in attraction to proximally recorded stimuli compared with those made farther from the focal male (Supplementary Fig. 3.12a-c). Moving from 100 m to 16 m did not significantly alter phonotaxis scores at any stimulus playback level, with all differences at or near 0. Advancing from 16 m to 8 m elevated the phonotaxis scores, although the positive changes at 70 dB were not significantly different from 0. Changes at 55 and 85 dB were also significantly larger in the transition from 32 m to 16 m (Kruskal-Wallis one-way ANOVA and Dunn's post-hoc pair-wise comparisons; 55 dB: $H_{6,217}$ = 33.12, p = 0.0051; 85 dB: $H_{8,224}$ = 89.91, p = 0.0297). Reducing the stimulus distance to 4 m resulted in decreases to phonotaxis scores, which were significantly less than 0

(Wilcoxon signed rank test, $p < 0.01$). Decreasing the distance to the focal male to 1 m generally resulted in increases in phonotaxis scores at all amplitudes, and these were significantly larger than 0 at 55 and 70 dB SPL. Overall, the largest changes (positive or negative) in female responses occurred when the stimulus recording distance was decreased from 16 m or closer, irrespective of the SPL.

3.3.3 ORIENTATION

Orientation to the synthetic control and 85 dB SPL experimental stimuli (Fig. 3.5a, Supplementary Table 3.3) were as described previously (Christie et al. 2010). Mean vector-length (*r* statistic) values were at or above 0.95 for all 85 dB SPL groups. Reducing the level of the experimental Hv call to 70 and 55 dB SPL increased the variability of wall contact points, but still elicited accurate orientation, with mean angles and vector lengths similar to the responses at 85 dB SPL, and larger than for all chorus stimuli, irrespective of playback level. Contact points were distributed non-randomly for the Hv call at all SPLs (*V*-test, $p \leq 0.01$), and the mean angles had lower standard deviations than in response to chorus stimuli, regardless of distance or playback level.

While not as accurately as with the experimental Hv stimuli, females still localized the loudspeaker broadcasting 1 m and 8 m chorus sounds (Fig. 3.5b, d, Supplementary Tables 3.3, 3.4). At all playback levels, wall contact locations were significantly non-random ($p \leq 0.01$) with mean angles always within ~11° of the speaker location and mean vector lengths between 0.46-0.73. Even at the lowest sound level (55 dB), vector lengths were greater than those for stimuli more distant than 8 m played at greater SPLs (Fig. 3.6). Unlike the phonotaxis score data, the best localization (showing the highest *r* value and smallest standard deviation and confidence interval about the mean

angle) was always observed at 85 dB SPL. Reduction of the SPL from 85 to 70 dB SPL resulted in moderate increases in contact point dispersion for the 1 and 8 m stimuli. The responses at 55 dB SPL, while non-random, showed a marked increase in standard deviation and reduction in mean-vector length, suggesting less accurate localization. Similar to the phonotaxis scores, the orientation to the 4 m stimulus was discontinuous, showing less accuracy compared with both the 1 m and 8 m stimuli at 85 and 70 dB SPL (Fig. 3.5c), with mean angles farther from 0°, as well as larger standard deviations and smaller *r*-values at the louder playback levels, particularly at 70 dB (0.16, compared with 0.66 for both 1 m and 8 m stimuli). Wall contacts were non-random only at 85 dB SPL, and the mean angles at both 70 and 55 dB SPL deviated significantly from the speaker location by >35° - amounts seen in responses to more distant stimuli.

With increasing distance, orientation responses became less directed (Fig. 3.6a-c, Supplementary Table 3.4). Mean angles deviated farther from the target, and no stimulus distance / SPL combination resulted in a mean vector length above 0.37. Unlike the 1 - 8 m data, responses to the stimuli from 16 m and beyond did not necessarily have the largest *r*-values or smallest standard deviations at 85 dB SPL, and the dispersions were high enough to prevent calculation of 95% confidence intervals. At 16 m, only the 85 and 70 dB SPL stimuli elicited non-random orientation. The responses at 32 m were randomly distributed at 85 dB SPL, but not at lower SPLs. For chorus sounds recorded at the furthest distances – 50 and 100 m - females oriented non-randomly only at the loudest stimulus playback level (Fig. 3.6c, d, Supplementary Table 3.4).

3.4 DISCUSSION

3.4.1 SUMMARY OF FINDINGS

Results of playback experiments, using synthetic calls and natural chorus noise recorded at increasing distances from single males, showed that temporal fine structure of calls was more important for female attraction compared to the sound level, and increasing stimulus intensity did not always improve signal attractiveness. Whenever significant changes in phonotaxis scores were observed, they were seen in calls recorded at closer distances (1 – 16 m) from the focal male. We found a linear relationship between signal attractiveness and playback SPL only for the synthetic calls. In contrast, female attraction was greatest for natural chorus sounds played at intermediate and high SPLs (70 and 85 dB SPL, respectively) and reduced at the lowest SPL (55 dB). Amplifying the amplitude of more distantly recorded chorus sounds did not linearly increase their attractiveness, suggesting that sound level plays a less important role than the presence of temporal structures. At all SPLs, positive and negative changes in stimulus attractiveness between two adjacent recording distances were larger in magnitude and significantly different from 0 when moving towards the focal male from < 32 m, while moving from > 32 m resulted in much smaller changes not significantly different from 0.

Localization to synthetic calls showed greater accuracy than any natural chorus stimuli at all SPLs (Figs. 3.5, 3.6). Females oriented towards stimuli recorded at 1 and 8 m at all SPLs, with accuracy increasing with SPL (Fig. 3.5). In contrast, accuracy of orientation to the acoustic stimuli from 16, 32, and 50 m was more idiosyncratic and generally less than for shorter-distance sounds (Fig. 3.6). Localization acuity was

improved at 85 dB SPL, except for the 32 m stimuli. These results suggest that an increase in sound level can enhance localization acuity even for male calls embedded in a dense chorus.

Compared with Beckers and Schul (2004), the mean female responses in the present study (Supplementary Tables 3.3, 3.4) had generally lower phonotaxis scores and *r* values. The phonotaxis scores and vector lengths for short stimulus distances (0.19 - 0.5, and 0.16 - 0.76 respectively for all SPLs at 1 – 8 m) were roughly equivalent to those seen toward synthetic calls broadcast at ~49-32 dB SPL. Behavioral measures for longer distances (≥16 m) correspond to responses to synthetic stimuli at ~37-25 dB SPL, near the estimated limits of female response (Beckers and Schul 2004). Bee and Schwartz (2009) also used a similar paradigm to study female *H. chrysoscelis* responses to synthetic calls of varying SNR presented with a noise masker with spectral characteristics identical to conspecific choruses. Although the actual SNRs of our stimuli were not determined, comparison with this study allows a rough estimate. *H. versicolor* responses in the current study to the 1 – 8 m calls were similar to model stimuli broadcast in noise with a SNR of -3 to -6 dB considering phonotaxis scores, and +3 to -3 dB for mean vector lengths. Phonotaxis scores and vector lengths in response to calls recorded from 16 – 100 m were similar to much less attractive stimuli with SNRs of -3 to -27 dB. The notably smaller standard deviations in our orientation data - including the more distant stimuli - may be due to residual natural temporal structure not present in the synthetic chorus noise when paired with calls at very low SNRs. These comparisons suggest that our natural stimuli, even those recorded closest to a calling male, show a markedly reduced attractiveness equivalent to model calls with low SNRs or amplitudes,

implying a significant challenge to the female auditory system in the tasks of call detection, recognition, and discrimination.

With increasing distance from the chorus, the fine temporal structure of individual calls becomes less well defined (Supplementary Figs. 3.3, 3.5-3.11; Kuczynski et al. 2010; Chapter 2, or Christie et al. 2010). Female gray treefrogs are sensitive to changes in these factors, as they represent salient species-recognition cues (Gerhardt and Doherty 1988; Diekamp and Gerhardt 1995; Gerhardt and Watson 1995; Gerhardt and Schul 1999; Gerhardt et al. 2000; Schwartz et al. 2010; Ward et al. 2013a, b). Differences in stimulus amplitude also affect *H. versicolor* female discrimination – in a two-choice paradigm, amplitude differences as small as 2-4 dB are enough to alter female treefrog preferences (Gerhardt et al. 2000; Vélez and Bee 2011, Bee et al. 2012). In our study, increasing the playback level did not improve attractiveness of the signal at long distances (32–100 m). This suggests that the degradation of the temporal call features was largely responsible for the unattractiveness of the stimuli.

Although our stimuli are derived from natural calls, a comparison with altered synthetic stimuli in previous studies using the no-choice paradigm highlights the significant effect of sound propagation in a chorus on call attractiveness. Female responses to the most proximal stimuli (< 16 m) with the highest phonotaxis score and vector lengths had equivalent attraction to synthetic stimuli with highly altered call pulse parameters. Proximal stimuli were as attractive as model calls with pulse rates of 4-7 Hz, rise-times $\leq$ 5 ms, durations of 8-17 ms, pulses-per-call of 4-8, and inter-pulse-intervals of 40-48 ms (Schul et al. 2002; Beckers and Schul 2004). These ranges of pulse temporal features have been shown to be significantly less attractive than

standard calls (Gerhardt et al. 1996; Gerhardt and Schul 1999; Schul and Bush 2002). and in some cases, are outside natural range of the local H. versicolor population at the tested temperature (Gerhardt 1991).

We found that whereas female *H. versicolor* demonstrated a monotonically increased attraction to the level of synthetic calls, the attractiveness of chorus sounds was *not* necessarily improved by increasing the playback level (Fig. 3.3b, 3.4, Supplementary Tables 3.2, 3.3). For chorus sounds, the mean phonotaxis scores typically peaked at 70 dB SPL, though the difference was not statistically significant for chorus distances beyond 16 m (Figs. 3.3, 3.4). Interestingly, this pattern has also been seen for synthetic stimuli played without masking in the range of 67-85 dB SPL (Beckers and Schul 2004). This increased attraction for an *intermediate* sound level using naturalistic stimuli was also seen in *H. chrysoscelis* when tested with both synthetic calls masked by noise with a 'chorus-shaped' frequency spectra (Bee and Schwartz 2009), and 'chorus-like' sinusoidal amplitude-modulated (SAM) synthetic stimuli with varied modulation depth - mimicking the degradation of pulse structure seen with increasing distance from a calling male (Kuczynski et al. 2010). Stimuli mimicking 'short' distances (high modulation depth) from a male showed the highest attraction at the loudest SPL (85 dB), while those representing farther distances, with more degradation (moderate modulation depth), showed the highest percentage of female response at 73 dB SPL (Kuczynski et al. 2010). A caveat with using natural chorus sounds as stimuli is that in propagation multiple temporal and spectral features are altered simultaneously. To elucidate which individual parameter is most critical in female

attraction and orientation, ideally one would want to manipulate that sound parameter and keep all other features constant (Wiley 2017).

Another potential explanation for this reduced attraction to chorus sounds is the difference in frequency spectra between the artificial calls and chorus. Spectral energy of the synthetic conspecific calls is concentrated in 1.1 and 2.2 kHz bands, whereas that of the chorus includes significant acoustic power in frequency bands flanking these spectral peaks in *H. versicolor* calls (Fig. 3.1; Supplementary Figs. 3.4, 3.5). The additional acoustic energy could inhibit neurons in central auditory nuclei via two-tone inhibition (TTI). Various physiological studies in the anuran hind- and midbrain have demonstrated inhibition of neurons that are sensitive to the dominant frequencies in species-specific vocalizations when the acoustic stimuli also contain significant energy in frequencies above and/or below those present in the species vocal signal (Fuzessery and Feng 1982; Fuzessery and Feng 1983). Although the best frequencies of these auditory neurons (from the Northern Leopard frog, *Lithobates pipiens*) had BFs generally around 500 Hz, studies on direction-dependent frequency tuning in the anuran midbrain showed TTI in cells with BFs > 1 kHz (Zhang and Feng 1998), opening the possibility of TTI in species with higher BFs.

An unexpected result of our study was the reduction in both stimulus attractiveness and orientation to the 4 m chorus stimuli, an effect also seen in a previous study (Christie et al. 2010). The origin of the reduced responses is unclear but could potentially be due to particular properties of the chorus background at this distance. In female *H. chrysoscelis* phonotaxis experiments using synthetic conspecific calls embedded in a fluctuating ‘chorus-shaped’ noise masker, Vélez and Bee (2011)

found significant improvement in call recognition (2-4 dB release from masking) when noise fluctuations were < 5 Hz. At greater masker fluctuation frequencies, particularly those approaching male call pulse rates (~45.5 pulses/s), the noise actually *increased* masking by ~6 dB, a phenomenon known as 'modulation masking' (Bacon and Grantham 1989, Vélez and Bee 2013). It is possible that at 4 m, our chorus stimuli have significant power in the range of ~20-25 Hz, approximately the pulse rates of *H. versicolor* at temperatures of ~20-24 °C, which are consistent with field and laboratory experimental environments (Gerhardt and Doherty 1988, Gerhardt and Schul 1999). The acoustic power in this frequency range may increase the masking of the focal and nearby males. Clearly, further research is necessary to determine the viability of this tenet.

3.4.2 CHORUS EFFECTS ON MALE/FEMALE MATING BEHAVIOR

As chorus density increases, so does the probability of call overlap from other males, which may have direct effects on female attraction (Schwartz and Marshall 2006). Although there have been many studies on female preferences for various call features, it is still unclear how these preferences are affected by signal degradation and 'naturalistic' masking. In *Hyla microcephala*, female choice between two alternative calls was impaired when a call was overlapped by another with <6 dB SPL difference between the two (Schwartz 1993). Female *Hyla ebraccata* failed to detect conspecific calls in chorus noise when the SNR was 1.5 dB or lower (Wollerman 1999) and had reduced frequency discrimination when the SNR was less than 9 dB (Wollerman and Wiley 2002). In *H. versicolor*, female preference functions for temporal call features presented without noise/maskers demonstrate remarkable sensitivity to small

differences (Gerhardt and Doherty 1988; Gerhardt et al. 2000; Bush et al. 2002). In *H. versicolor,* selectivity for call duration was reduced when stimuli were broadcast with chorus noise at ~7 dB lower than the level of synthetic call (Schwartz et al. 2001). Under low SNR conditions in dense choruses, the chorus background may restrict the isolation and discrimination of call features, impeding mate selection and resulting in smaller effective *communication distances* (Lang 2000).

Our results and those of others suggest that under field conditions, the ability of females to isolate and discriminate among male calls is likely limited to short distances (<32 m), beyond which the conspicuous temporal features of calls evident at high SNRs are degraded (Schwartz 1993; Gerhardt and Schul 1999; Kuczynski et al. 2010; Christie et al. 2010). This is supported by studies of female *H. chrysoscelis,* demonstrating increased signal recognition thresholds when artificial conspecific calls were broadcast concurrently with 'chorus-shaped' noise from above (Bee and Schwartz 2009), and preference for longer calls was reduced under the same conditions (Vélez et al. 2013). Furthermore, comparison with previous studies in the *H. chrysoscelis* - *versicolor* complex suggests that responses to stimuli ≥ 32 m have extremely-low to no- attraction for *H. versicolor* females. Female *H. versicolor* showed no attraction to temporally modulated and unmodulated 'chorus-shaped' noise stimuli, with vector lengths (0.15-0.31) similar to those when presented with calls recorded from 16 – 100m, irrespective of SPL (Vélez and Bee 2010). When tested with synthetic calls in 'chorus-shaped' noise with SNRs < -9 dB, Bee and Schwartz (2009) found an asymptotic relationship with SNR and mean phonotaxis score. Across the range of -9 to -33 dB SNR, scores varied only from ~0.15-0.25, a very small range compared with larger SNRs – suggesting little

attraction to females from lack of call detection or recognition. In the present study, mean responses to 4 m (55 dB SPL), 16 m and 32 m (55 and 85 dB SPL), and 50 m and 100 m (all SPLs) had similar attraction. Considering the chorus itself as a signal, 'soundscape' stimuli designed to have identical spectral characteristics as natural *H. chrysoscelis* choruses, but which lacked natural chorus temporal structures (modulation spectrum and amplitude fluctuations) did not elicit attraction, even when played at 70 dB SPL – well within the range of natural choruses and those used in anuran bioacoustics experiments (Vélez et al. 2017; Christie et al. 2010). Taking into account the similarity of behavioral responses to 16 m – 100 m stimuli and those of synthetic calls with highly degraded/altered temporal features, as well as stimuli lacking temporal structure characteristic of the natural chorus as a whole, our results suggest that the stimuli beyond ~16 – 32 m had sufficient degradation of temporal features to not elicit significant female recognition and orientation, even at ethologically significant SPLs approximating the sound levels at distances of $\leq$ 1 m from a calling male in a dense chorus.

3.4.3 STRATEGIES TO ASSIST CALL DETECTION & DISCRIMINATION

The fact that *H. versicolor* females responded to calls recorded at 1 m from a calling male - presented in a spatially simple environment - with behavioral responses similar to model calls at ~50 dB SPL or with pulse temporal parameters outside species norms and consistent with low female attractiveness underlines the difficult task performed by the female CNS in the wild. Nevertheless, the successful female detection and evaluation of male call is critical for their reproductive success. While these tasks are

made more difficult in dense choruses, the anuran auditory system has evolved to mitigate the effects of call masking. To reduce the effect of call overlap, males of many frog species time their calls during acoustic 'gaps' of choruses (Zelick and Narins 1982; Narins and Zelick 1988; Schwartz 1993; Schwartz and Bee 2013). Females of some species are somewhat resilient to the problem of call overlap. Call choice of female *H. versicolor* was minimally affected if male calls overlap was approximately one-third or less (Schwartz and Marshall, 2006).

In nature, acoustic backgrounds are often not constant, but fluctuate at varying modulation rates. Humans can take advantage of acoustic 'gaps' when modulated background noise amplitudes drop temporarily below the target signal/speech's level, allowing brief, acoustic 'glimpses' – a release from masking termed 'dip-listening' (Cooke 2006; Vestergaard et al. 2011). Previous behavioral studies have found mixed results – they failed to find significant evidence for dip-listening in female *H. cinerea*, while female *H. chrysoscelis* showed lowered signal recognition thresholds under acoustic conditions when the 'gaps' allowed for the perception of several consecutive call pulses (Vélez and Bee 2011; Vélez et al. 2012). Neurophysiological studies in *Lithobates pipiens* have revealed that some midbrain neuronal subpopulations exhibit a release from masking in detecting a 'call-like' tone-pulse train in a SAM masker (Goense and Feng 2012). Due to the use of recorded chorus stimuli, we did not test for 'dip-listening' in *H. versicolor*, but it would be valuable to determine whether they behave similarly to *H. chrysoscelis*, especially since *H. versicolor* female call preferences are strongly affected by not just pulse-rate, but also by duration and shape. Lee et al. (2017) showed that female *H. chrysoscelis* could exploit another statistical

feature - *comodulated* frequencies in the chorus background. Females had better call detection and increased attraction to calls presented in *comodulated* chorus-shaped' noise, compared to calls in uncorrelated or unmodulated noise. As with the 'dip-listening' hypothesis, it would be interesting to test *H. versicolor* as the significant differences in pulse structure and female selectivity for call features suggests that the utility of this tactic may be *more* valuable in this species.

Another strategy to increase the effective SNR of a call is to take advantage of spatial separation of the signal/masker. Ward et al. (2013a) found that female *H. chrysoscelis* were better able to discriminate calls with varying pulse rates (encompassing both *H. chrysoscelis* and *H. versicolor* average pulse rates) when the signal and 'chorus-shaped' noise masker were spatially separated. In the current study, the stimuli contained both 'signal' and chorus 'masker', such that playback always represented a 'co-localized', non-separable condition, which may decrease signal attractiveness with distance more than females in a natural chorus with a more complex spatial configuration of acoustic stimuli. This might also explain why in our study orientation to the chorus stimuli was improved by increasing playback amplitude, while the inherent attractiveness of the stimuli was not affected.

3.4.4 SPECIES/ECOLOGICAL CONSIDERATIONS

While the chorus background may alter female perception of several spectral/temporal features of male calls, there is evidence that different call features might not be affected equally by the chorus background, allowing some discrimination by females even in high noise conditions. When responding to SAM calls which

mimicked the effect of the chorus background acoustically ‘filling in’ the inter-pulse intervals, *H. chrysoscelis* females showed significant tolerance to the degraded temporal structure (Kuczynski et al. 2010). It is important to note that the most salient call feature for *H. chrysoscelis* females is the pulse-rate, while females of the species in the present study, *H. versicolor*, are most sensitive to pulse shape and duration (Schul and Bush 2002) – which may result in differential responses to calls embedded in natural or synthetic ‘chorus-like’ noise. Beckers and Schul (2004) suggested that for *H. versicolor* females, reduced call levels result in the audibility of only the peaks of individual pulses, whereas their assessment of pulse duration is impaired reducing the call attraction. This may also hold for natural conditions of low SNR, in which *H. versicolor* females face difficulties assessing pulse duration and/or rise/fall times but may be less deleterious to pulse rate detection in *H. chrysoscelis*. Future behavioral experiments with synthetic stimuli, allowing for both the fine manipulation of temporal degradation and SNRs (Bee and Schwartz 2009), and exploring the chorus noise *gestalt* as a relevant stimulus (Lee et al. 2017) are necessary to explore these questions.

3.4.5 NEUROPHYSIOLOGICAL CONTEXT

How signal degradation is represented in the auditory system is not clear. Gerhardt and Schul (1999) suggest that the amphibian papilla, which is sensitive to the 1.1 kHz dominant energy in conspecific calls, may be critical for pulse rise-time encoding. Examining the effects of call feature alteration and degradation in a frequency-dependent manner might also serve to link behavioral responses to

naturalistic stimuli and subsequent processing in the auditory periphery. Feng et al. (1991) and more recently, Schrode and Bee (2015) demonstrated that sensitivity to species-specific call temporal features arises in the auditory periphery, suggesting an evolutionary adaptation for detection and discrimination of conspecific vocalizations at the level of auditory nerve fibers, in addition to previously known selectivity in more central structures (Walkowiak 1984; Eggermont 1990; Gooler and Feng 1992; Diekamp and Gerhardt 1995; Edwards et al. 2002; Rose et al. 2015).

In the anuran CNS, previous studies have shown populations of frequency and temporally selective neurons in the torus semicircularis and thalamic nuclei, some of which may be involved in the processing of species-specific vocalizations (Aertsen et al. 1981; Fuzessery and Feng 1982; Hall and Feng 1986, 1988). Recently, neurons with differential responses to conspecific *H. versicolor* and heterospecific *H. chrysoscelis* call features have been described (Rose et al. 2015). Alteration of encoding call features under naturalistic noise conditions has the potential to explain observed behavioral responses to degraded calls. Future studies should compare neuronal responses to synthetic calls with high signal-to-noise stimuli with responses to calls embedded in a chorus. *In vivo* intracellular studies (e.g. Leary et al. 2008) may elucidate a role of inhibitory connections in the processing of call stimuli.

Intriguingly, the ‘degraded’ acoustic stimulus of the frog chorus might itself play a significant role in localization behavior beyond that of a localization and orientation target. Recent work in *H. cinerea* suggests that playback of chorus recordings over the course of several days had an enhancement effect on auditory midbrain activity, as measured by the immediate-early gene *egr-1* expression (Gall and Wilczynski 2014).

Furthermore, such pre-exposure resulted in both a priming effect (increased response to masker + target vs. target alone) and *decreased* forward masking of synthetic calls as measured via midbrain auditory-evoked potentials (AEPs) (Gall and Wilczynski 2016). Gall et al. (2019) further demonstrated that fluctuating hormones, in particular estradiol, correlated with changes in female behavioral receptivity to male calls post-oviposition and also with a *decrease* in AEP responses compared to pre-oviposition females. This suggests a more short-term, proximal role of the neuroendocrine axis in processing of reproductively-relevant auditory stimuli. Whether this interaction is present in the *H. chrysoscelis/versicolor* complex remains to be determined.

3.5 ACKNOWLEDGEMENTS

We thank the University of Missouri-Columbia and the Missouri Dept. of Conservation for site access and capture permits. Carl Gerhardt provided advice and material support, and Oliver Beckers, Sarah Bush, Noah Gordon and Sarah Humfeld provided valuable advice and comments. Paul Kimball, Stephanie Kleyman, and Leah Reynolds aided in animal collection and running experiments. Atulya Iyengar provided valuable spectral analysis advice, and Daniel Eberl and Bernd Fritsch provided indispensable advice, comments, and feedback on the manuscript.

Research funding was provided by a National Science Foundation Collaborative Research in Computational Neuroscience (CRCNS-0422073) grant to ASF and a National Science Foundation grant (IOB-0445286) to JS. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois.

3.6 COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest

The authors declare no conflict of interest.

3.7 FIGURES

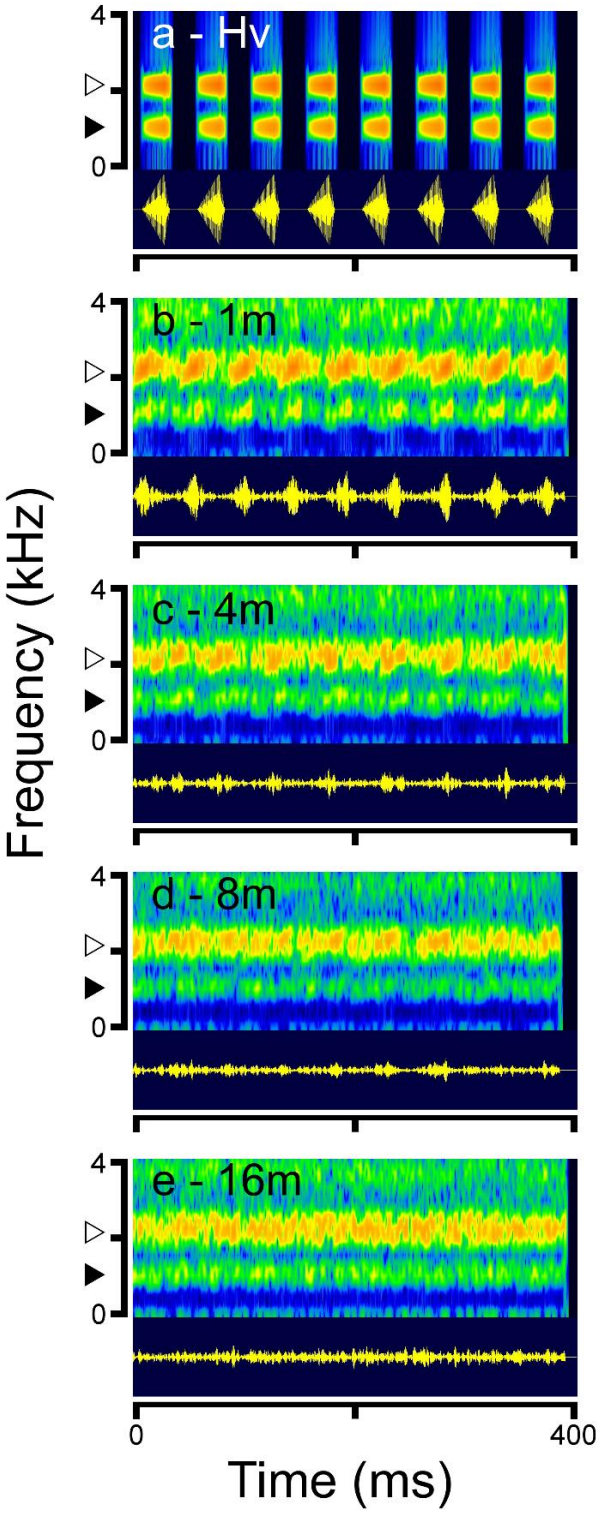


Fig. 3.1 Oscillograms and spectrograms of 400 ms sections of (a) a synthetic *H. versicolor* call and recordings of natural chorus made at distances of (b) 1 m, (c) 4 m, (d) 8 m, and (e) 16 m from a focal male. Black and white triangles indicate 1.1 and 2.2 kHz, respectively. Note the gradual loss of power in the ~1.1 kHz band and the attenuation combined with 'smearing' of the 2.2 kHz band with increasing distance

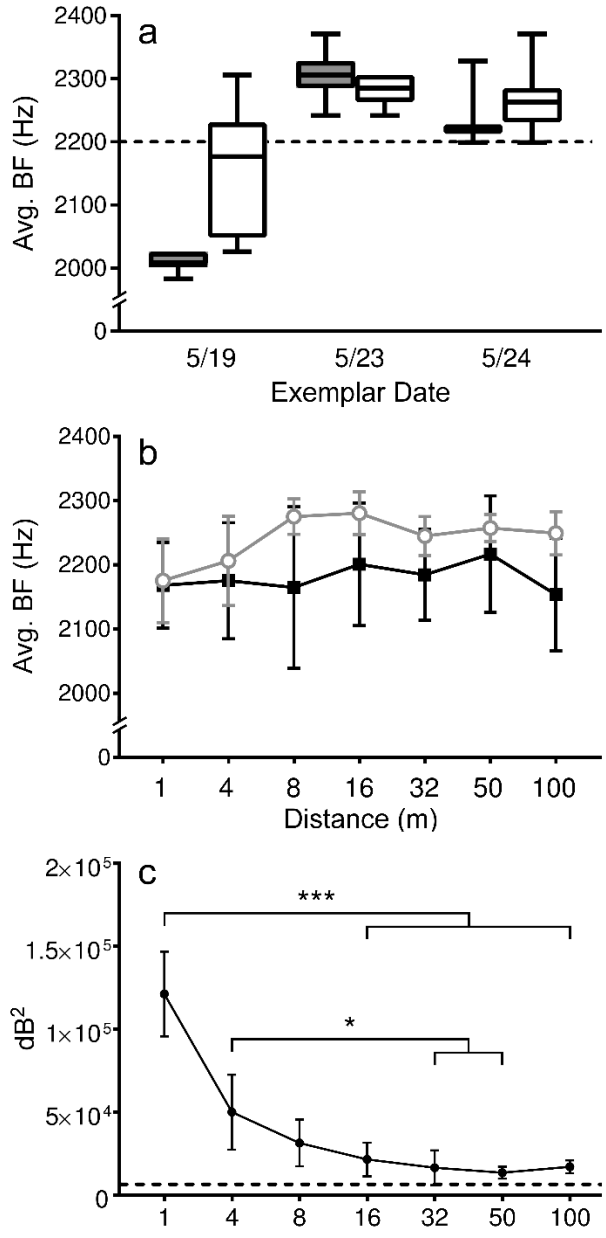


Fig. 3.2 Spectro-temporal characteristics of field-recorded *H. versicolor* advertisement calls. (a) Distribution of 'best frequency' (BF) bands for calls recorded on 3 different dates, with recordings from ALL distances pooled. Boxes and whiskers represent 25th-75th percentiles and minimum-maximum values, respectively, while bars indicate medians. Grey and white plots show data from 'focal' and 'distant' microphone recordings, respectively. The dotted line indicates the frequency band containing power at 2200 Hz, the BF for the synthetic phonotaxis stimuli. Note the discontinuous ordinate. (b) Mean

Fig. 3.2 (cont.) ± 95% CI of 'best frequency' (BF) bands vs. recording distance. Calls recorded from all 3 exemplar nights were pooled. Black squares and grey circles represent data from 'focal' and 'distant' microphone recordings, respectively. Note the discontinuous ordinate. (c) Maximum spectrogram cross-covariance vs. recording distance from a focal calling male. Values indicate median ± 95% CI of maximum cross-covariance between spectrogram 'slices' made at the BF band for calls simultaneously recorded by the 'focal' and 'distant' microphones at the indicated distance. The dashed line indicates the median maximum cross-covariance between calls recorded via the 'focal' microphone at all distances and samples of unmodulated white noise. Horizontal bars and asterisks indicate significant differences with Dunn's multiple comparison tests (* $p \leq 0.05$; **** $p \leq 0.0001$) after Kruskal-Wallis one-way ANOVA

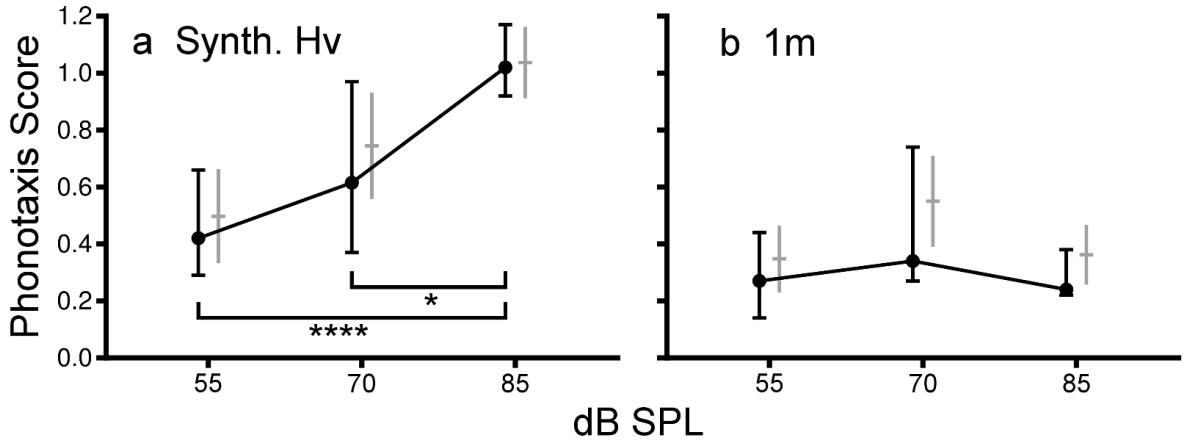


Fig. 3.3 Phonotaxis scores (black circles and error bars represent medians ± 95% CI; grey dashes and error bars represent means ± 95% CI) for the (a) synthetic *H. versicolor* call, (b) 1 m chorus. The data were pooled for all chorus exemplar / SPL pairs. Phonotaxis scores were calculated using the time to reach the loudspeaker. Horizontal bars and asterisks indicate significant differences with Dunn's multiple comparison tests (* $p \leq 0.05$; **** $p \leq 0.0001$) after Kruskal-Wallis one-way ANOVA on the speaker-derived scores

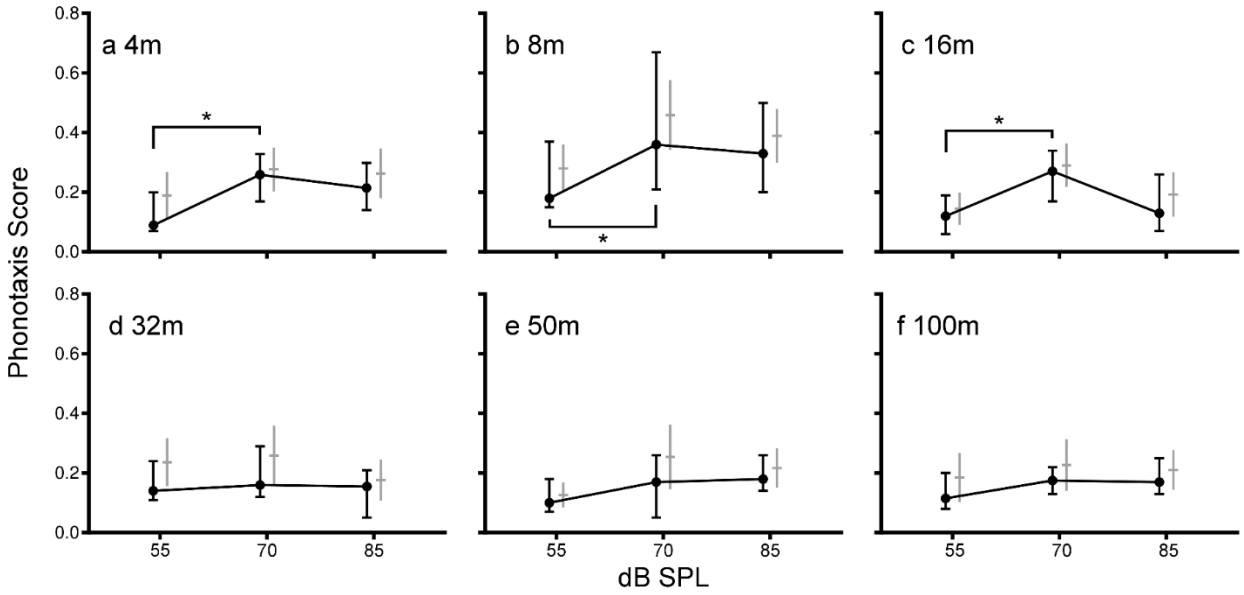


Fig. 3.4 Phonotaxis scores for the (a) 4 m chorus, (b) 8 m chorus, (c) 16 m chorus, (d) 32 m chorus, (e) 50 m chorus, (f) 100 m chorus. Horizontal bars and asterisks indicate significant differences with Dunn's multiple comparison tests (* $p \leq 0.05$) after Kruskal-Wallis one-way ANOVA on the speaker-derived scores. For other details, see caption for Figure 3.3

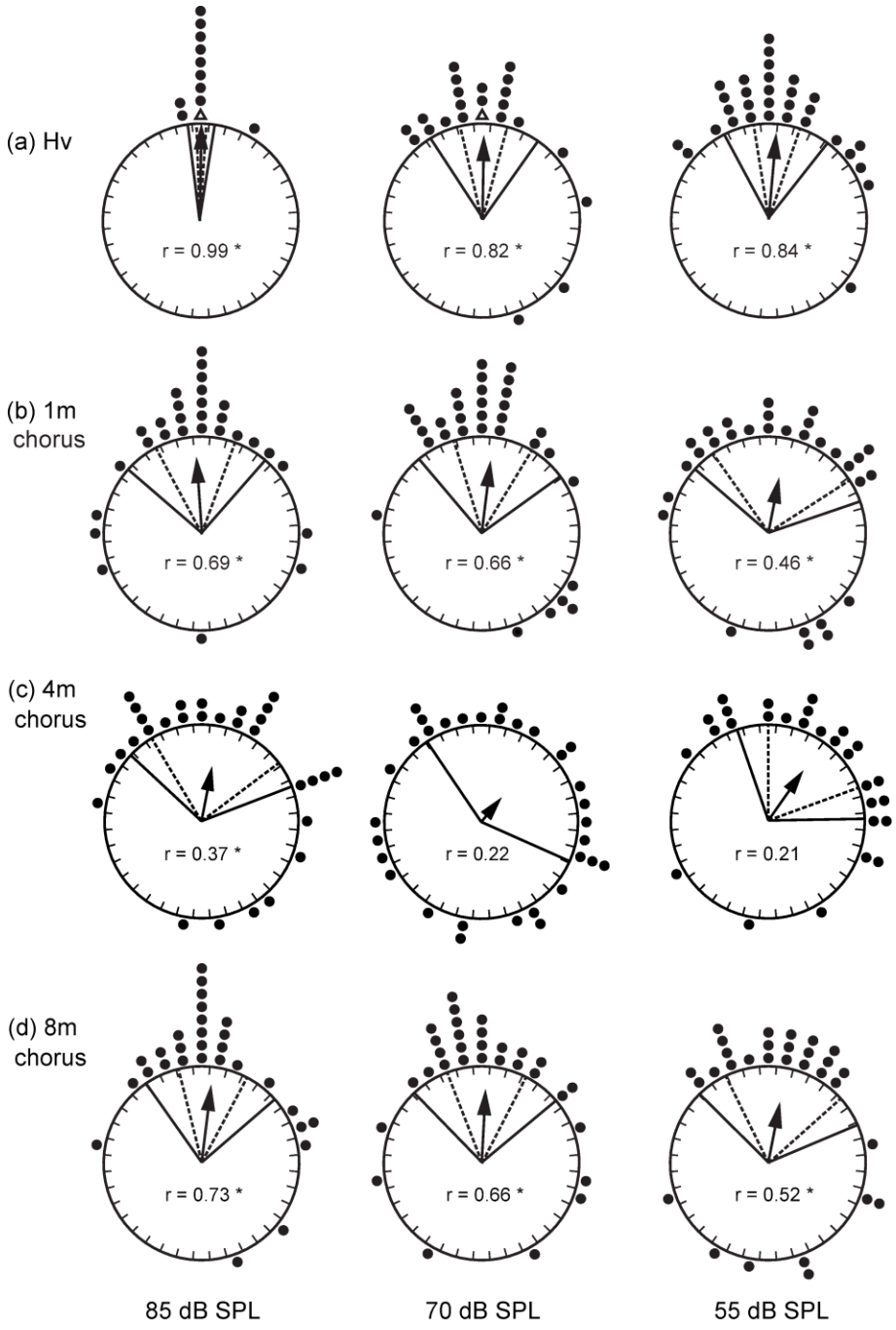


Fig. 3.5 Circular histograms showing the distributions of the locations of the arena wall contacted by females. Whereas the stimulus was presented from different directions during the experiment, in each figure the stimulus source is shown at the top of the figure for ease of comparison. Shown are results of phonotactic responses at 85, 70, and 55 dB SPL to synthetic (a) Hv call, (b) 1 m chorus, (c) 4 m chorus, (d) 8 m chorus. Each dot represents a single wall contact in the corresponding 10° arena wall segment.

Fig. 3.5 (cont.) Each open triangle represents 10 contacts. Arrows represent the mean directions of all contacts, with the length corresponding to the r statistic (shown inside each histogram). Solid and dotted lines represent the SD and 95% CI, respectively, for the mean angle. Asterisks denote contact distributions that significantly differ from random (V-test, $p \leq 0.05$)

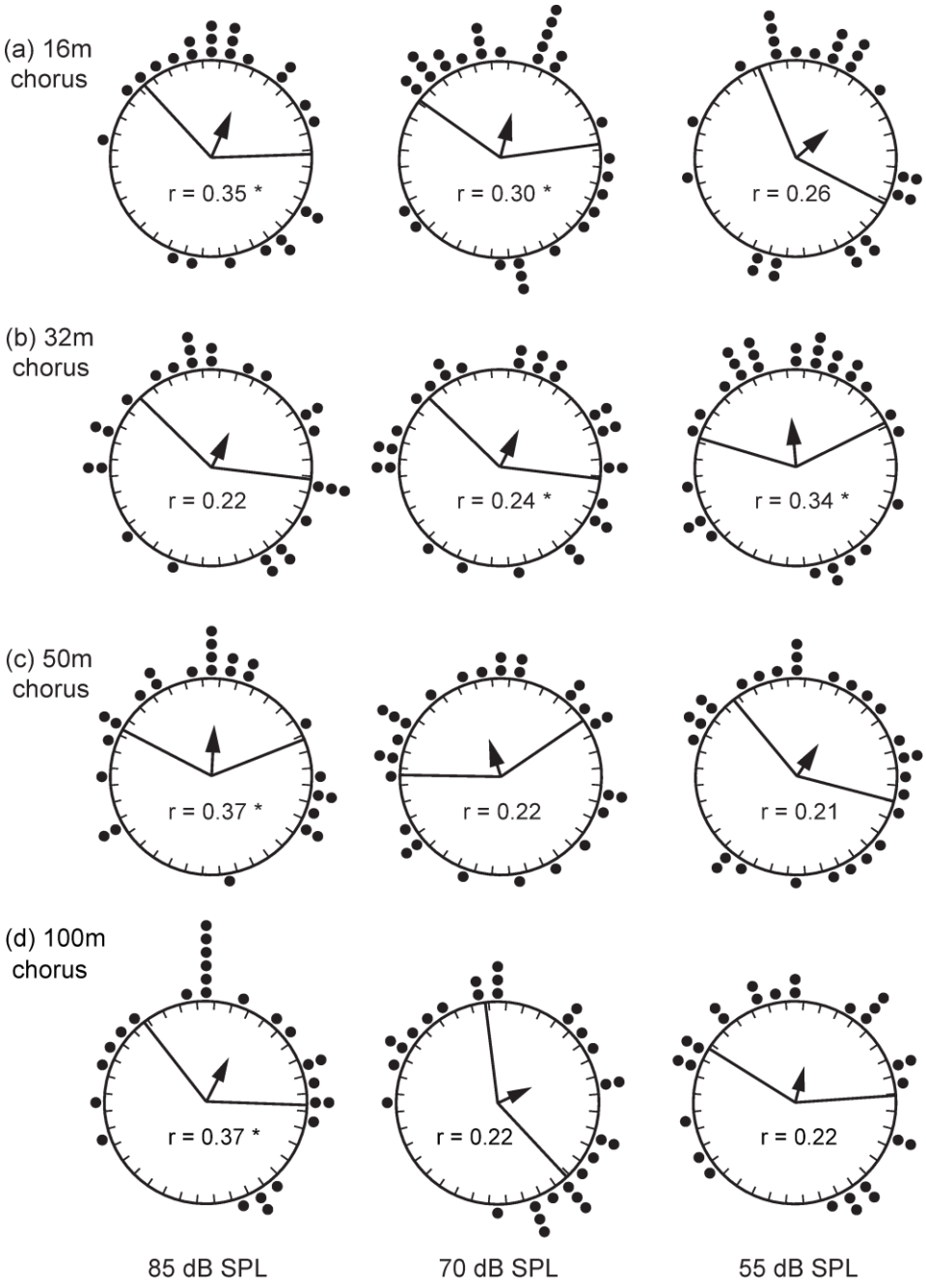


Fig. 3.6 Circular histograms showing arena wall contact data at 85, 70, and 55 dB SPL for (a) 16 m chorus, (b) 32 m chorus, (c) 50 m chorus, (d) 100 m chorus. For other details, see caption for Figure 3.5

3.8 SUPPLEMENTARY TABLES

Exemplar	5/19	5/23	5/24	Grand Avg.
Mean BF (Hz)	2013	2309	2224	2181
95% C.I.	6	16	11	28
n	25	23	29	77
Mean Pulses/Call	19.58	18.44	20.58	19.61
95% C.I.	0.93	1.47	0.87	0.63
n	26	25	31	82
Mean Call Length (ms)	1025	914	916	939.9
95% C.I.	49	59	42	34.3
N	27	34	31	77
Mean ICI (ms)	4093	3223	3683	3624
95% C.I.	591	419	484	281
n	20	27	24	71

Supplementary Table 3.1 Call statistics for exemplars used to generate phonotaxis stimuli. Calls for each exemplar date were recorded from a single male treefrog. Shown are the best frequency (BF; in Hz), mean pulses per call, call duration (in ms), and inter-call-interval (ICI; in ms), with corresponding 95% confidence intervals of the mean and n values of calls recorded 1 m from the male. Synthetic *H. versicolor* call stimuli had parameters similar to recorded male calls. Only calls fully attributable to the focal male (no overlapping by calls of similar amplitude) were used to count pulses per call. All data were taken from 'focal' microphone recordings made at 1 m distance from the calling male

Distance (m)	1	4	8	16	32	50	100	Uncorr
Mean X-cov (dB^2)	121280	50022	31410	21455	16471	13513	17051	6517
95% C.I.	14747	15189	13004	7282	6669	2303	2633	190
Median X-cov (dB^2)	125886	47528	27085	20874	14119	12969	17132	6507
n	14	11	7	10	12	12	11	77
***p*-value**	0.0001	0.001	0.0156	0.0039	0.0005	0.0005	0.001	n/a

Supplementary Table 3.2 Maximum cross-covariance of spectrogram vectors from the distant microphone recordings and a minimally cross-covariant negative control ('Uncorr'). Shown are means with 95% Cls, medians, n, and *p*-values corresponding to the one-sample Wilcoxon's signed-rank test (α = 0.05) of median values compared to the negative control

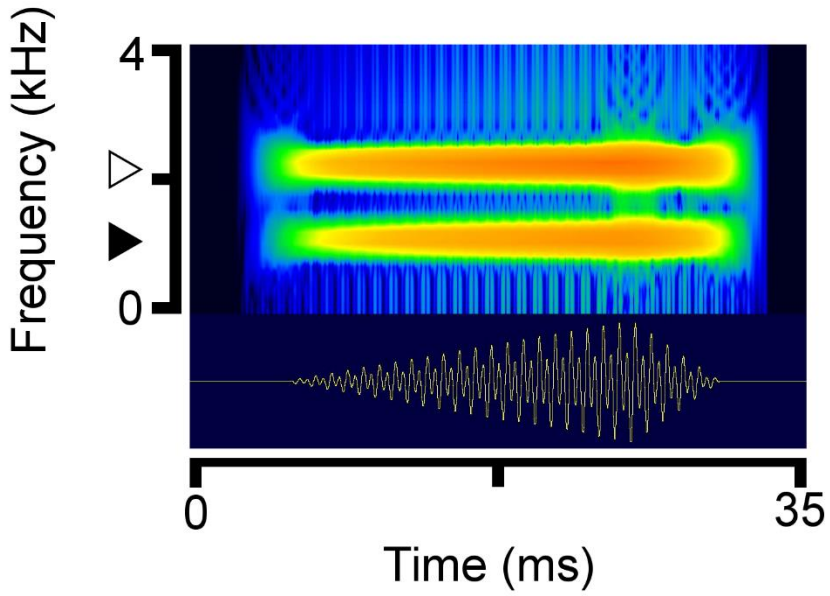
Stimulus	Hv control			Hv experimental*			1 m			4 m		
SPL (dB)	**55**	**70**	**85**	**55**	**70**	**85**	**55**	**70**	**85**	**55**	**70**	**85**
n	34	34	29	32	32	29	31	31	28	31	31	28
***μ* (deg.)**	358	359	0	5	1	1	11	7	356	35	40	11
S.D. (deg.)	24	4	4	33	34	8	60	47	45	54	74	58
r	0.91	1.00	1.01	0.84	0.82	0.99	0.46	0.66	0.69	0.55	0.16	0.49
Mean score	n/a	n/a	n/a	0.5	0.74	1.04	0.35	0.55	0.36	0.19	0.28	0.26
±95% C.I.	n/a	n/a	n/a	0.17	0.19	0.13	0.12	0.16	0.11	0.08	0.07	0.08
Median Score	n/a	n/a	n/a	0.42	0.62	1.02	0.27	0.34	0.24	0.09	0.26	0.22
+95% C.I.	n/a	n/a	n/a	0.24	0.36	0.15	0.17	0.4	0.14	0.11	0.07	0.09
-95% C.I.	n/a	n/a	n/a	0.13	0.25	0.1	0.13	0.07	0.02	0.02	0.09	0.08

Supplementary Table 3.3 Phonotaxis score and orientation data for the final control Hv, experimental Hv, 1 m chorus, and 4 m chorus stimuli. The mean orientation angles are shown with the SD of the mean and associated mean vector length *r*, and phonotaxis scores are shown with SEM of the mean. The mean, (± 95% CI) and median (± 95% CI) phonotaxis scores are also shown. *Regardless of experimental group assignment, control Hv stimuli had a playback level of 85 dB SPL

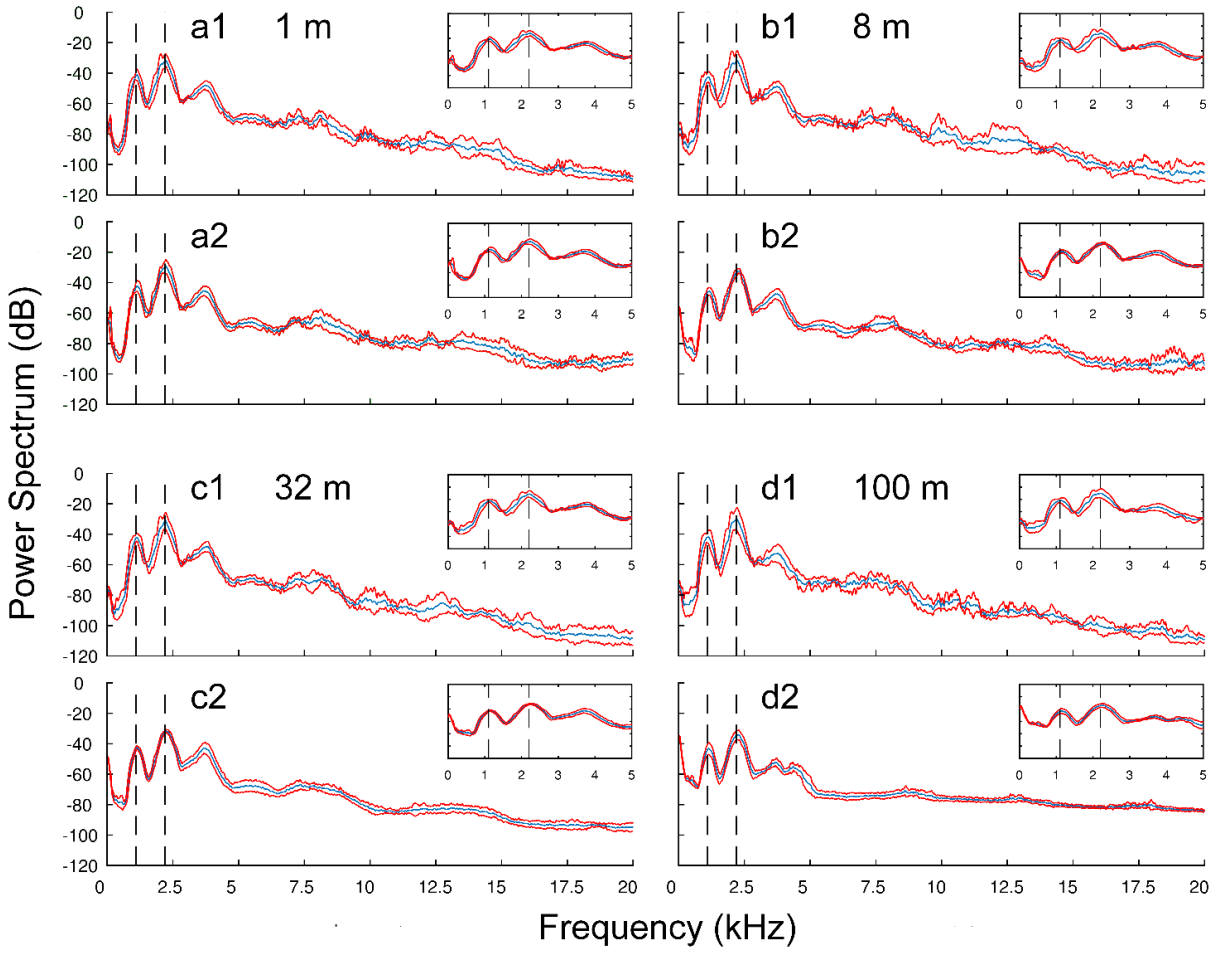
Stimulus	8 m			16 m			32 m			50 m			100 m		
SPL (dB)	**55**	**70**	**85**	**55**	**70**	**85**	**55**	**70**	**85**	**55**	**70**	**85**	**55**	**70**	**85**
n	31	31	29	31	31	27	31	30	28	29	31	28	30	30	28
***μ* (deg.)**	11	3	7	48	14	23	355	26	25	331	343	3	14	65	27
S.D. (deg.)	56	47	42	70	68	65	66	71	72	72	72	66	72	72	65
r	0.52	0.66	0.73	0.26	0.3	0.35	0.34	0.24	0.22	0.21	0.22	0.37	0.22	0.22	0.37
Mean score	0.28	0.46	0.39	0.15	0.29	0.2	0.24	0.26	0.18	0.13	0.25	0.22	0.18	0.23	0.21
±95% C.I.	0.08	0.12	0.09	0.05	0.07	0.07	0.08	0.10	0.07	0.04	0.11	0.07	0.08	0.09	0.07
Median score	0.18	0.36	0.33	0.12	0.27	0.13	0.14	0.16	0.16	0.1	0.17	0.18	0.12	0.18	0.17
+95% C.I.	0.19	0.31	0.17	0.07	0.07	0.13	0.1	0.13	0.06	0.08	0.09	0.08	0.09	0.05	0.08
-95% C.I.	0.03	0.15	0.13	0.06	0.1	0.06	0.03	0.04	0.11	0.03	0.12	0.04	0.04	0.05	0.04

Supplementary Table 3.4 Phonotaxis score and orientation data for the 8 m chorus, 16 m chorus, 32 m chorus, 50 m chorus, and 100 m chorus stimuli. For a description for the data shown, see Supplementary Table 3.3, and Materials and Methods

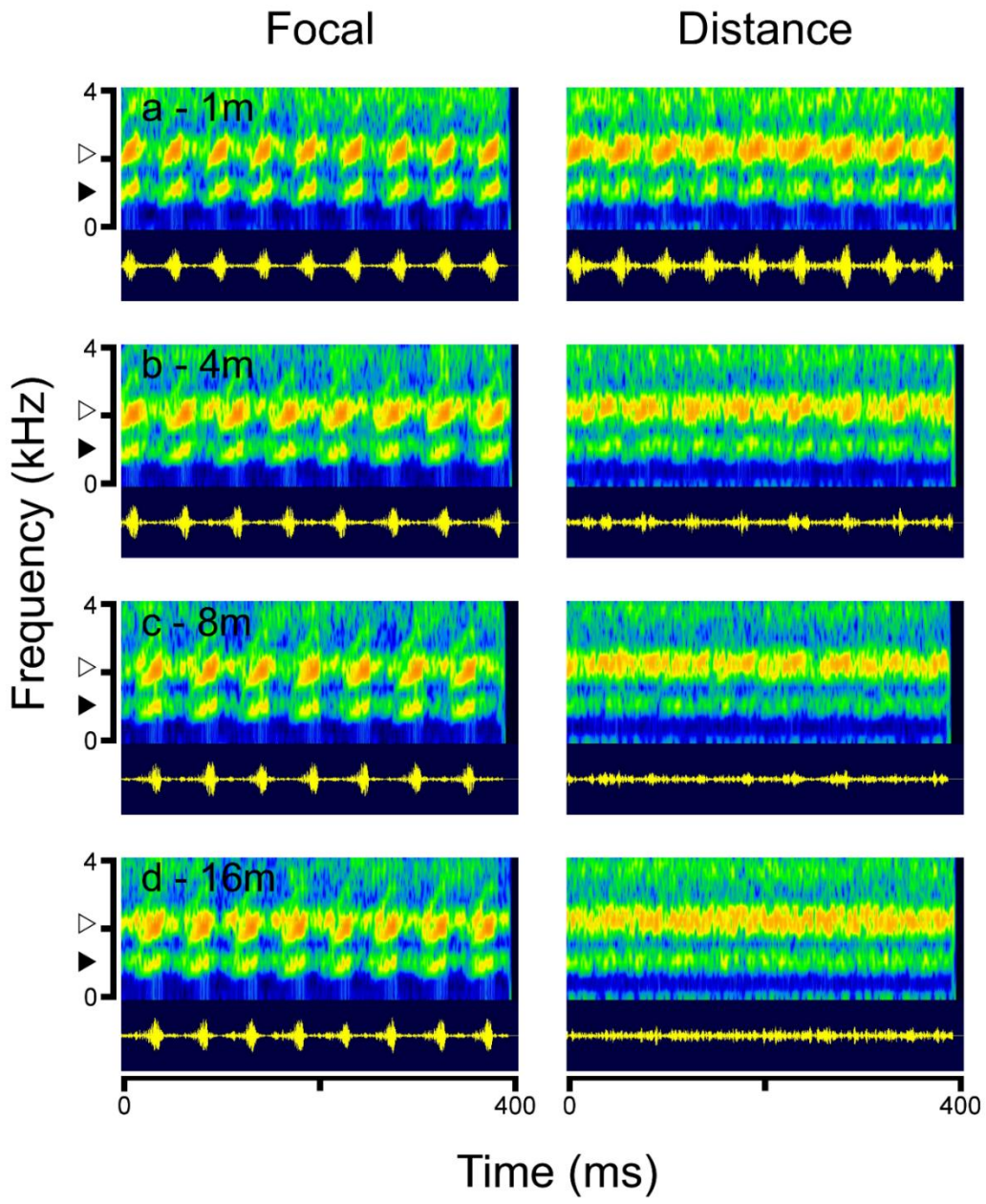
3.9 SUPPLEMENTARY FIGURES



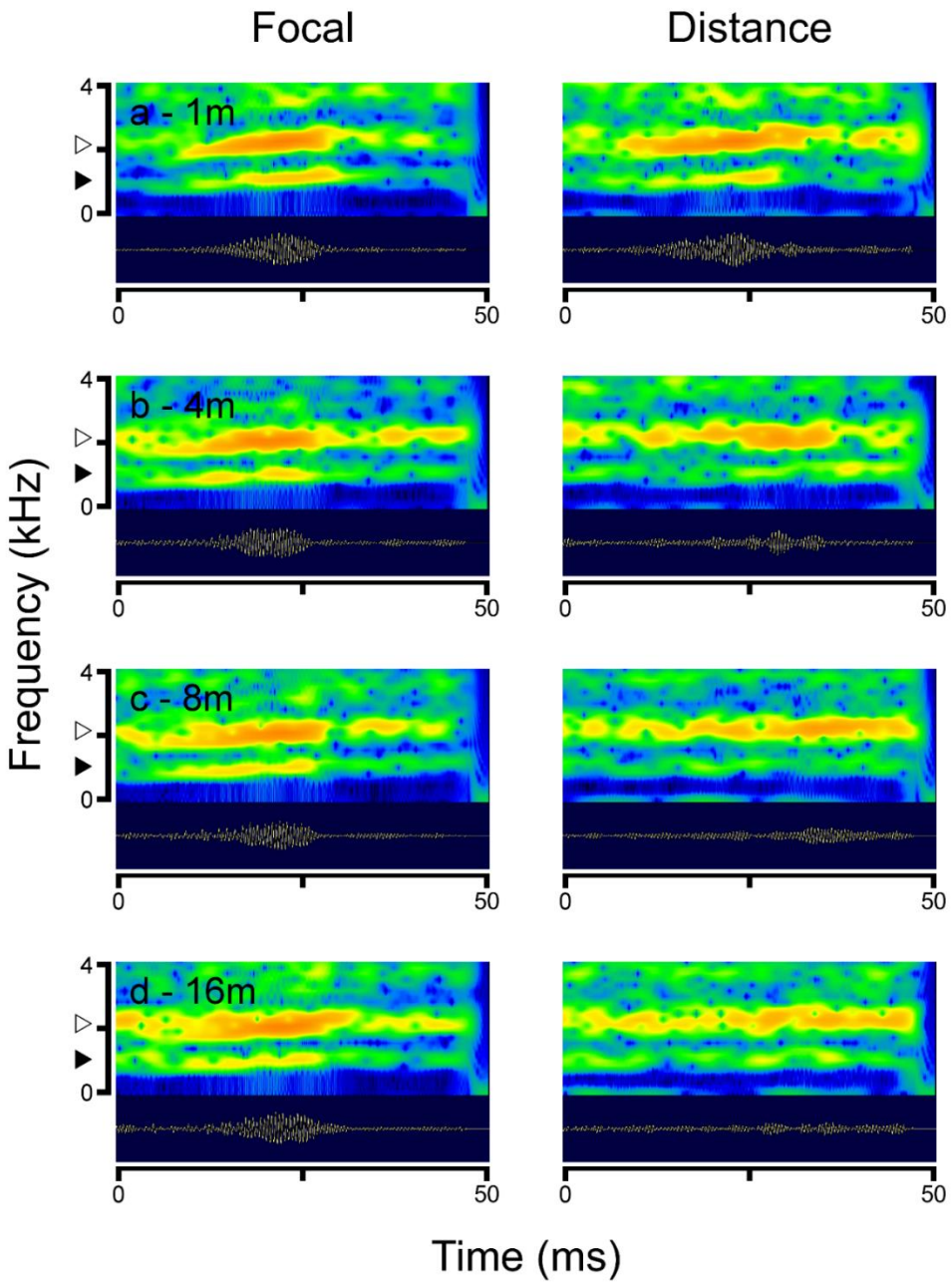
Supplementary Fig. 3.1 Oscillogram and spectrogram for a single 'pulse' from the synthetic *H. versicolor* call stimulus, showing the linear rise/fall times and prominent spectral peaks centered at 1.1 and 2.2 kHz, indicated by black and white triangles, respectively



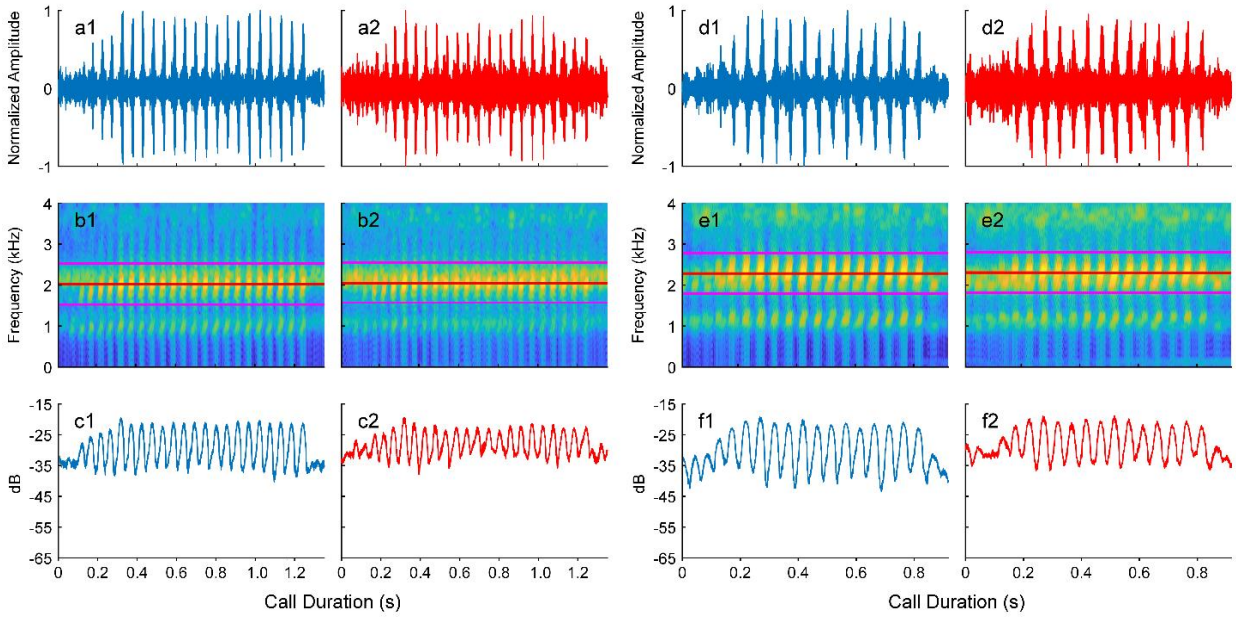
Supplementary Fig. 3.2 Mean (blue) ± SD (red) *H. versicolor* call spectra recorded at (a) 1m, (b) 8m, (c) 32m, (d) 100m. For each distance, the upper trace (1) represents the spectra of calls recorded by the 'focal' microphone, while the lower trace (2) represents those from the 'distant' microphone. Insets show a magnified view of the 0-5 kHz region. Dotted lines at 1.1 and 2.2 kHz indicate the 1st and 2nd harmonic regions of *H. versicolor* calls



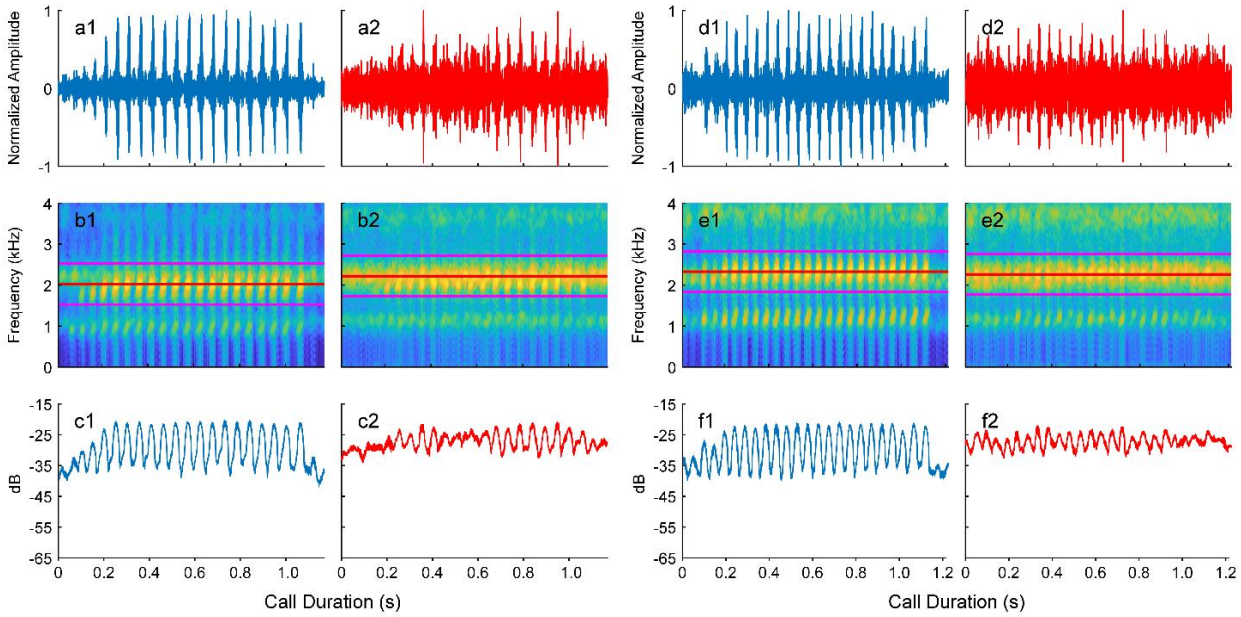
Supplementary Fig. 3.3 Oscillograms and spectrograms of simultaneously recorded calls from a focal *H. versicolor* male (left), and chorus noise (right) at increasing distances from the male: (a) 1m, (b) 4m, (c) 8m, (d) 16m. Each call consists of multiple 'pulses', which are shown in more detail in Supplementary Figure **3.**4



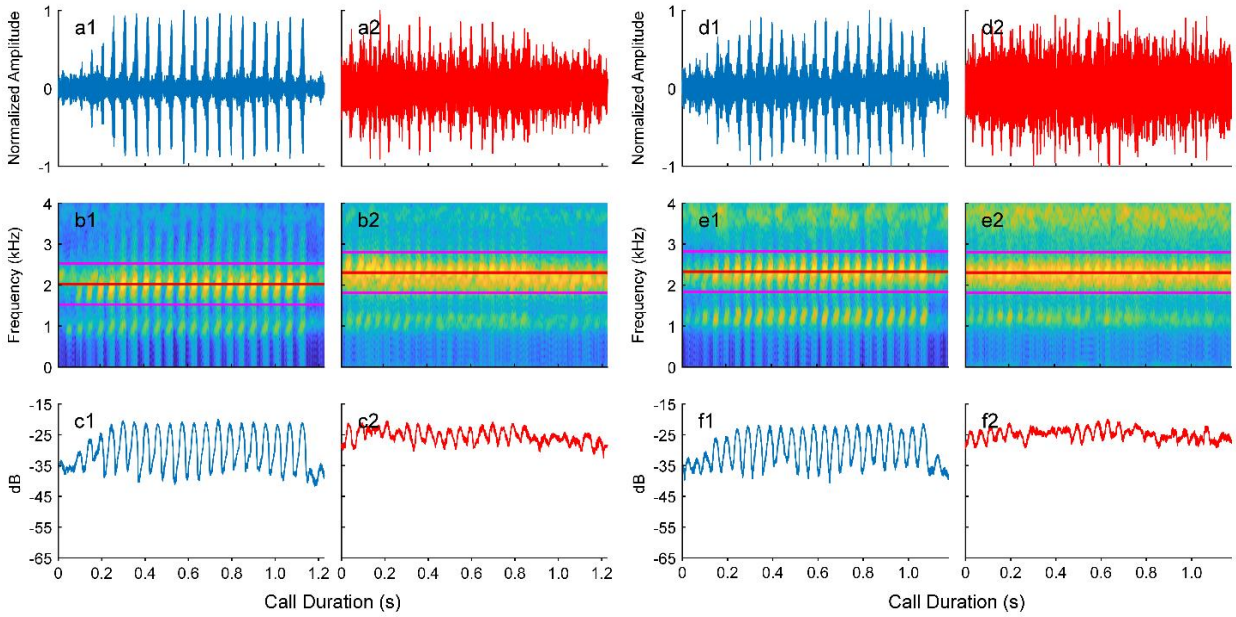
Supplementary Fig. 3.4 Oscillograms and spectrograms of simultaneously recorded single call 'pulses' of a focal *H. versicolor* male (left), and chorus noise (right) at increasing distances from the focal male: (a) 1m, (b) 4m, (c) 8m, (d) 16m



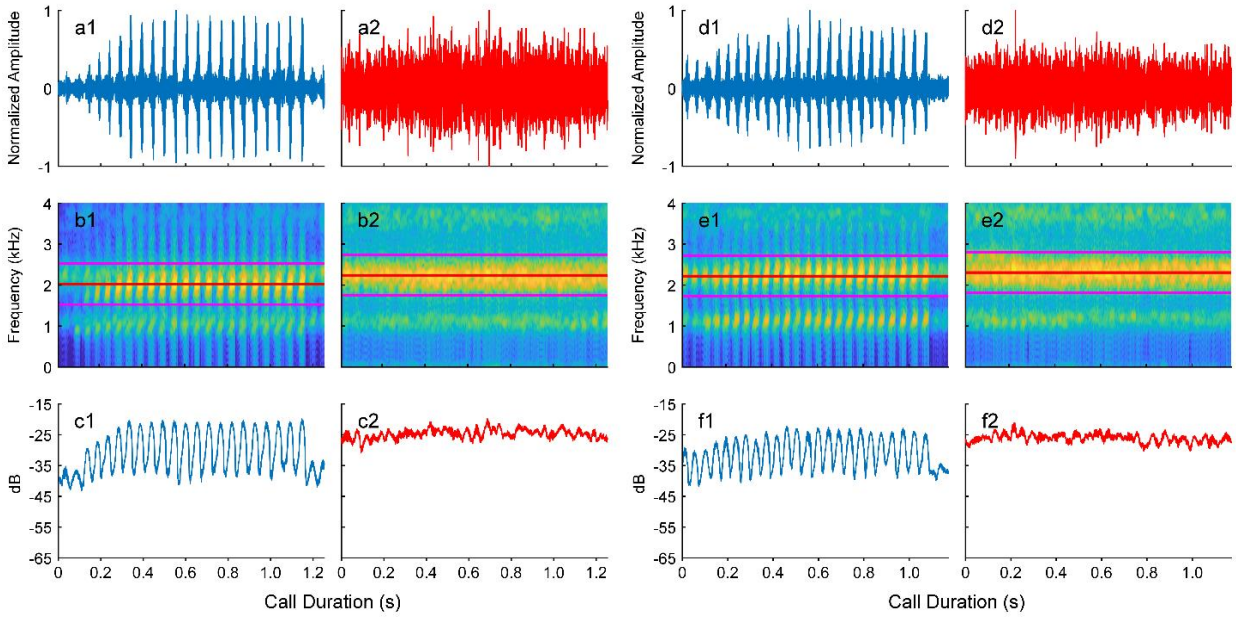
Supplementary Fig. 3.5 Spectro-temporal properties of two representative natural *H. versicolor* calls recorded at 1m. (a1-2) Oscillograms of calls simultaneously recorded by the 'focal' (blue) and 'distant' (red) microphones for the first call. (b1-2) Spectrograms of 'focal'/'distant' calls. Red lines indicate the calculated best frequency (BF) band for the call, while magenta lines show the BF ± 500 Hz, used to calculate spectrogram vectors shown in (c). (c1-2) 'Slices' calculated from spectrograms in (b) – each slice represents mean spectral power about the call's BF ± 500 Hz (between magenta lines in (b). Blue and red lines indicate calls recorded by the 'focal' and 'distant' microphones, respectively. (d-f) Same as (a-c) but for the second call



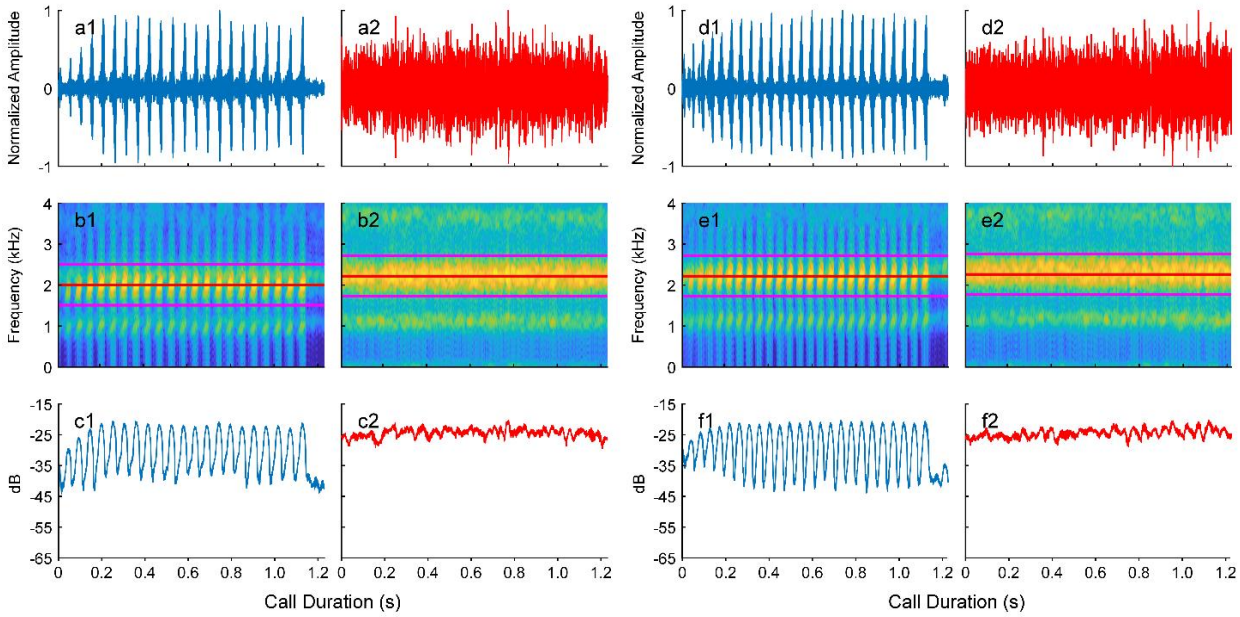
Supplementary Fig. 3.6 Spectro-temporal properties of two representative natural *H. versicolor* calls recorded at 4 m. For details and explanation, see caption for Supplementary Figure 3.5



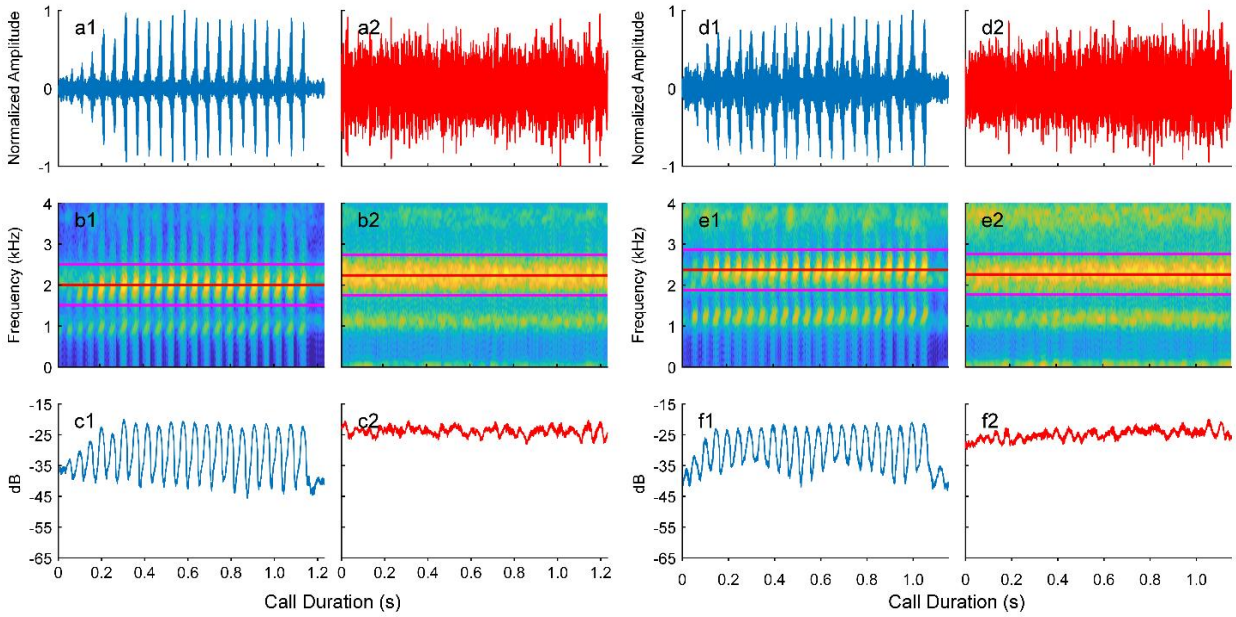
Supplementary Fig. 3.7 Spectro-temporal properties of two representative natural *H. versicolor* calls recorded at 8 m. For details and explanation, see caption for Supplementary Figure 3.5



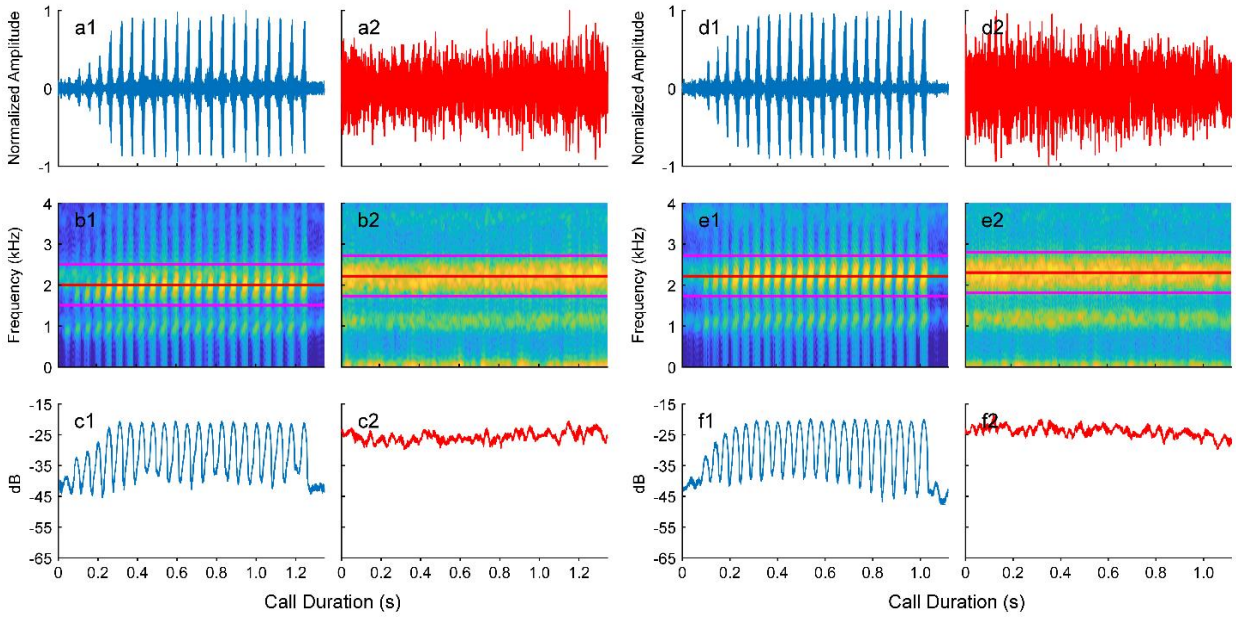
Supplementary Fig. 3.8 Spectro-temporal properties of two representative natural *H. versicolor* calls recorded at 16 m. For details and explanation, see caption for Supplementary Figure 3.5



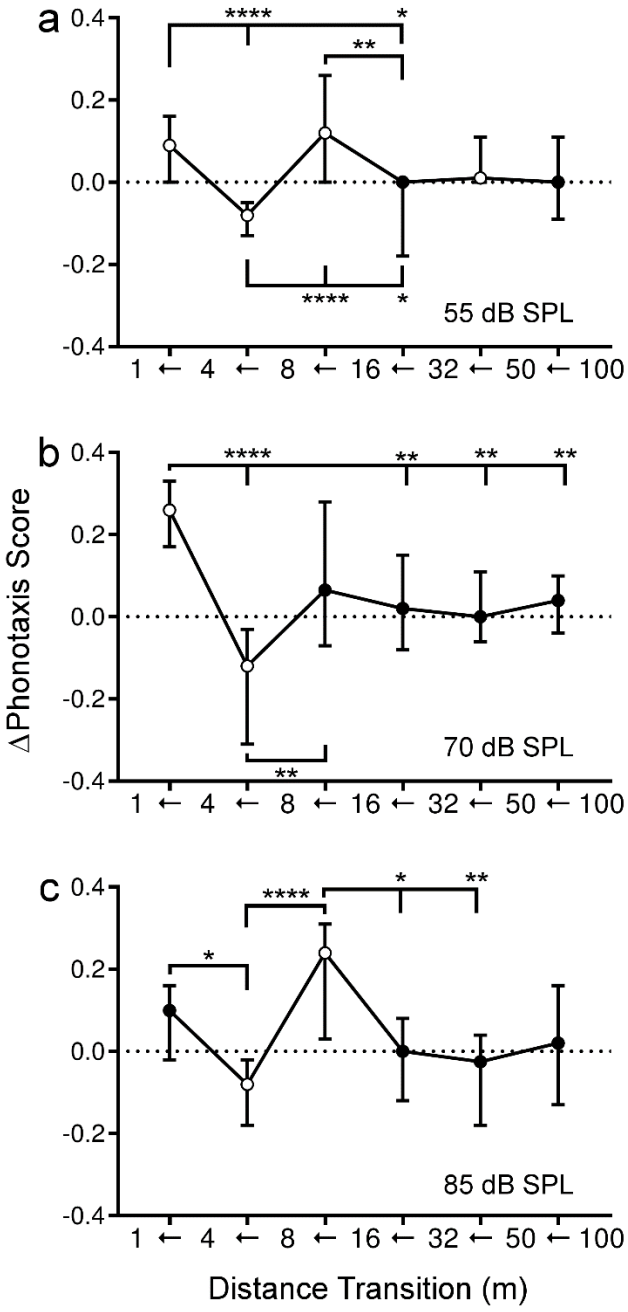
Supplementary Fig. 3.9 Spectro-temporal properties of two representative natural *H. versicolor* calls recorded at 32 m. For details and explanation, see caption for Supplementary Figure 3.5



Supplementary Fig. 3.10 Spectro-temporal properties of two representative natural *H. versicolor* calls recorded at 50 m. For details and explanation, see caption for Supplementary Figure 3.5



Supplementary Fig. 3.11 Spectro-temporal properties of two representative natural *H. versicolor* calls recorded at 100 m. For details and explanation, see caption for Supplementary Figure 3.5



Supplementary Fig. 3.12 Changes in phonotaxis score with decreasing recording distance to the calling male. Values indicate medians ± 95% CI for stimulus playback at (a) 55 dB, (b) 70 dB, (c) 85 dB SPL. Open circles indicate values differing significantly from 0 (Wilcoxon signed rank test, $p \leq 0.05$). Arrows indicate score changes are when transitioning from a stimulus recorded at the more *distant* to the more *proximal* position from the focal male. Horizontal bars and asterisks indicate significant differences with Dunn's multiple comparison tests (* $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$) after Kruskal-Wallis one-way ANOVA test (55 dB: $H_{6,217} = 33.12$; 70 dB: $H_{6,179} = 39.43$; 85 dB: $H_{6,165} = 28.12$)

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CHAPTER 4: REPRESENTATION OF ADVERTISEMENT CALL AND CHORUS SOUNDS IN THE MIDBRAIN OF THE GRAY TREEFROG, *HYLA VERSICOLOR*

4.1 INTRODUCTION

Acoustic communication is critical for the survival and successful reproduction in numerous taxa. A major challenge in auditory processing is to detect and discriminate acoustic signals of biological relevance, as in predator/prey detection (Arlettaz et al. 2001; Hartbauer et al. 2010), offspring recognition (Charrier et al. 2002; Aubin and Jouventin 2002), and mate selection (Wollerman 1999; Ronacher and Hoffman 2003; Pohl et al. 2009), in the presence of background noise or competing (a)biotic sounds. The challenge is greater when the background consists of conspecific signals having similar spectral and temporal characteristics as the signal of interest (Wollerman and Wiley 2002; Marshall et al. 2006; Vélez and Bee 2010). In addition to interference or masking from competing acoustic sources, the signals of interest may themselves be modified and distorted by propagation and interactions with the atmosphere, substrate, and flora (Dabelsteen et al. 1993; Forrest 1994; Kime et al. 2000; Coulridge and van Staaden 2004).

Most anuran amphibians use acoustic signaling for mate attraction and evaluation (Narins et al. 2007). In many species, males gather in dense choruses and emit species-specific advertisement calls. Females must then detect, localize, and discriminate amongst these calls, eventually choosing a mate. Some choruses can comprise hundreds of individual males, resulting in significant call overlapping and potential masking of spectral/temporal features of individual male calls (Marshall et al. 2006; Feng and Schul 2007). In dealing with such significant acoustic 'clutter', female

anurans might increase energy and/or time expenditure in signal discrimination. These costs, which in some systems also include potential matings with related heterospecific congeners may have significant consequences for survival and reproductive success (Gerhardt et al. 1994; Grafe 1997).

With clear behavioral choices, simple acoustic signals, and easily accessible nervous systems, anurans provide tractable systems for the study of information processing in 'noisy' environments. Most studies of anuran auditory behavior and physiology have used simple, often synthetic signals which aid the execution and interpretation of behavioral and physiological experiments but may not accurately represent conditions experienced by the animal 'in the field' (Feng and Schul 2007). Natural environments typically contain complex acoustic scenes and provide a relevant ecological and evolutionary context for hearing. Few physiological studies have attempted to closely mimic naturalistic contexts and compare auditory system performance to both laboratory and field-derived stimuli (Rheinlaender and Römer 1986; Römer et al. 1989).

Although there have been a few studies which have used recorded anuran calls to study the representation of conspecific vocalizations in the auditory system (Walkowiak 1980; Eggermont and Epping 1986; Diekamp and Schneider 1988), how natural auditory 'scenes' such as the breeding chorus are represented in the auditory system remains unknown. Previous behavioral studies (Gerhardt and Klump 1988; Swanson et al. 2007; Christie et al. 2010; Buxton et al. 2015; Vélez et al. 2017) show that 'noisy' stimuli with degraded or altered advertisement calls can act as an attractive stimulus. In this study we examined the responses of single neurons in the torus

semicircularis (TS) of gray treefrogs, *Hyla versicolor*, to natural chorus sounds recorded in the field and broadcast at natural SPLs. The TS, a homolog of the mammalian inferior colliculus, is a highly connected region of the central auditory system with many connections to both higher and lower auditory processing areas (Feng 1983; Feng and Lin 1991; Luksch and Walkowiak 1998). It is an important integration center, processing both temporal and spectral information from the ascending auditory pathway and is critical for directed phonotaxis to advertisement calls (Gooler and Feng 1992; Hall 1994; Endepols and Walkowiak 2001; Endepols et al. 2003). We investigated single TS neurons' responses to chorus sounds recorded at increasing distances from a focal male, as well as synthetic calls used previously for characterizing neuronal properties in *H. versicolor* (Diekamp and Gerhardt 1995). We examined the strength of a unit's response to the stimuli, as well as the temporal properties and variability of spike trains. An earlier behavioral study demonstrated that female *H. versicolor* were not attracted to chorus stimuli recorded at distances beyond ~32 m (Christie et al. 2010). We hypothesized that chorus sounds recorded up to distances of 32 m would elicit distance-dependent responses in a population of TS units. In addition, we predicted that the variability of neural responses would increase proportionally with increasing chorus distance. As synthetic conspecific calls played at SPLs approximating 1 m distance from a calling male regularly elicit strong phonotaxis (Gerhardt and Doherty 1988; Diekamp and Gerhardt 1995), we also predicted that TS neurons would respond strongly to synthetic calls approximating natural calls recorded at short ranges (~1 m).

4.2 MATERIALS AND METHODS

4.2.1 SUBJECTS AND COLLECTION/STUDY SITES

Experimental subjects comprised 61 (48 males and 13 females) gray treefrogs (*Hyla versicolor*) of the northwestern mitochondrial lineage (Ptacek et al. 1994). Animals were collected from breeding choruses at the Thomas Baskett Wildlife Area and Three Creeks State Park, near Ashland, Missouri. Animals were caught in amplexus by hand and kept in plastic containers with water at room temperature.

4.2.2 SURGICAL PREPARATION

Animals were anesthetized by immersion in 0.4% tricaine methanesulfonate (MS-222) with a pH of 7.4. After application of lidocaine (4%) on the dorsal surface of the head, an incision was made in the skin overlying the skull. A small opening was made in the skull with a dental drill, and the meninges were removed exposing the tectal surface, which was kept moist with buffered Ringer's solution. After the surgery, animals were allowed to recover from anesthesia and placed in a sound-proof room (Industrial Acoustics Company no. 404) whose inner walls were covered with 6" (15.24 cm) anechoic foam; the temperature was kept at 21 ± 1 °C. Animals were covered with moistened gauze to facilitate cutaneous respiration. Frogs were immobilized during the recording session with periodic intramuscular injections of *d*-tubocurarine chloride (10 µg/g body weight). Afterward, the frog was killed by an overdose of MS-222. These procedures are in compliance with the Guiding Principles for Research Involving Animals and Human Beings and have been evaluated and approved by the Institutional Animal Care and Use Committee of the University of Illinois.

4.2.3 EXPERIMENTAL PROTOCOL

Single-unit recordings were made from the left torus semicircularis (TS) with glass microelectrodes (WPI 1B100f-4) having a tip diameter of 1-2 µm; the electrodes were filled with 0.05M potassium acetate with Tris buffer (pH 7.4), resulting in impedances of ~15-30 MΩ. Electrodes were positioned on the surface of the optic tectum (overlying the TS) and advanced ventrally into the left TS via a remote-controlled piezo-electric microdrive (RSF Electronik). Neurons were recorded from multiple regions of the TS, but predominantly from the lateral regions of the principal and magnocellular nuclei (Feng 1983; Feng and Lin 1991).

Action potentials were amplified with an extracellular preamplifier (Dagan 2400), band-pass filtered (Krohnite 3700; cut-off frequencies at 100 Hz and 10 kHz) and monitored with a digital oscilloscope (Yokogawa DL1200A). Action potentials were digitized and stored on a PC using BrainWare32 software (Tucker-Davis) for off-line analysis. Stimulus generation and acquisition of neural data were made with Tucker-Davis (TDT) System-II hardware and software. Acoustic signals (see Fig. 4.1), once generated, were amplified with an audio amplifier (Sony GX59ES) and broadcast through a free-field loudspeaker (ADS 200LC). The loudspeaker was placed at a horizontal distance of ~0.55 m from the right eardrum of the frog, contralateral to the recording electrode.

4.2.4 ACOUSTIC STIMULI

Search stimuli were presented at 70 dB SPL and consisted of a series of 4 tone pulses (300, 600, 1100, 2200 Hz), each with a duration of 50 ms, followed by a natural *H. versicolor* call (17 pulses; 680 ms total duration), and a band-limited noise burst

having a flat spectrum over 250 Hz - 4 kHz and a duration of 400 ms. SPLs were measured beforehand with a SPL measuring amplifier (Bruel and Kjær 2610**,** fast RMS, high-pass 22.4 Hz filtered, re: 20 µPa) and a free-field condenser microphone (Bruel and Kjær 4165) placed at the position of the frog's head.

Acoustic stimuli consisted of chorus sounds (pre-recorded in the field at distances of 1, 4, 8, 16, 32, 50, and 100 m from a calling male on two nights when the chorus was most intense; detailed descriptions of chorus recordings and apparatus are given in Chapter 2 (or Christie et al. 2010) as well as synthetic frog calls and noise stimuli. To approximate ambient/substrate effects as perceived by female treefrogs approaching a breeding chorus, ground-level recordings of chorus sounds were used (see Chapter 2 or Christie et al. (2010)). We isolated 1.5 s segments containing the entire un-interrupted call of the focal male (Fig. 4.1; note that at long distances the focal male's call is not discernible on the oscillograms). All chorus and call stimuli were created using sound editing software (Adobe Audition). All chorus stimuli were played at the natural sound pressure levels encountered in the field (Table 4.1; see Chapter 2 or Christie et al. (2010) for details). To determine a neuron's responses to the chorus stimuli, the inter-pulse-intervals (IPIs) of the focal male's call were measured for each stimulus and used in subsequent analysis (see below).

The synthetic conspecific *Hyla versicolor* ('Hv') call was similar to those used in previous behavioral studies (Gerhardt and Doherty 1988; Bush et al. 2002; Beckers and Schul 2004; Christie et al. 2010; Christie et al. 2019). Each call contained 29 pulses of 25 ms duration, with an IPI of 25 ms. Combined with an initial 50 ms silent period, the total call length was 1.5 s. While this duration was somewhat long compared to the

population average (Gerhardt and Doherty 1988; Gerhardt 1991), we felt that the increased acoustic energy enabled a better comparison to the chorus stimuli. Each pulse consisted of 2 phase-locked sinusoids of 1.1 and 2.2 kHz, with the former being 6 dB less intense than the latter. The pulses had rise- and fall-times of 80% and 20% of the total pulse duration, respectively.

We also presented a synthetic call of the diploid sibling species *H. chrysoscelis*. In phonotaxis experiments, female *H. versicolor* are often attracted to heterospecific calls (Gerhardt and Doherty 1988), and thus we wanted to compare the responses of midbrain neurons to the calls of both species. The heterospecific call consisted of 66 sound pulses of ~11 ms duration, and an inter-pulse interval of ~11 ms. With a 60 ms initial silent period, the total call length was 1.5 s. Each sound pulse had the same power spectrum and exponential rise- and fall-times as natural *H. chrysoscelis* call pulses described previously (Gerhardt and Doherty 1988).

To control for responses to acoustic energy in general, a band-limited noise stimulus (duration = 1.5 s) was also presented – this had a flat spectrum over 0.1 - 22 kHz. The noise, conspecific, and heterospecific calls were initially presented at 85 dB SPL. In order to compare units' responses to natural chorus sounds with the responses to the synthetic Hv call, we presented a series of conspecific calls (see above) at SPL levels that matched those of the chorus sounds which frogs would encounter at various distances in the field (see Table 4.1).

When a single unit was isolated, we first presented the synthetic conspecific call, followed by the 1 m and 100 m chorus sounds. All remaining chorus stimuli were then presented in random order. Upon completion of the stimulus set, we presented the

synthetic Hv call again, to ensure that the neuron's response properties had not changed significantly during the recording session. Each stimulus presentation (chorus or synthetic) consisted of 20 repetitions with an inter-stimulus-interval of 3 s. We then broadcast the synthetic conspecific calls at the SPL values used for each chorus recording stimulus, with each call/SPL combination repeated 5 times.

Frequency-tuning curve estimation

Once the responses to chorus and synthetic acoustic stimuli were acquired, we quickly assessed the unit's frequency-tuning curve (FTC) using a series of 25 ms tone pips (5 ms rise/fall times, 300 ms inter-stimulus interval, 3 repetitions per tone), created with SigGen (Tucker-Davis Technologies). Tone frequencies ranged from 100-4000 Hz. From the FTC curves we determined the unit's 'Best Frequency' (BF) – the stimulus frequency with the lowest response threshold (dB SPL).

Histology

To ascertain that recording loci were in the TS, at the end of a recording session, we iontophoretically injected horseradish peroxidase (100-200 nA positive current for 3-5 min., with 1-min. intervals without current following each minute of injection) to the last recording locus. The frog was then allowed to recover in a container lined with a moistened substrate, at room temperature. After a survival period of 24-48 hours, the frog was anesthetized with MS-222, and perfused transcardially with buffered saline, followed by 2.5% glutaraldehyde in phosphate buffer (pH 7.4), and later 5% sucrose in phosphate buffer. The brain was removed and stored in 10% sucrose in phosphate

buffer, and later embedded in egg yolk, sectioned, and reacted with 1-1,3-3 diaminobenzidine (Feng and Lin 1991; Penna et al. 1997).

4.2.5 DATA ANALYSIS

Data were analyzed on a PC using MATLAB (The Mathworks, Inc.). We constructed peri-stimulus time histograms (PSTHs) for units' responses to all acoustic stimuli and cumulative PSTHs for selected stimuli (see below). We compared the cumulative distribution functions of the power spectra (FFT, Hanning window, 512 points) of the chorus samples used in stimulus construction using Kolmogorov-Smirnov (K-S) tests in MATLAB (The Mathworks, Inc; $\alpha = 0.05$) to justify pooling scores for chorus stimuli over the two nights. We could not falsify the null hypothesis of equal distributions, and therefore the results for the 2 nights were pooled, enabling population level analyses.

To more accurately quantify the responses of units possessing varied basic response characteristics, we classified neurons based on their responses to band-limited noise (a control stimulus). Specifically, we examined a neuron's temporal discharge patterns in response to the noise burst by examining the unit's PSTH as well as its cumulative PSTH. The overall scheme was similar to that used by Megala and Capranica (1981) to distinguish midbrain neurons on the 'peakedness' of their PSTHs. In the present study, neurons with a more 'peaked' PSTH tended to show strong onset responses, and thereafter the firing rates adapted very quickly. Units with less conspicuously peaked PSTHs tended to show sustained responses throughout stimulus presentation with slower adaptation. TS neurons could therefore be classified by

differences in firing adaptation rates. The number of cumulative PSTH bins (width = 1 ms) from stimulus onset until $1 - e^{-1}$ (≈ 0.632) of the total cumulative spike count were determined. Neurons which reached the latter within 100 ms (or in the last 100 ms if starting from stimulus *offset*) were classified as *rapidly-adapting*. Cells which reached this threshold after 100 ms, but less than 750 ms were classified as *intermediate-adapting.* Cells that reached the threshold after 750 ms were considered *slowly-adapting*. A value of 750 ms (50% of stimulus duration) was used as a cut-off for the *intermediate-* / *slowly*-adapting classification. For each adaptation-response class, we constructed a population PSTH for each of the acoustic stimuli using a bin width of 1 ms.

To quantify units' responses to various acoustic stimuli, we computed z-scores and average spike counts for each stimulus. Z-scores (Coleman and Mooney 2004) were calculated as the difference in activity (measured as spike counts) during stimulus presentation (S, mean $\bar{S}$) and a baseline silent period (B, mean $\bar{B}$) over the S.D. of the difference: $$z = \frac{\bar{S} - \bar{B}}{\sqrt{\text{Var } S - \text{Var } B - 2*\text{Covar}(S,B)}}$$

A higher z-score indicates more activity during each stimulus presentation than spontaneous firing. Spike counts, rather than firing rates, were used, as TS neurons typically have lower spontaneous activity compared with those of the auditory periphery (Hall and Feng 1990; Gooler and Feng 1992; Penna et al. 1997).

To examine the variability in response to natural and synthetic sounds, we measured the latency (in ms) of the first spike in response to acoustic stimulation, as well as the standard deviation (S.D.) of the first-spike latency. Differences in response magnitude (z-scores and average spike counts) and response variability (latency to first

spike and S.D. of the latency) to chorus and SPL-matched conspecific call stimuli were tested using Kruskal-Wallis non-parametric analysis of variance with Bonferroni post-hoc comparisons (α = 0.05, Zar 1999). For each adaptation-response type (rapidly-, intermediate-, or slowly-adapting), analysis of variance was performed on responses within all chorus stimuli and all conspecific synthetic calls, but not between these groups.

To look for regularities in spike timing, we computed first-order inter-spike-interval (ISI) histograms from the population PSTHs (bin width = 1 ms). To isolate activity (ISIs) in response to the focal male calls, we measured the average (± S.D.) inter-pulse-intervals (IPIs) from the calls of the focal male for all chorus recording distances on both exemplar nights.

4.3 RESULTS

We recorded the responses of 146 TS units from 61 animals. Of the 96 units whose frequency-tuning curves (FTCs) were determined, 61% (*n* = 59) had unimodal U- or V-shaped (Fig. 4.2a-c) FTCs. The remainder had bi- ('W'; *n* = 27 or 28%) or tri-modal ('W+'; *n* = 10 or 10%) curves (Fig. 4.2d-f). Thresholds ranged from approximately 30 – 80 dB SPL and showed no correlation with BF (Supplementary Fig. 4.1, top panel). Most cells had BFs <900 Hz (66%), 14% of cells had BFs between 900 and 1300 Hz, and the remaining 21% had BFs >1300 Hz (Supplementary Fig. 4.1, bottom panel). These values roughly correspond to the low- and mid-frequency ranges of the amphibian papilla, and the high-frequency fibers of the basilar papilla (Feng et al. 1975; Hillary 1984). Nearly two-thirds of torus units (N = 92 or 64%) did not show spontaneous

activity. Most TS neurons (68%) responded to both con- and heterospecific synthetic calls. About 17% of cells responded to neither synthetic call; only 8% responded to conspecific calls only, and the same proportion to heterospecific calls only. Practically all cells responded to the band-limited noise or tone stimuli presented at the unit's BF (~98% to either).

The number of units responding to natural chorus stimuli was a function of the recording distance / SPL (Table 4.1, Fig. 4.3, dark bars). Chorus sounds recorded at the shortest distances (1 and 4 m) elicited responses in 81% and 91% of cells, respectively. As the distance from the chorus increased, the proportion of cells responding to chorus sounds dropped rapidly, especially beyond 32 m. For the most distantly recorded chorus sounds (100 m), less than one-fifth (17%) responded. About one-fifth of TS units did not respond to *any* chorus stimulus, regardless of recording distance. The trend was similar (with the absolute percentage generally being slightly higher) when the frog was presented with synthetic calls having the same SPLs as the recorded chorus (Fig. 4.3, white bars).

The temporal discharge patterns of TS neurons in response to band-limited noise were diverse and could be classified into: *rapidly-adapting* units which demonstrated prominent onset (and/or offset) responses and rapid reductions in firing rate (usually to 0 spikes/s) within 100 ms of the stimulus (Fig. 4.4a, 4.5a, left column), *slowly-adapting* units which displayed sustained, tonic-like responses over the stimulus duration (Fig. 4.4c, 4.5a, right column), and *intermediate-adapting* ('intermediate') cells which had neither the robust onset/offset responses of the rapidly-adapting neurons, nor true sustained responses throughout the stimulus of the slowly-adapting cells (Fig. 4.4b,

4.5a, center column). The majority (76%) of TS neurons were slowly adapting, and only 10% were rapidly adapting and 14% intermediate adapting (Supplementary Fig. 4.2).

Responses to chorus and SPL-matched synthetic stimuli

The three classes of TS neurons showed different population PSTHs in response to the synthetic Hv call (Fig. 4.5b). Rapidly-adapting cells showed pronounced onset-like responses, followed by much weaker but steady responses to each call pulse. Slowly-adapting cells had weaker onset responses similar in magnitude with respect to the steady-state response; responses to individual pulses were more robust and consistent throughout the duration of the stimulus. Intermediate-adapting cells showed the weakest onset response and demonstrated strong time-locked firing to the first few 5-10 call pulses only, and thereafter the responses waned rapidly, especially during the last 500 ms of the call.

For chorus sounds at short distances (1-8 m, Fig. 4.5c, d), all cell classes displayed pronounced onset-responses, with the rapidly- and slowly-adapting cells having the largest magnitudes. At 1 and 4 m, all TS neurons showed some ability to follow the individual call pulses (Fig. 4.5c, data not shown for 4 m), with rapidly- and slowly-adapting cells showing better pulse-following responses than intermediate-adapting neurons. Chorus pulse-following responses were reduced at 8 m (Fig. 4.5d), but rapidly- and slowly-adapting cells gave time-locked responses to sound pulses in SPL-matched synthetic calls (Fig. 4.5e, insets).

With increasing distance from the focal male and the chorus (Fig. 4.5e, f), overall spike activity was markedly reduced; even the onset responses were attenuated

compared to the proximal stimuli. The three classes of TS neurons still showed some differences in response characteristics in terms of adaptation rate and response magnitude, at least up to 50 m (data not shown). For the SPL-matched synthetic stimuli, both rapidly- and slowly-adapting cells were able to respond to call pulses at the 16 m SPLs (data not shown), while the intermediate cells did not. At lower SPLs the rapidly-adapting cells failed to respond to the majority of call pulses, leaving only a weak onset response for the 50 and 100 m SPLs. In contrast, the slowly-adapting cells responded to pulses at SPLs from 16-50 m, only losing time-locking ability at 100 m (Fig. 4.5e, f, insets).

Response strengths

Rapidly-adapting cells showed higher z-scores and spike counts for conspecific synthetic calls than noise bursts (Fig. 4.6a, b), but these differences were not statistically significant. In contrast, intermediate- and slowly-adapting cells showed the opposite trend – the difference was only significant for the intermediate cells (Fig. 4.6c-f).

For rapidly- and intermediate-adapting neurons, an increase in chorus distance or reduction in SPL beyond 4 m appeared to only marginally affect the z-scores and spike counts (Fig. 4.6a-d). On the other hand, responses of slowly-adapting neurons to chorus sounds showed systematic reductions in both z-scores and average spike counts with increasing recording distance (Fig. 4.6c). For all cell classes, responses to the SPL-matched synthetic calls at 1 m had the highest z-scores and spike counts, though these measures were quite variable in rapidly- and intermediate-adapting cells

(Fig. 4.6a-d). Beyond 1 m, both response metrics tended to attenuate progressively with decreasing SPL of the synthetic calls. For the rapidly-adapting cells, analysis of variance tests found no significant differences within responses to chorus stimuli and Hv calls at increasing distances. For the intermediate adapting cells, band-limited noise stimuli had significantly higher z-scores and spike counts than the Hv controls (Fig. 4.6c, d). Slowly-adapting cells showed significant differences in responses to chorus sounds (Fig. 4.6e, f). Compared to the Hv call, activity was significantly lower for the 8-100 m chorus sounds, whereas responses to noise and 1-4 m chorus did not differ.

Response Variability

In all cell classes, the first spike latency (and S.D.) was generally lower for SPL-matched synthetic Hv calls compared to the chorus. As with response magnitudes, the response latencies and their variability differed between the three cell groups. For chorus stimuli, both the latency and its S.D. were more variable in rapidly-adapting cells (Fig. 4.7a, b) than intermediate-adapting cells (Fig. 4.7c, d), which were in turn, more variable than the slowly-adapting cells (Fig. 4.7e, f). Rapidly-adapting cells displayed no consistent trends with increasing chorus distance or decreasing synthetic call SPL. For intermediate- and slowly-adapting cells, increasing chorus distance or reducing the SPL of the Hv call increased the first spike latency and its S.D. (Fig. 4.7c-f). For intermediate-adapting cells, the first spike latencies for 100 m chorus were significantly longer than either for the Hv control or noise stimulus, but otherwise there were no other differences. The variability of slowly-adapting cells' latencies to chorus and synthetic stimuli were similar, and generally less than for the other groups. Slowly-adapting cell

latencies for the 100 m chorus were greater than the synthetic Hv control, while those for both the 1 m and noise stimuli were less (Fig. 4.7e). For latency S.D., values for the 8-100 m chorus were larger than the Hv control stimulus, while there were no differences between the Hv, noise, and 1-4 m chorus stimuli (Fig. 4.7f).

Spike and call timing

Inter-spike-interval (ISI) histograms were constructed to examine periodicity in spike timing and to determine how closely it corresponded to the periodicity in the natural and synthetic stimuli. In response to the control Hv call, all cell response classes exhibited strong periodicity with at least two prominent peaks (Fig 4.8a; Supplementary Fig. 4.3a). The first (~4-8 ms) represents the driven firing rate of the cell, while the second (~50 ms) corresponds to the synthetic call inter-pulse interval (IPI, see Materials and Methods). This was confirmed by the responses to noise (Fig. 4.8b; Supplementary Fig. 4.3b): here the ISI histograms displayed only the first, driven-rate peak; the second peak was absent. At 1 m, all three cell groups had peaks corresponding to the IPIs of the focal male (mean IPI: 50.37 ms, S.D.: 4.68 ms; mean shown as a dotted line, with the ±S.D. indicated by the grey bar). With increasing distance both the driven and IPI peaks declined in size for both chorus and synthetic stimuli (Fig. 4.8d, e; Fig. 4.9a-d; Supplementary Fig. 4.3d, e; Supplementary Fig. 4.4a-d). This was most noticeable in the intermediate-adapting cells, whose responses retained significant IPI peaks only at 1 and 4 m (Fig. 4.8c, d; Supplementary Fig. 4.3c, d), whereas rapidly-adapting cells' IPI peaks were visible for chorus and synthetic stimuli up to 32 m (Fig. 4.9b; Supplementary Fig. 4.4b). For slowly-adapting cells, the chorus responses displayed an IPI peak up to

50 m (Fig. 4.9b, c; Supplementary Fig. 4.4b, c), and the SPL-matched artificial calls still elicited an IPI peak at the lowest SPL, corresponding to a recording distance of 100 m (Fig. 4.d; Supplementary Fig. 4.4d).

Looking at population spike train autocorrelation shows strong periodicity matching call IPIs for the synthetic control call (Supplemental Fig. 4.5a), with the rapidly- and slowly-adapting cells showing the least 'jitter' compared to the intermediate-adapting cells. Conversely, no periodicity was seen in response to the noise stimulus, regardless of adaptation type (Supplemental Fig. 4.5b). For the chorus stimuli, at the most proximal recording distances (1-8 m; Supplementary Fig. 4.5c-e), rapidly-adapting cells quickly lost clear periodicity around 4-8 m, while remaining sensitive to the amplitude-matched synthetic Hv calls. Intermediate-adapting cells showed some periodicity for the 1st order interval at 1 m, which attenuated by 8 m. At 8 m, even the response to the SPL-matched Hv call was reduced, though still present (Supplementary Fig. 4.5e, inset). The slowly-adapting neurons showed periodical firing to both chorus and Hv stimuli out to 8 m. For the most distantly recorded stimuli (16-100 m; Supplementary Fig. 4.6a-d), both rapidly- and intermediate-adapting units showed little, if any periodicity to the natural chorus. Rapidly-adapting cells followed the SPL-equalized Hv calls out to 32 m, while intermediate-adapting cells showed marginal responses at 50 and 100 m. Again, the slow-adapting neurons demonstrated the most periodic activity, with natural stimulus responses at 16 m, and synthetic at 100 m.

4.4 DISCUSSION

The present investigation represents the first study of how natural chorus sounds from various distances are encoded in the frog CNS, and how those responses compare to SPL-matched synthetic Hv stimuli. We found that TS neurons are non-homogeneous in their temporal discharge patterns in response to noise – which is unsurprising given the diversity of their morphology (Feng 1983), connectivity (Feng and Lin 1991; Endepols and Walkowiak 2001; Luksch and Walkowiak 1998), chemoarchitecture (Endepols et al. 2000), biophysical characteristics (Yang et al. 2009), and frequency and temporal coding properties (Fuzessery and Feng 1982; Rose and Gooler 2007).

Most neurons (61%) had unimodal ‘U’/’V’ FTCs, with the remainder possessing bi- or tri-modal ‘W’/’W+’ FTCs. This is a lower unimodal percentage than seen in the grass frog *Rana temporaria*, with 83% unimodal cells, and 17% bi/tri-modal neurons (Hermes et al. 1982). Most recorded cells had BFs <900 Hz (66%), with fewer cells having mid- (900-1300 Hz; 14%) and high-range (>1300 Hz; 21%) BFs. These values differ significantly from those of Diekamp and Gerhardt (1995), who found a more equal BF distribution, with a much higher proportion of mid- (34%) and high-range (36%) neurons. Our thresholds were slightly narrower, ~10 dB higher at the low, and ~10 dB lower at the high ends of the distribution (30-80 dB vs, 20-90 dB). It is difficult to completely account for these differences. One potential factor is the season in which the recordings were performed. Walkowiak (1980) demonstrated that multiple response properties, including spontaneous activity, BF distribution, responsiveness to stimuli, and temporal response patterns vary significantly across the mating and quiescent

seasons. Diekamp and Gerhardt (1995) presumably performed most recordings during the mating season (May-June), while our recordings spanned the post-mating season time through the early spring. Similar to other studies, we found low rates of spontaneous activity in the TS, with only 36% of the recorded neurons demonstrating significant activity. This proportion corresponds closely to those reported for the European fire-bellied toad (65% during the resting season), *Bombina bombina* (Walkowiak 1980), and the grass frog (56-64%), *R. temporaria* (Epping and Eggermont 1985, 1986a), but is a significantly higher percentage than that seen in the leopard frog, *Lithobates pipiens* (Goense and Feng 2005) and in both *Hyla versicolor* and *Hyla chrysoscelis* (Rose et al. 1985). Comparisons between species are complicated by potential species differences and the known seasonal variabilities (Walkowiak 1980).

Over 90% of units responded to the synthetic Hv call at 83-85 dB SPL, while more than two-thirds of recorded neurons responded to both con- and heterospecific synthetic mating calls. This demonstrates a comparative lack of specificity also seen in other studies of *H. versicolor* (Diekamp and Gerhardt 1995) and other species, including the marsh frog, *Rana* (*Pelophylax*) *ridibunda* (Diekamp and Schneider 1988). More *H. versicolor* neurons (80.8%) responded to recorded natural calls (chorus recordings at 1 m) than found previously in *B. bombina* (51.6%, Walkowiak 1980), but proportions were similar to those found in *R. temporaria* (74.1%, Walkowiak 1980; 80.6%, Epping and Eggermont 1986b), and *R. ridibunda* (75%; Diekamp and Schneider 1988). Almost all isolated TS units responded to noised bursts and/or tone pips, which was also found in both *B. bombina* and *R. temporaria*, although in both cases the proportion of units responding to *noise* bursts were significantly lower (57.1 - 85.6%, Walkowiak 1980).

We observed three classes of TS neurons based on the unit's firing rate adaptation to broadband noise bursts: rapidly-, intermediate-, and slowly-adapting (Fig. 4.4). Rapidly-adapting cells had strong onset/offset behavior and generally only fired a few spikes before becoming quiescent. Intermediate-adapting cells had slightly less prominent onset responses but also tended to fire several hundred ms into stimulus presentation, with moderate rate adaptation. Slowly-adapting cells had the least conspicuous onset responses and tended to fire at relatively stable steady-state rates through the noise burst duration. Even the majority of slowly-adapting cells tended to have some onset responsiveness (Fig. 4.4c, left column), similar to auditory nerve the dominant PSTHs from auditory nerve fibers of bullfrogs (Megala and Capranica 1981), cane toads (Carlile and Pettigrew 1984), *R. ridibunda* (Diekamp and Schneider 1988), and leopard frogs (Feng et al. 1991). Inter-study and interspecies comparisons are fundamentally complicated by the (often simultaneous) use of differing species, stimulus parameters (noise burst vs. tone pip, stimulus duration, rise-time, ISI, SPL, etc.), and delivery methods (tympanically-coupled vs. free-field). Nonetheless, our results bear some functional similarity to those of other studies. The rapidly-, intermediate-, and slowly-adapting neuron responses resemble ‘pure-phasic’, ‘phasic / tonic’, and ‘pure-tonic’ cell responses to noise bursts of Walkowiak (1980). In response to tone pips, Gooler and Feng (1992) categorized TS cells in *L. pipiens* as ‘phasic’, ‘phasic-burst’, and ‘tonic’, with firing patterns closely resembling those in the present study. The relative abundance of slowly-adapting cells (76%, compared to 14% intermediate- and 10% rapidly-adapting) is greater than what has been reported in the TS of other anuran species (48.4%, Walkowiak 1980), but similar proportions were reported in *L. pipiens*

(67% tonic, 14% phasic-burst, 19% phasic, Gooler and Feng 1992). Stimulus type and structure might account for some of these differences. Walkowiak (1980) suggested that unit responses to noise stimuli differ from those obtained by tones, leading to a higher instance of tonic-like responses. The physiological origins of TS cell response differences are not fully clear. It has been suggested that response properties of torus cells are the result of different afferent connections from the lower brainstem auditory nuclei (Carlile and Pettigrew 1984; Diekamp and Schneider 1988, Endepols and Walkowiak 2001).

Compared to mammalian studies using natural vocalization stimuli, anuran midbrain neurons have relatively low selectivity for conspecific sounds (Šuta et al. 2003; Pincherli et al. 2007). TS neurons exhibited very little species call selectivity, with over two-thirds of neurons responding to both the *H. versicolor* and *H. chrysoscelis* synthetic calls, and only 8% of neurons were sensitive to the conspecific call alone. Female *H. versicolor* will make phonotactic approaches to the heterospecific mating call (Chapter 3, or Christie et al. 2019; Gerhardt and Doherty 1988). Rose et al. (1985) demonstrated that TS unit AM rate tuning curves were generally broad, and only slightly biased to conspecific rates. Diekamp and Gerhardt (1995) found that <30% of neurons had modulation rate band-pass selectivity that spanned conspecific call pulse rates, with units generally having broad modulation rate preferences, spanning both treefrog species. A lack of mating call specificity has also been reported for several other frog species, including *B. bombina* (Walkowiak 1980), *R. ridibunda* (Diekamp and Schneider 1988), and *R. temporaria* (Walkowiak 1980; Epping and Eggermont 1986b; Eggermont and Epping 1986). Our results provide further evidence for the hypothesis that

discrimination between signals with different temporal properties may take place in the thalamus (Hall and Feng 1987; Diekamp and Schneider 1988; Penna et al. 1997). In this model, the TS is the first auditory processing center where neurons become selective 'feature detectors' for individual call parameters including pulse duration, rise/fall times, and AM rate / duration (Walkowiak 1984; Gooler and Feng 1992). These units then project to upstream centers in the di- and/or telencephalon, onto 'coincidence' detecting neurons. There is additional evidence that some of this computation may start as early as the TS, as neural activity-dependent *egr-1* gene expression in the laminar nucleus of the TS was the highest and most specific to stimuli with spectral call features known to be sufficient for species recognition and female phonotaxis in the túngara frog, *Engystomops* (fr. *Physalaemus*) *pustulosus* (Mangiamele and Burmeistser 2011). Most electrophysiological studies of the frog TS have been based on recordings made from the principal and magnocellular nuclei, as opposed to the laminar nucleus, which provide the bulk of TS output to the thalamus and telencephalon (Feng and Lin 1991).

Of the three TS neuron classes described, the slowly-adapting cells show the highest potential for detecting a male's calls in a chorus. Compared to rapidly- and intermediate-adapting cells, slowly-adapting units show systematically graded time-locked responses (with ISIs that match the average IPI of the chorus stimuli and SPL-matched synthetic calls) and first-spike latencies with low variability with increasing distance (Fig. 4.6e-f; Fig. 4.7e-f). Furthermore, the time-locked responses extend to greater distances from the focal male and lower SPLs compared to the other two classes (Fig. 4.9b-d; Supplementary Fig. 4.4b-d; Supplementary Fig 4.6a-d). Our previous behavioral study (Chapter 2, or Christie et al. 2010) has shown that female

treefrogs orient toward chorus sounds recorded from distances of 1 m up to 32 m from a calling male; beyond this distance, the frog's orientation is largely random. Considering the various response metrics described in the present study, there are no large or sudden changes between responses to the chorus at 32 m and those at 50-100 m. All response adaptation groups demonstrated some population activity to both stimuli. How might the various subpopulations of TS units behave in the field? As female *H. versicolor* approach the breeding chorus from daytime resting sites, the population of slowly-adapting cells would likely be the first to respond. At some threshold (presumably based on net population activity) the responses would be of sufficient magnitude to enable the female to 'detect' the chorus and allow orientation. As the female nears the chorus, the chorus background would get louder, and males on the edge of the chorus - closest to the female - might be perceptible above males calling from further away. More cells would respond, including higher-threshold rapidly- and intermediate-adapting cells. At close distances to the focal male (1-8 m), females would be able to detect individual call pulses, and eventually discriminate and evaluate their temporal and spectral properties.

The relative contributions of the TS response types to call discrimination is not currently known, although *H. versicolor* females are extremely sensitive to small changes in the temporal structure of conspecific calls (Gerhardt and Doherty 1988; Diekamp and Gerhardt 1995; Bush et al. 2002). Future studies examining the effects of noise on TS responses and call encoding would be valuable, including those using 'naturalistic' synthetic chorus noise which would allow for the fine-tuning of signal and noise properties (Bee and Schwartz 2009; Kuczynski et al. 2010). In Chapter 3 (or

Christie et al. 2019), we demonstrated that the degradation of *timing* cues and information present in *H. versicolor* calls is significantly more effective in reducing the attractiveness of chorus stimuli than reducing the SPL. In addition, previous studies have demonstrated that masking noise (with spectral energy in the frequency bands of male calls) can 'fill in' call inter-pulse intervals, disrupting discrimination (Kuczynski et al. 2010). The effect of stimuli using natural or artificial 'chorus-shaped' noise on the preferences for individual temporal features of the *H. versicolor / chrysoscelis* complex have yet to be examined in detail. There is some evidence that females of both species may use different fine temporal characteristics in call evaluation (Bush and Schul 2002). How noise affects these mechanisms and their representation in the auditory system at the neural level is equally unclear. Also unknown is the relative contribution of different TS cell types to the various stages of auditory-guided behavior (i.e. detection, orientation, discrimination, etc.). Recent advances in whole-brain immunohistochemical labeling of immediate-early gene activity in anurans (Chakraborty et al. 2010) are well-placed to begin to answer these questions. In addition, investigations at the whole organism (phonotaxis experiments) as well as at the single cell levels (*in vivo* slice studies, patch-clamping, e.g. Yang et al. 2009) are required if we are to gain a better understanding of how the nervous system processes noisy but relevant acoustic signals, and to link neural activity to whole-animal behavior.

4.5 ACKNOWLEDGEMENTS

We thank the University of Missouri-Columbia and the Missouri Dept. of Conservation for site access and capture permits. Carl Gerhardt provided advice and material support, and Paul Kimball, Stephanie Kleyman, and Leah Reynolds aided in animal collection. Research funding was provided by a National Science Foundation Collaborative Research in Computational Neuroscience (CRCNS-0422073) grant to ASF and a National Science Foundation grant (IOB-0445286) to JS. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois.

4.6 TABLE

Chorus stimulus responsiveness				
Stimulus	dB SPL	Total cells	# of units	% of total
1 m	83-86	146	118	80.82
4 m	77-82	111	101	90.99
8 m	72-78	136	84	61.76
16 m	70-75	109	72	66.06
32 m	65-69	109	61	55.96
50 m	59-64	112	44	39.29
100 m	47-53	145	24	16.55
Non-responsive	--	146	28	19.18
Synthetic call at chorus SPL stimulus responsiveness				
Chorus stimulus SPL	dB SPL	Total cells	# of units	% of total
1 m	83-86	68	65	95.59
4 m	77-82	67	59	88.06
8 m	72-78	64	57	89.06
16 m	70-75	65	56	86.15
32 m	65-69	66	42	63.64
50 m	59-64	66	34	51.52
100 m	47-53	67	17	25.37
Non-responsive	--	68	3	4.41

Table 4.1 Proportion of torus units responding to synthetic and natural chorus stimuli

4.7 FIGURES

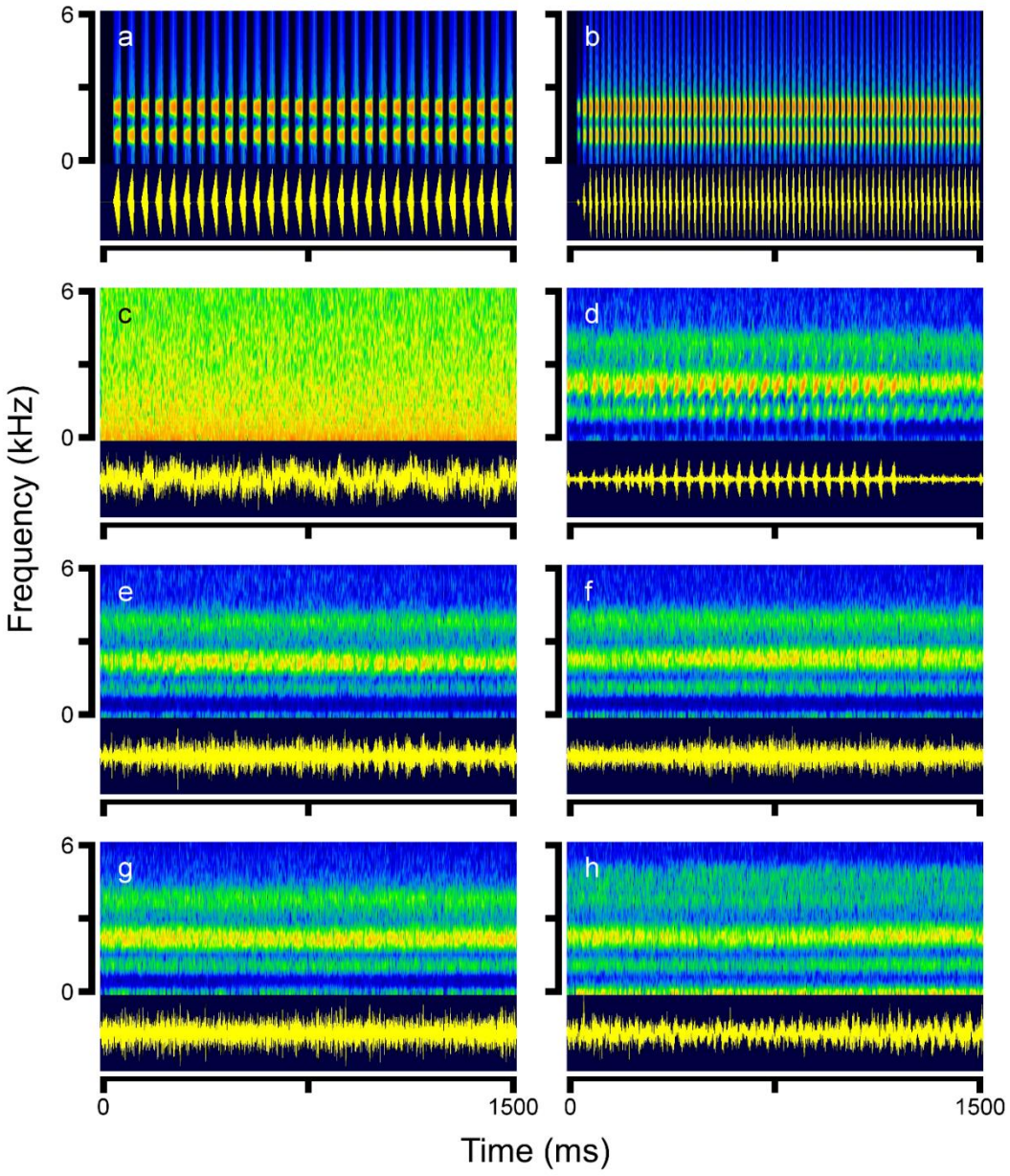


Fig. 4.1 Oscillograms (bottom) and spectrograms (top) of synthetic and chorus stimuli examples. (a) Synthetic *Hyla versicolor* call, (b) Synthetic *Hyla chrysoscelis* call, (c) Band-limited noise, (d) 1 m chorus, (e) 8 m chorus, (f) 16 m chorus, (g) 32 m chorus, (h) 100 m chorus

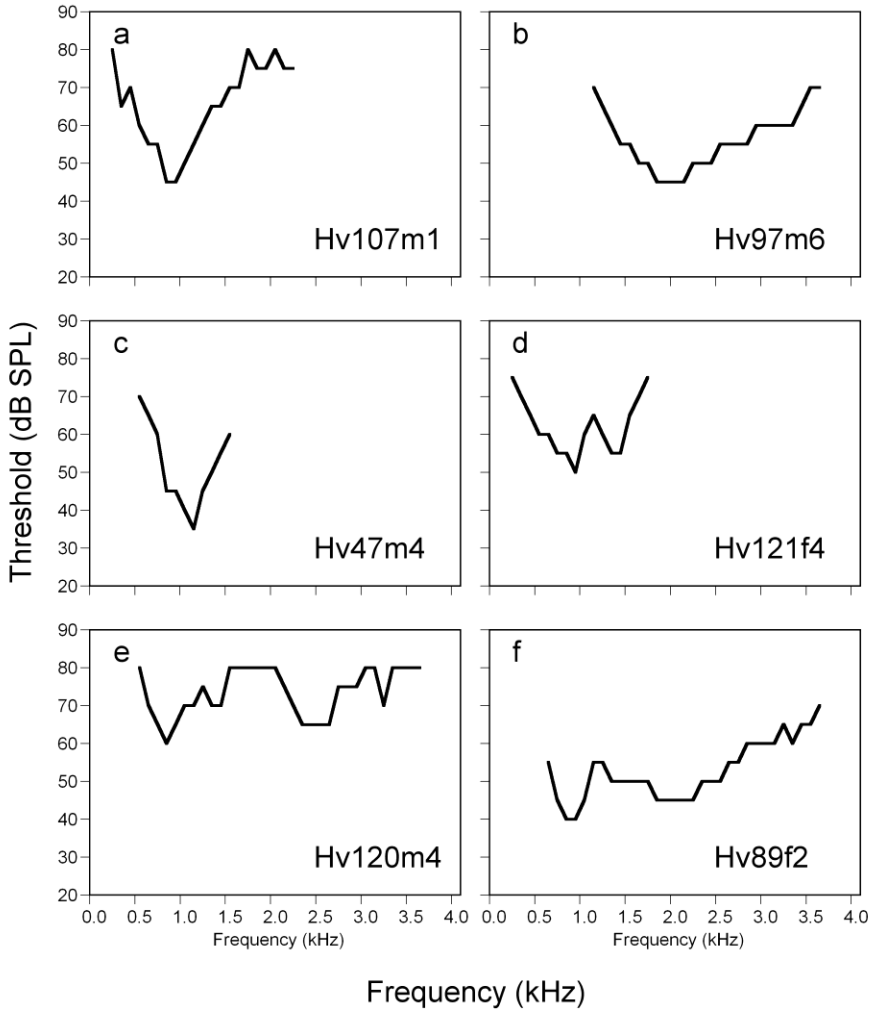


Fig. 4.2 Representative frequency-tuning curves (FTCs) of selected torus cells. (a-c) U/V-shaped FTCs, (d-f) W and W+ FTCs

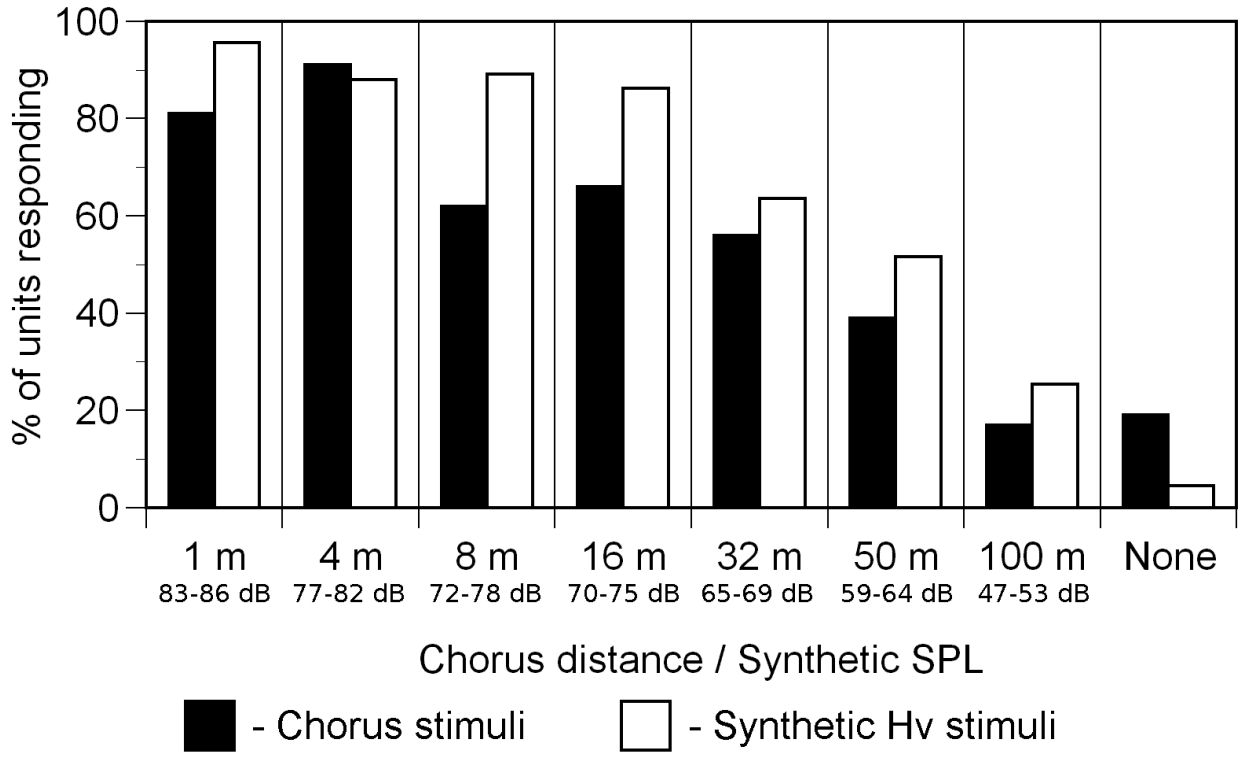


Fig. 4.3 Number of cells (expressed as % of total) responding to chorus stimuli recorded at specified distances (dark bars), or to synthetic conspecific calls at the same SPL as chorus sounds at the recording distance (white bars). Total cell counts for chorus ('X m') and SPL-matched synthetic calls ('Hv'): 1 m (*n* = 143), Hv (*n* = 69); 4 m (*n* = 108), Hv (*n* = 68); 8 m (*n* = 133), Hv (*n* = 66); 16 m (*n* = 107), (*n* = 67); 32 m (*n* = 107), (*n* = 67); 50 m (*n* = 109), Hv (*n* = 67); 100 m (*n* = 143), Hv (*n* = 68); Non-responsive to chorus (*n* = 143), Non-responsive to Hv (*n* = 69). SPLs for each stimulus are displayed below bars

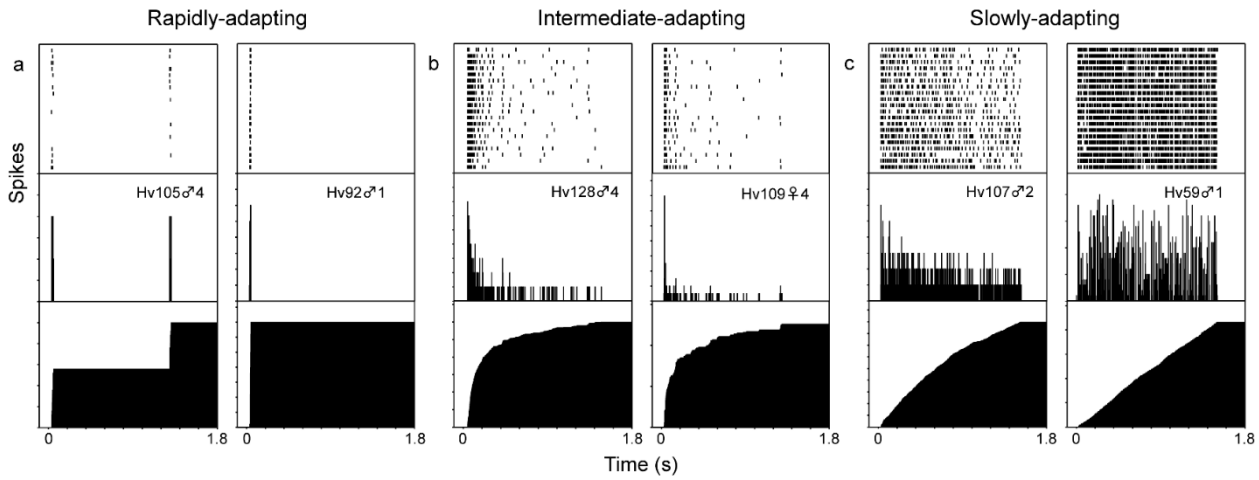


Fig. 4.4 Representative raster plots (top), PSTHs (middle), and cumulative PSTHs (bottom) of the three adaptation classes of temporal discharge patterns in response to band-limited noise (1500 ms duration). (a) Two rapidly-adapting cells, (b) Two intermediate-adapting cells, (c) Two slowly-adapting cells. PSTH bin width = 1 ms. Response magnitudes are not to scale

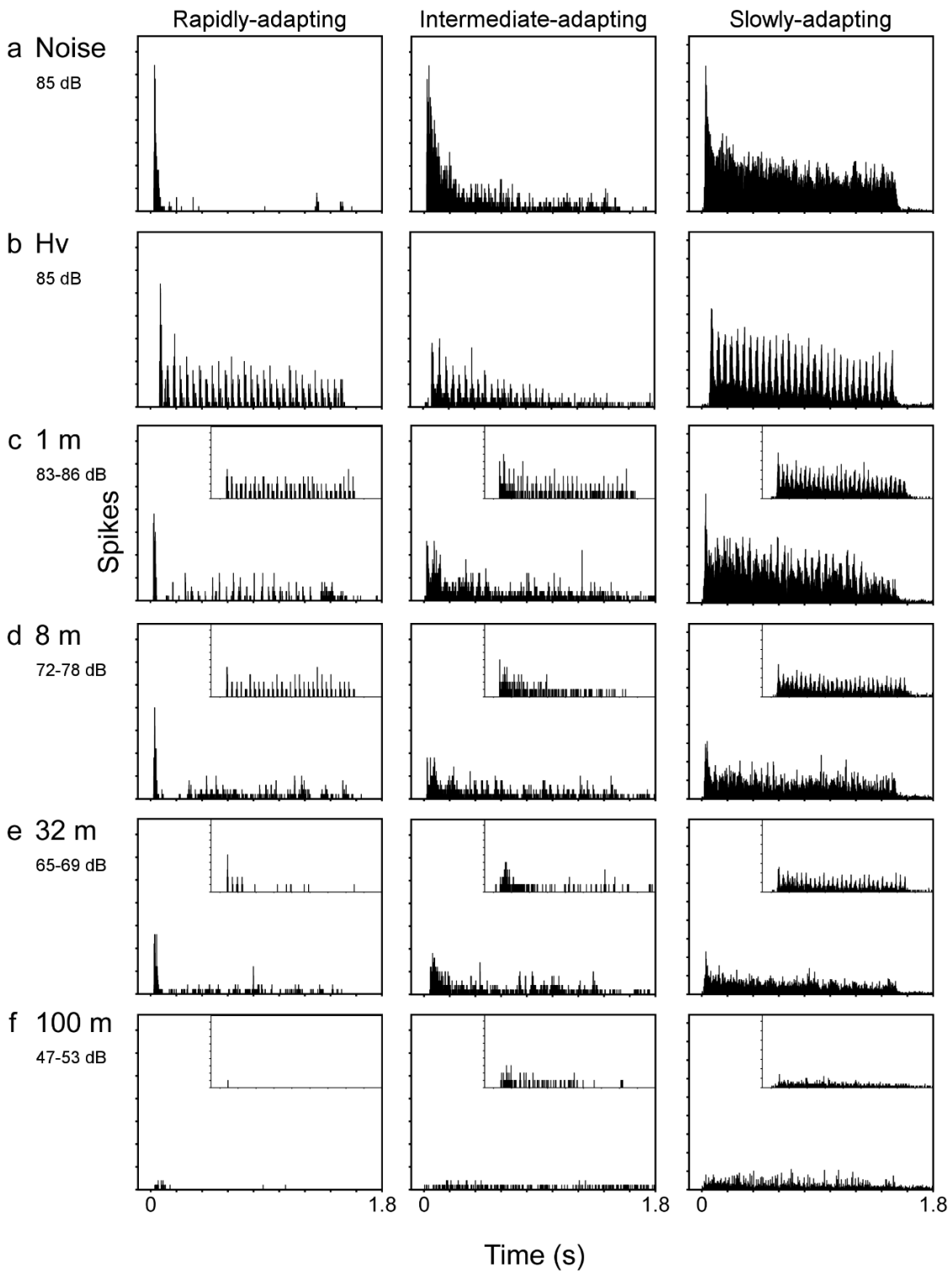


Fig. 4.5 Population PSTHs of rapidly-, intermediate-, and slowly-adapting TS cell responses to noise, chorus, and synthetic call stimuli. Chorus stimuli are represented by the larger histograms, while insets show responses to synthetic conspecific calls broadcast at the same SPL as the corresponding chorus

Fig. 4.5 (cont.) stimulus. Sound levels are in dB SPL. Response magnitude (ordinate) scales for chorus responses of rapidly-, intermediate-adapting, and slowly-adapting cell insets are 5x. Scales for rapidly- and intermediate-adapting cell insets are 20x. (a) Band-limited noise (*n* = 13, 21, 101), (b) Control synthetic conspecific call (*n* = 13, 21, 102). Total cell counts for chorus ('X m') and SPL-matched synthetic calls ('Hv'): (c) 1 m chorus (*n* = 13, 12, 102), Hv (*n* = 5, 7, 55) (d) 8 m chorus (*n* = 13, 21, 95), Hv (*n* = 5, 7, 51), (e) 32 m chorus (*n* = 9, 11, 84), Hv (*n* = 5, 7, 53), (f) 100 m chorus (*n* = 12, 21, 101), Hv (*n* = 5, 7, 55). Numbers represent *n*-values for rapidly-, intermediate-, and slowly-adapting response groups, respectively

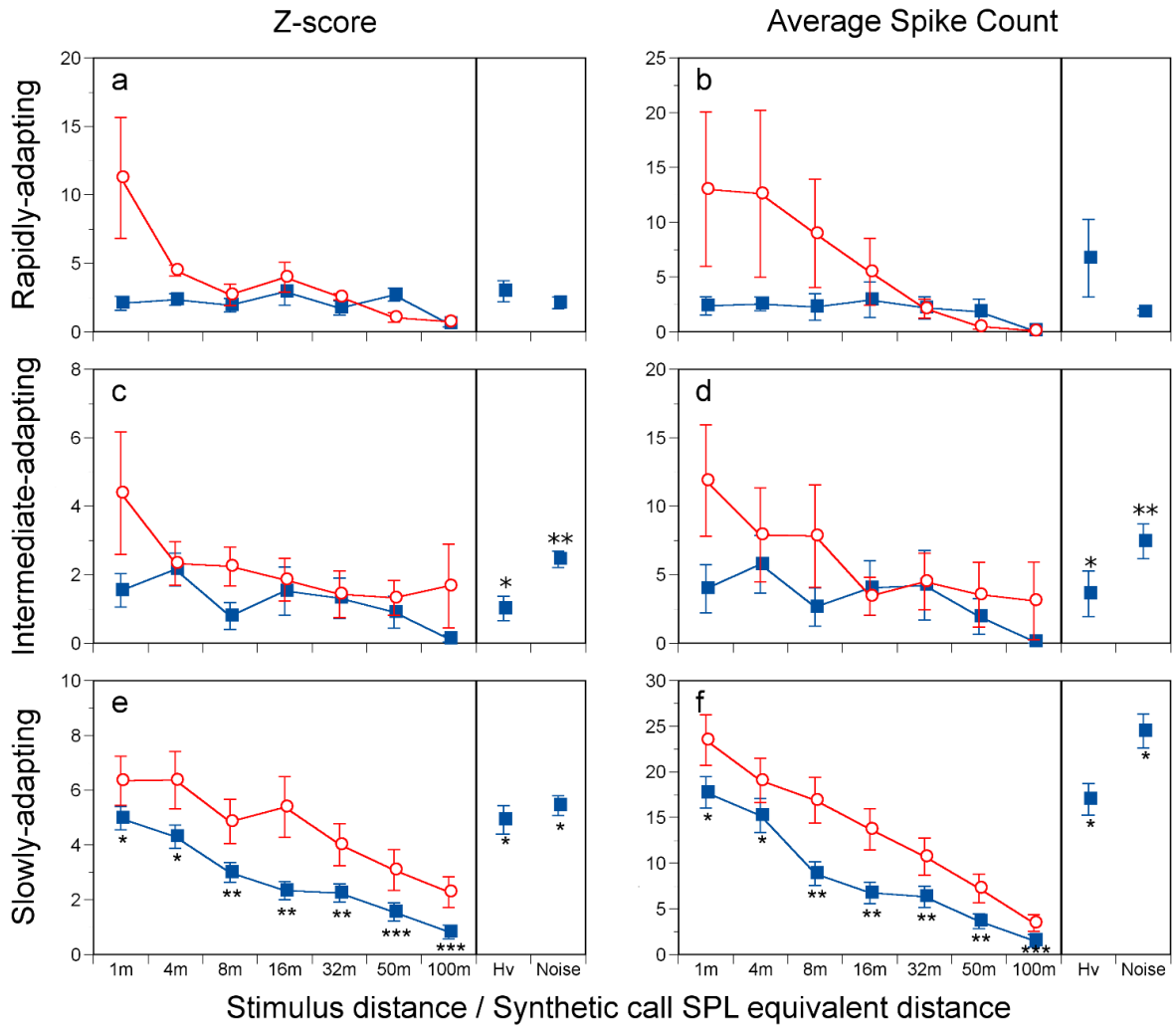


Fig. 4.6 Response strengths (z-scores) to chorus and synthetic stimuli. Blue squares (■) indicate values for chorus and control synthetic stimuli; red circles (O) indicate values for SPL-matched synthetic conspecific calls. Left column shows z-scores for rapidly- (a), intermediate- (c), and slowly-adapting (e) cells. Right column shows average spike counts for rapidly-adapting (b), intermediate-adapting (d), and slowly-adapting (f) cells. All values are shown ± SEM. Asterisks indicate chorus stimulus Bonferroni post-hoc comparison groups with significantly different medians with respect to the control conspecific 'Hv' stimulus (Kruskal-Wallis one-way ANOVA, $p \leq 0.05$)

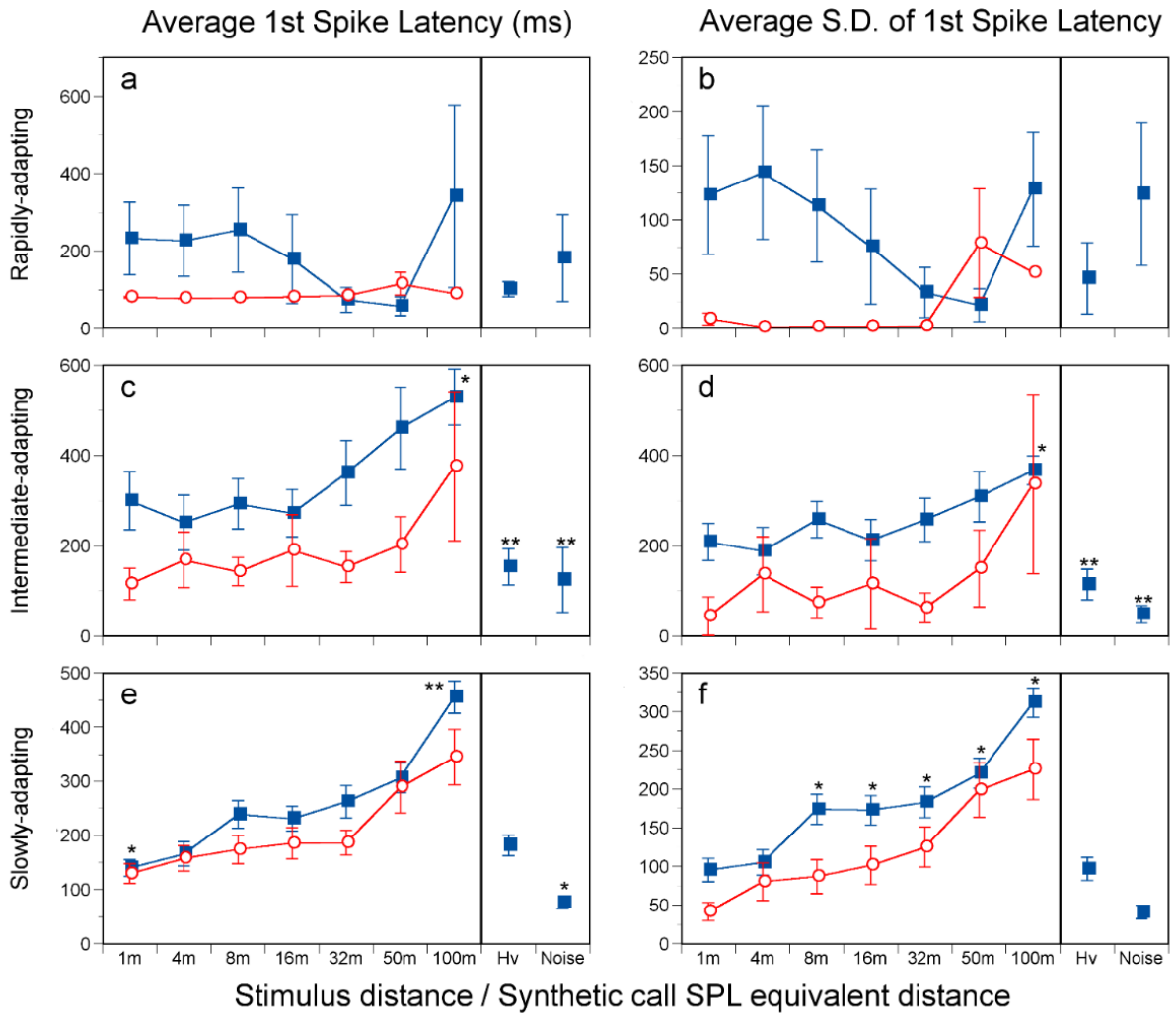


Fig. 4.7 Response variability to chorus and synthetic stimuli. Blue squares (■) indicate values for chorus and control stimuli; red circles (O) indicate values for synthetic conspecific calls broadcast at equivalent SPLs as the chorus stimuli. Left column shows average latency to the first spike (in ms) for (a) rapidly-, (c) intermediate-, and (e) slowly-adapting cells. Right column shows the standard deviation of first spike latency for (b) rapidly-adapting, (d) intermediate-adapting, and (f) slowly-adapting cells. All values are shown ± SEM. Asterisks indicate chorus stimulus Bonferroni post-hoc comparison groups with medians significantly different with respect to the control conspecific 'Hv' stimulus (Kruskal-Wallis one-way ANOVA, $p \leq 0.05$)

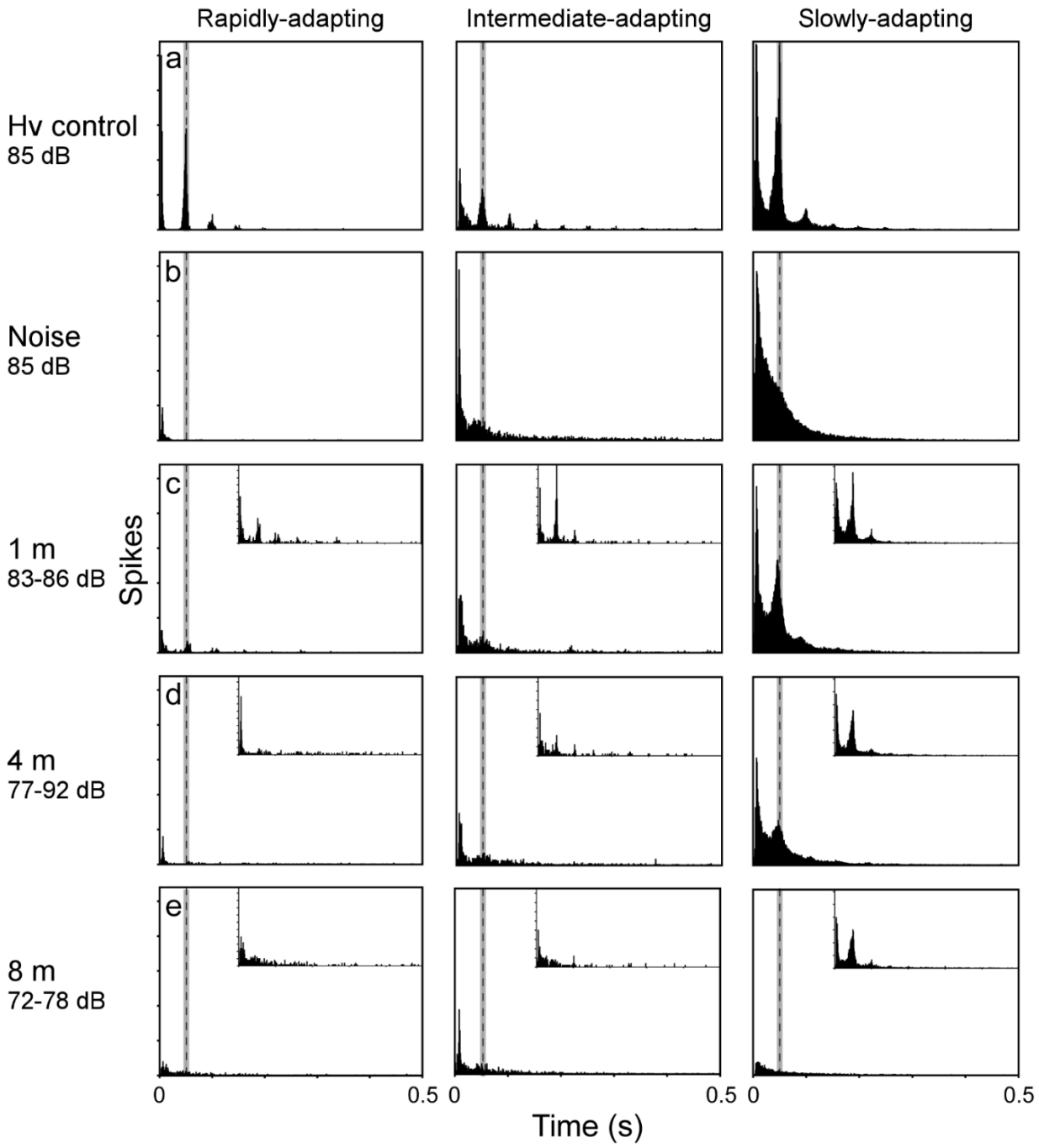


Fig. 4.8 First-order ISI histograms for rapidly-, intermediate-, and slowly-adapting cells in response to chorus and synthetic stimuli. The dotted lines and gray bar areas represent the mean ± S.D. of the IPIs of the focal males recorded by the hypercardioid microphone at that distance – see Materials and Methods. Chorus stimuli are represented by the larger histograms, while insets show responses to synthetic conspecific calls broadcast at the same SPL as the corresponding chorus stimulus. Inset ISI histogram

Fig. 4.8 (cont.) magnitude axis is 5x for all stimuli. Sound levels are in dB SPL. (a) Control synthetic conspecific call (*n* = 13, 21, 102), (b) Band-limited noise (*n* = 13, 21, 101), Total cell counts for chorus ('X m') and SPL-matched synthetic calls ('Hv'): (c) 1 m chorus (*n* = 13, 12, 102), Hv (*n* = 5, 7, 55), (d) 4 m chorus (*n* = 9, 12, 84), Hv (*n* = 5, 7, 54), (e) 8 m chorus (*n* = 13, 21, 95), Hv (*n* = 5, 7, 51). Numbers represent *n*-values for rapidly-, intermediate-, and slowly-adapting response groups, respectively

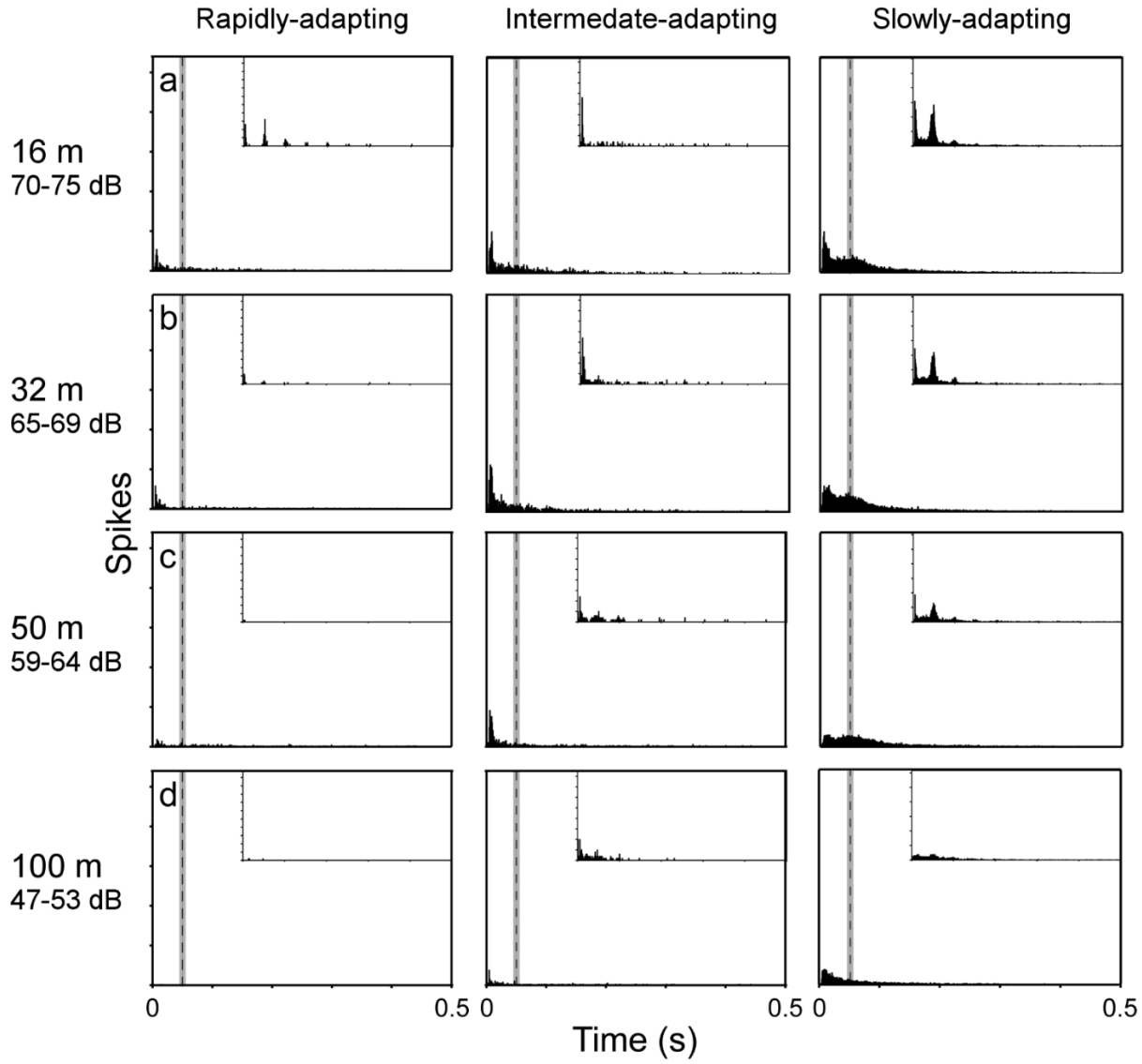
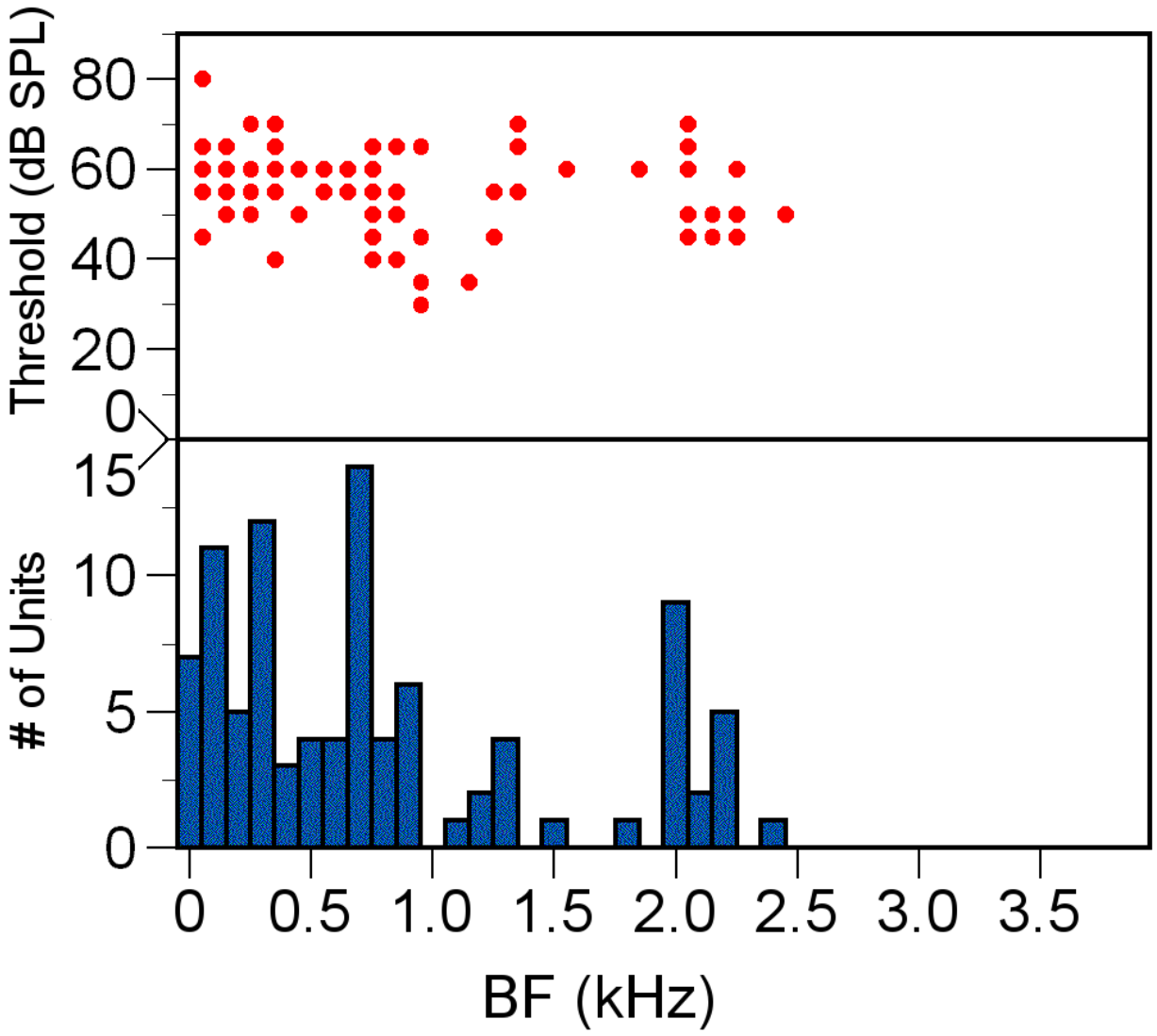
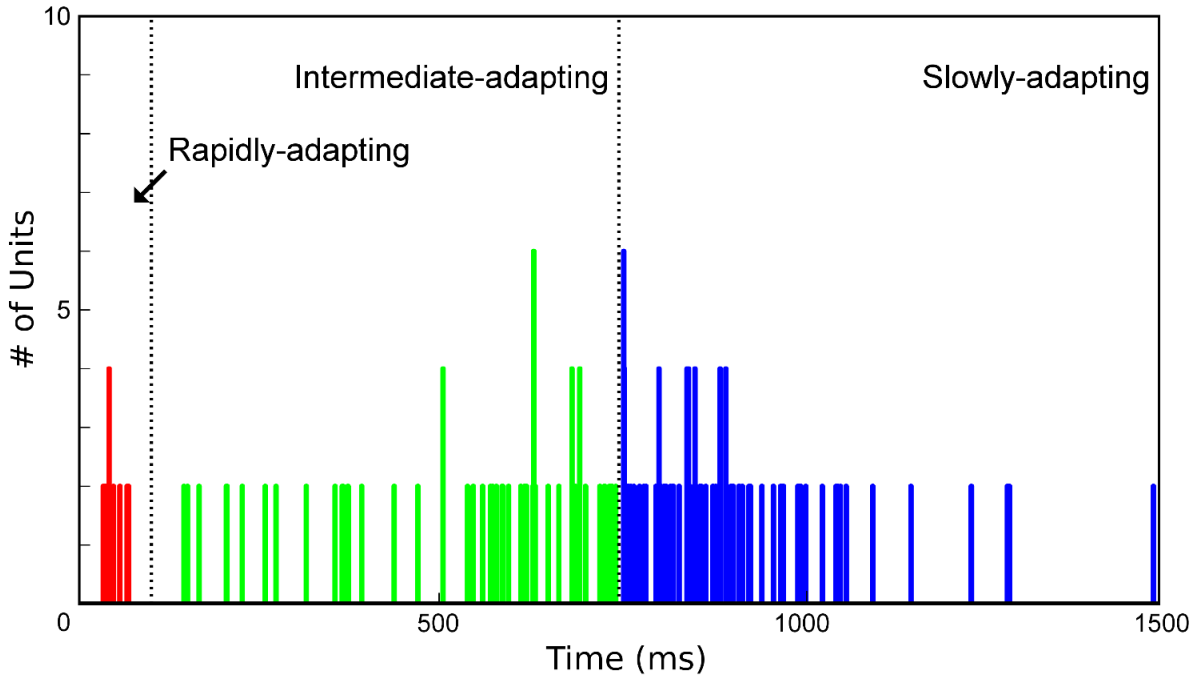


Fig. 4.9 First-order ISI histograms for rapid-, intermediate-, and slow-adapting cells in response to chorus and synthetic stimuli. See Figure 4.8 for further details. Total cell counts for chorus ('X m') and SPL-matched synthetic calls ('Hv'): (a) 16 m chorus (*n* = 8, 12, 82), Hv (*n* = 5, 7, 52), (b) 32 m chorus (*n* = 9, 11, 84), Hv (*n* = 5, 7, 53), (c) 50 m chorus (*n* = 8, 12, 84), Hv (*n* = 5, 7, 53), (d) 100 m chorus (*n* = 12, 21, 101), Hv (*n* = 5, 7, 55). Numbers represent *n*-values for rapidly-, intermediate-, and slowly-adapting response groups, respectively

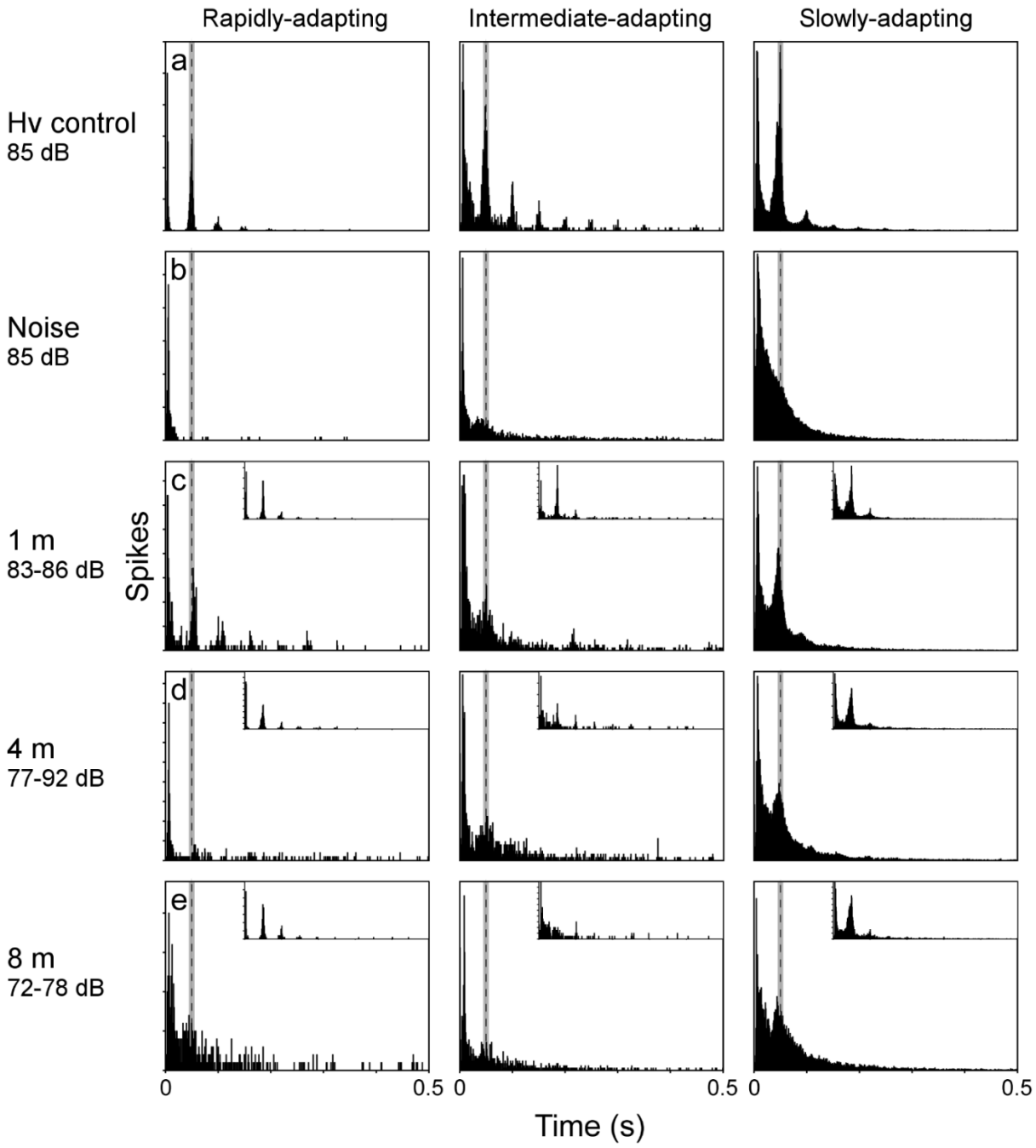
4.8 SUPPLEMENTARY FIGURES



Supplementary Fig. 4.1 BF vs. threshold level (top panel) and BF histogram (bottom panel)

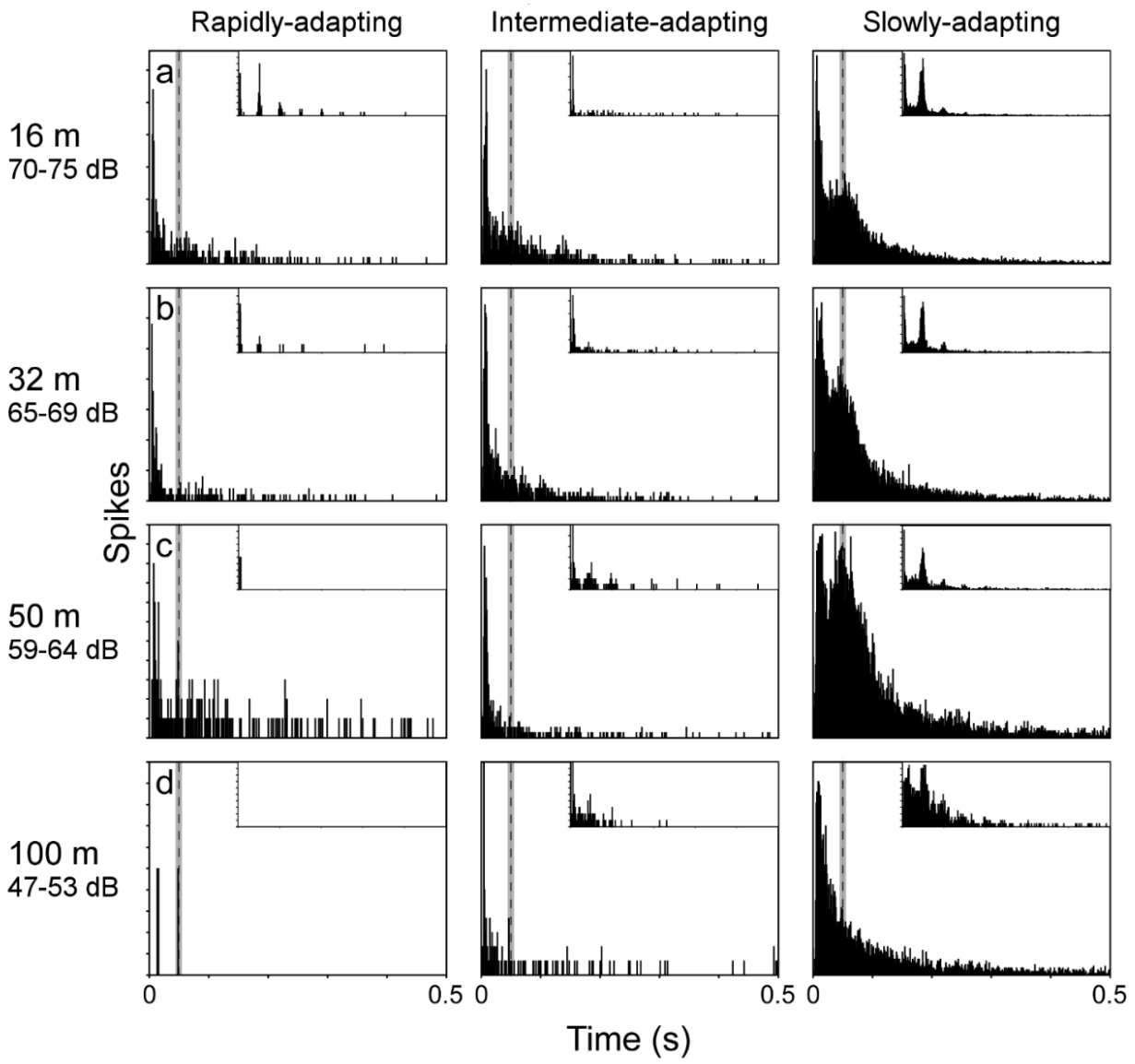


Supplementary Fig. 4.2 Histogram displaying PSTH bin times at which the cumulative spike count for the noise stimulus reaches $1 - e^{-1}$ (≈ 0.632) of the total spike count. Dotted lines indicate bins corresponding to 100 and 750 ms, respectively. Colors indicate temporal response type classifications: red = rapidly-adapting; green = intermediate-adapting; blue = slowly-adapting

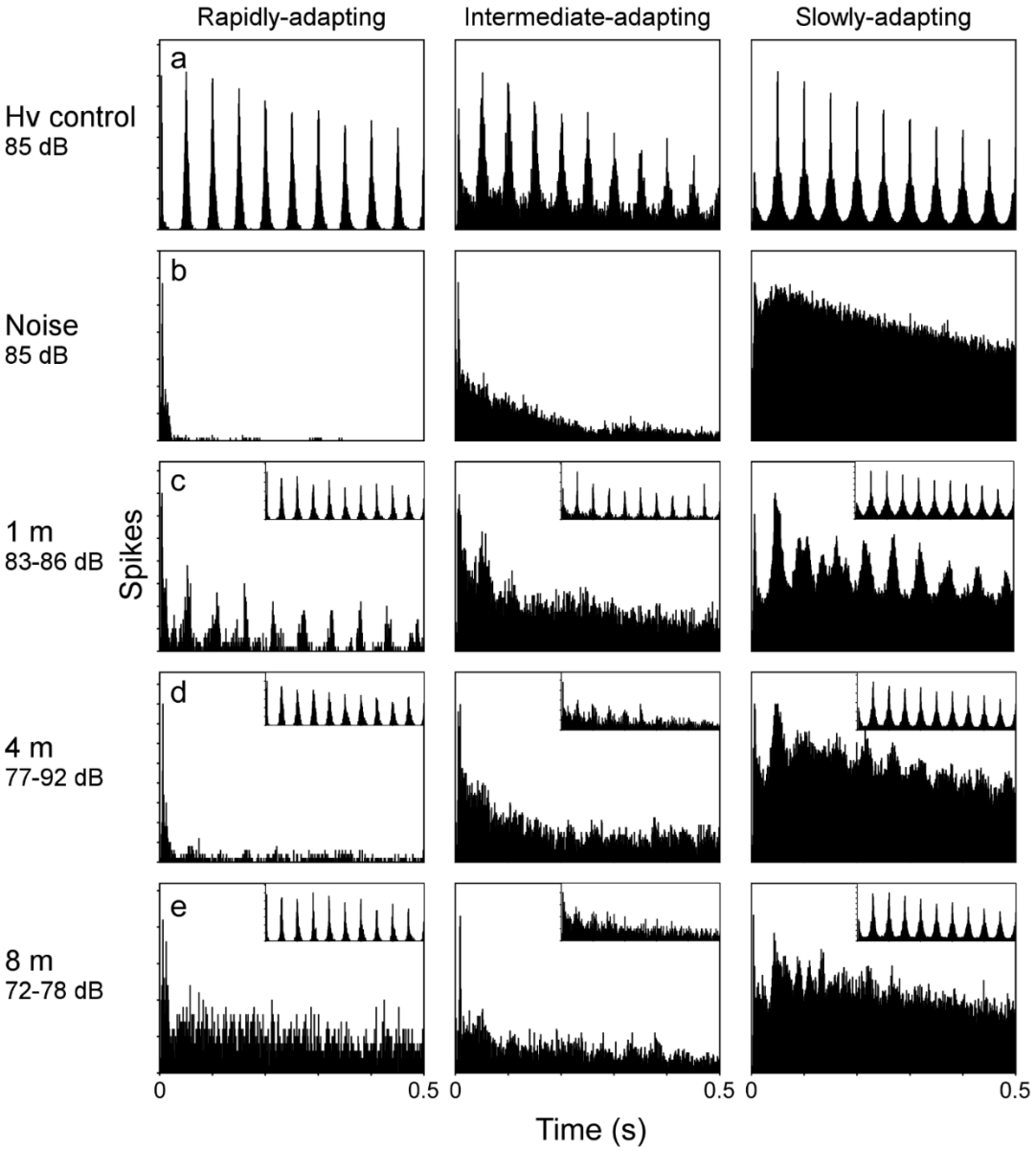


Supplementary Fig. 4.3 First-order ISI histograms for rapidly-, intermediate-, and slowly-adapting cells in response to chorus and synthetic stimuli. The dotted lines and gray bar areas represent the mean ± S.D. of the IPIs of the focal males recorded by the hypercardioid microphone at that distance – see Materials and Methods. PSTH magnitude axes are scaled to show responses. (a) Control synthetic conspecific call (*n* = 13, 21, 102), (b) Band-limited noise (*n* = 13, 21, 101). Total cell counts for chorus ('X m') and SPL-matched synthetic calls ('Hv'): (c) 1 m chorus (*n* = 13, 12, 102), Hv (*n* = 5, 7, 55), (d) 4 m chorus (*n* = 9,

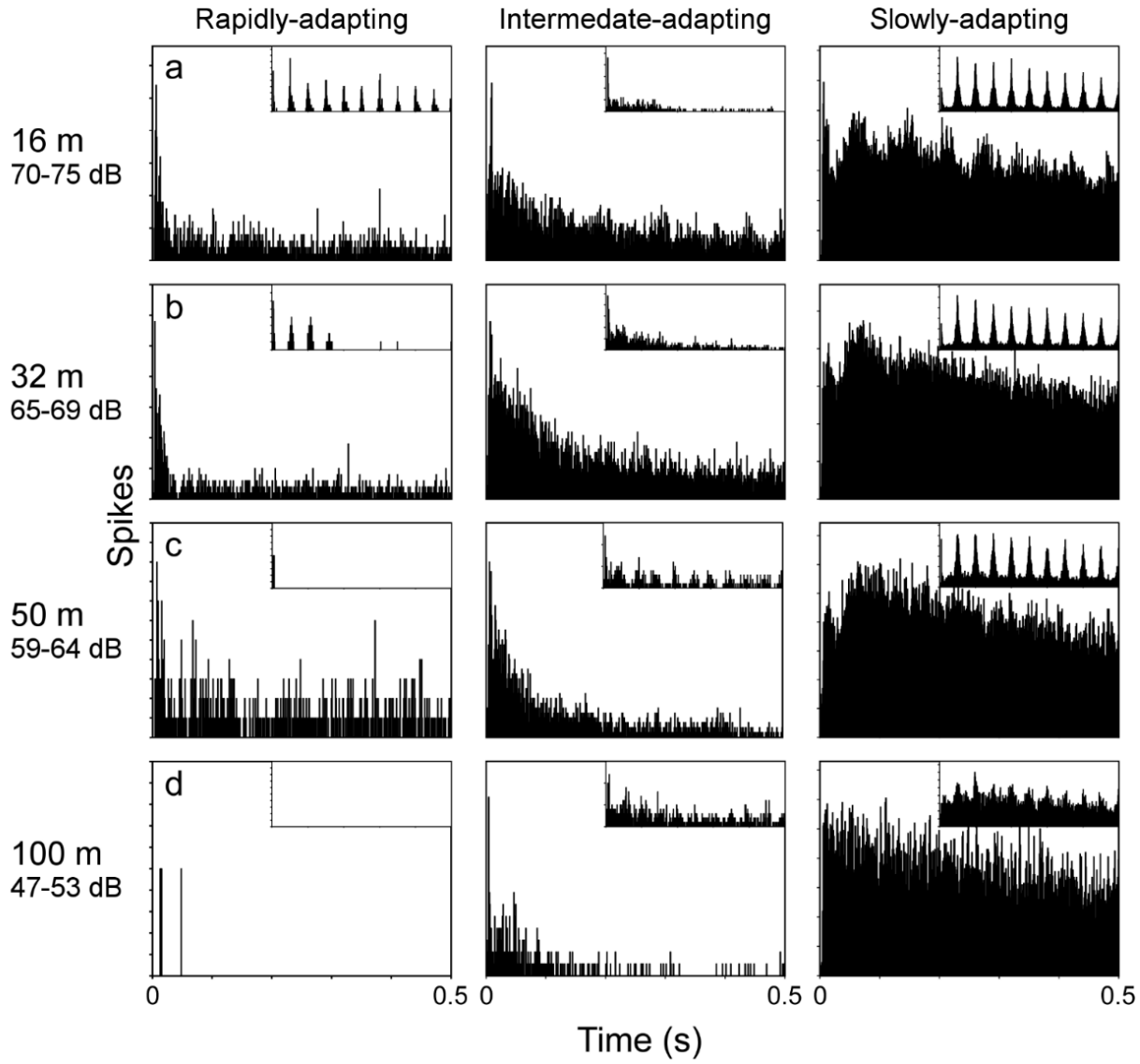
Supplementary Fig. 4.3 (cont.) 12, 84), Hv (*n* = 5, 7, 54), (e) 8 m chorus (*n* = 13, 21, 95), Hv (*n* = 5, 7, 51). Numbers represent *n*-values for rapidly-, intermediate-, and slowly-adapting response groups, respectively



Supplementary Fig. 4.4 First-order ISI histograms for rapidly-, intermediate-, and slowly-adapting cells in response to chorus and synthetic stimuli. See Supplementary Fig. 4.3 for details. PSTH magnitude axes are scaled to show responses. Total cell counts for chorus ('X m') and SPL-matched synthetic calls ('Hv'): (a) 16 m chorus (*n* = 8, 12, 82), Hv (*n* = 5, 7, 52), (b) 32 m chorus (*n* = 9, 11, 84), Hv (*n* = 5, 7, 53), (c) 50 m chorus (*n* = 8, 12, 84), Hv (*n* = 5, 7, 53), (d) 100 m chorus (*n* = 12, 21, 101), Hv (*n* = 5, 7, 55). Numbers represent *n*-values for rapidly-, intermediate-, and slowly-adapting response groups, respectively



Supplementary Fig. 4.5 Auto-correlation histograms for rapidly-, intermediate-, and slowly-adapting cells in response to chorus and synthetic stimuli. See Supplementary Fig. 4.4 for details. PSTH magnitude axes are scaled to show responses. (a) Control synthetic conspecific call (*n* = 13, 21, 102), (b) Band-limited noise (*n* = 13, 21, 101). Total cell counts for chorus ('X m') and SPL-matched synthetic calls ('Hv'): (c) 1 m chorus (*n* = 13, 12, 102), Hv (*n* = 5, 7, 55), (d) 4 m chorus (*n* = 9, 12, 84), Hv (*n* = 5, 7, 54), (e) 8 m chorus (*n* = 13, 21, 95), Hv (*n* = 5, 7, 51). Numbers represent *n*-values for rapidly-, intermediate-, and slowly-adapting response groups, respectively



Supplementary Fig. 4.6 Auto-correlation histograms for rapidly-, intermediate-, and slowly-adapting cells in response to chorus and synthetic stimuli. See Supplementary Fig. 4.4 for details. PSTH magnitude axes are scaled to show responses. Total cell counts for chorus ('X m') and SPL-matched synthetic calls ('Hv'): (a) 16 m chorus (*n* = 8, 12, 82), Hv, (*n* = 5, 7, 52), (b) 32 m chorus (*n* = 9, 11, 84), Hv (*n* = 5, 7, 53), (c) 50 m chorus (*n* = 8, 12, 84), Hv (*n* = 5, 7, 53), (d) 100 m chorus (*n* = 12, 21, 101), Hv (*n* = 5, 7, 55). Numbers represent *n*-values for rapidly-, intermediate-, and slowly-adapting response groups, respectively

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CHAPTER 5: CONCLUDING REMARKS AND FUTURE RESEARCH

5.1 CONCLUDING REMARKS

Through the use of natural chorus and synthetic call stimuli, I have found that female *Hyla versicolor* probably do not use the chorus background as an orientation cue at approach distances greater then ~32 m (Chapter 2). It is most likely that females use one or many alternate sensory modalities to successfully navigate to an active chorus (Sinsch 1990a; Sinsch 1990b). The results of this study are similar to another with the diploid sibling species of *H. versicolor*, *H. chrysoscelis* (Swanson et al. 2007). To get a better sense of general limits on the use of degraded acoustic cues for orientation, more comparative work is needed in other species. Previous studies of chorus attraction with unrelated species have varying results (Gerhardt and Klump 1988; Bee 2007; Buxton et al. 2015), which may suggest that a species' breeding ecology (such as permanence of water bodies, etc.) might correlate with the ability to use clustered signals for orientation.

While investigating what signal features are most relevant with regards to stimulus attraction, I tested chorus stimuli in which I independently altered SPL and the level of signal degradation to see which had a more profound effect on female phonotaxis (Chapter 3). When a female treefrog, moving from her daytime resting and foraging grounds, approaches an active chorus, the calls she hears of the constituent males undergo physical changes – the overall SPL increases, and the temporal structures of call pulses become progressively less degraded and masked. The increased signal-to-noise ratio of these features and their increased resolvability by the

female's auditory system seem to be the dominant factor in the transition from an unattractive to an attractive phonotaxis stimulus (Chapter 3). This also suggests that it is unlikely that females discriminate the finer temporal features of male calls beyond 4-8 m. In addition, I found female *orientation* to natural chorus stimuli, but not *attraction*, was proportional to stimulus SPL, unlike responses to synthetic calls commonly used in behavioral experiments in which both attraction and orientation improved with increasing sound amplitude. A potential factor in the reduction in attraction of chorus vs. synthetic call stimuli I considered is the effect of acoustic energy in 'flanking' regions to those important for attraction and selective orientation. Behavioral and *in vivo* neurophysiological studies, with naturalistic artificial stimuli with altered frequency distributions compared to male calls and the chorus sounds, are required to further understand the effect of altering individual call spectral and temporal parameters to mimic levels of degradation seen in nature. The different effect that SPL increases have on orientation/attraction, as well as female responses to the calls of the diploid sibling species *H. chrysoscelis* tantalizingly suggest that the females' ability to *localize* and to *discriminate* stimuli might be separable to some degree. Experiments to test these additional hypotheses are needed, though their designs are non-trivial in a species that, to date, seems to lack any strong audio-spatial neural map similar to those seen in owls (Knudsen and Konishi 1978). Investigating the sensory ecology and evolution in the *H. chrysoscelis/versicolor* species complex is an obvious follow-up experimental series. Since both species use different call parameters to orient and evaluate calls (Schul and Bush 2002), it would be useful to quantify in depth the alteration in signal temporal parameters and compare the behavioral effects of call degradation through the use of

altered synthetic calls and naturalistic ‘chorus-shaped’ noise (Bee 2009; Kuczynski et al. 2010).

Finally, I studied the representation of clustered signals – the chorus – in the auditory midbrain of treefrogs (Chapter 4). During the course of the experiments I found that torus semicircularis cell response properties were significantly non-homogeneous and derived three temporal adaptation classes based on responses to band-limited noise stimuli. *Rapidly*-adapting cells shared several characteristics of ‘phasic’ cells seen in other anuran midbrain studies (Walkowiak 1980; Gooler and Feng 1992), including strong onset behavior, and responded mostly to chorus sounds recorded close to a focal male – stimuli with a high SPL and less blurring of temporal structure. *Slowly*-adapting cells’ responses strongly resembled ‘tonic’ – and similar - cells (Walkowiak 1980; Gooler and Feng 1992) and tended to fire throughout the stimulus’ duration and to stimuli at most recording distances - usually further than the other classes (32 – 100 m in some cases). A population of cells with behavior in-between those of rapidly- and slowly-adapting cells, termed *intermediate*-adapting, resembled ‘phasi-tonic’ or ‘phasic-burst’ cells described in previous studies (Walkowiak 1980; Gooler and Feng 1992). These neurons showed significantly stronger response attenuation than slowly-adapting cells, yet had more sustained responses than rapidly-adapting cells. Interestingly, at the closest chorus distances and for the loudest synthetic calls, this group of cells had the poorest pulse-following ability. I hypothesized that in the field, the slowly-adapting cells would be the first population with significant activity in females approaching a chorus. Sufficient numbers of responding cells may then allow the use of the chorus as an orientation cue at the furthest ‘attractive’ distances found in the first experiment (~32 m;

Chapter 2). At closer distances the remaining cell types would also show activity, which may aid in more precise localization and possibly call discrimination when call/pulse features are then resolvable. An obvious next step would be to better quantify the biophysical properties that underlie the response differences in TS cells. Yang et al. (2009) suggest similar biophysical response types in the mammalian auditory system are present in anurans, even at the level of the TS. Newer genetic techniques such as those developed by Melendez (2008) and others (Chakraborty et al. 2010) may elucidate physiology at the network level, bridging the gap between whole animal (behavioral phonotaxis) and single cell (neurophysiological) studies.

Perhaps the most valuable outcomes of these studies are the insights that can be gained using natural (and naturalistic) stimuli. These provide a 'reality check' to other studies looking at auditory perception and behavior. Often, these studies demonstrate the limits of auditory system performance – what it *can* do. Studying sensory systems under conditions as close to the field as possible tells us more about what the system in question *does* do. A major goal and outcome of proposed experiments described above is the determination and delineation of different spatio-temporal behavioral 'regimes' under which the treefrog auditory system operates, similar to studies done with orthopteran insects (Lang 2000). This has useful comparative value, with the ultimate goal of discovering general acoustic communication principles – at the evolutionary, anatomical, and functional levels.

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APPENDIX A: SUPPLEMENTARY FILES

3.11.1 Supplementary File S1 Synthetic conspecific *H. versicolor* stimulus ‘Hv’ (32-bit mono .wav format with 44100 Hz sample rate).

3.11.2 Supplementary File S2 Compressed file containing 20 s chorus recordings made on 5-19-2006 at the following distances from the focal male: 1 m, 4 m, 8 m, 16 m, 32 m, 50 m, and 100 m (32-bit stereo .wav format with 44100 Hz sample rate). Each file contains simultaneous recordings, with the left channel representing the hypercardioid microphone 1 m from the focal male, and the right channel the omnidirectional microphone at the indicated distance. The distant microphone recording has not been altered or adjusted to compensate for the delay in sound propagation from the focal male. At each distance, the distant, omnidirectional recordings were looped to create the chorus stimuli presented to females.

3.11.3 Supplementary File S3 Compressed file containing individual calls extracted from the chorus recordings (File S2) from 5-19-2006, at all recording distances from the focal male: 1 m, 4 m, 8 m, 16 m, 32 m, 50 m, and 100 m (32-bit stereo .wav format with 44100 Hz sample rate). For each call, the left channel represents the call as recorded by the hypercardioid microphone 1 m from the focal male, while the right channel represents the simultaneous recording by the omnidirectional microphone at the indicated distance. The distant microphone recording has not been altered or adjusted to compensate for the delay in sound propagation from the focal male.

3.11.4 Supplementary File S4 Compressed file containing 20 s chorus recordings made on 5-23-2006 at the following distances from the focal male: 1 m, 4 m, 8 m, 16 m, 32 m, 50 m, and 100 m (32-bit stereo .wav format with 44100 Hz sample rate). For other details, see the description for supplementary file S2.

3.11.5 Supplementary File S5 Compressed file containing individual calls extracted from the chorus recordings (File S2) from 5-23-2006, at all recording distances from the focal male: 1 m, 4 m, 8 m, 16 m, 32 m, 50 m, and 100 m (32-bit stereo .wav format with 44100 Hz sample rate). For other details, see the description for supplementary file S3.

3.11.6 Supplementary File S6 Compressed file containing 20 s chorus recordings made on 5-24-2006 at the following distances from the focal male: 1 m, 4 m, 8 m, 16 m, 32 m, 50 m, and 100 m (32-bit stereo .wav format with 44100 Hz sample rate). For other details, see the description for supplementary file S2.

3.11.7 Supplementary File S7 Compressed file containing individual calls extracted from the chorus recordings (File S2) from 5-24-2006, at all recording distances from the focal male: 1 m, 4 m, 8 m, 16 m, 32 m, 50 m, and 100 m (32-bit stereo .wav format with 44100 Hz sample rate). For other details, see the description for supplementary file S3.