ABSTRACT

Title of dissertation: ECHOLOCATION, HIGH FREQUENCY HEARING, AND GENE EXPRESSION IN THE INNER EAR OF BATS

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Bats are the only mammals capable of true flight, and are the second-most speciose mammalian radiation, represented by over 1200 extant species. Key to their evolutionary success was echolocation, which is a complex trait requiring specializations for vocalization, hearing, and echo processing. Because they rely on detecting and analyzing echoes that may return greatly attenuated relative to their outgoing calls, interference from non-target 'clutter' echoes poses a challenge for echolocating bats. Here, I demonstrate that the echolocating bat *Eptesicus fuscus* alters its echolocation behavior to ameliorate the impact of clutter echoes when tracking a moving target, and that the magnitude of its behavioral adjustments depended on the distance and angular offset of two symmetrically placed 'distracter' objects. Furthermore, I found that individual bats make different adjustments to their calls, call timing, or head movements, suggesting that multiple strategies for echolocating in clutter may exist. In my second chapter, I examined the expression patterns of hearing-related genes in juvenile bats. Biomedical

research establishing the functional roles of hearing genes rarely examines gene expression beyond the early post-natal stage, even though high frequency hearing does not mature until late in development. I show that several key hearing genes implicated in human deafness are upregulated in juvenile bats relative to adults, or exhibit sustained upregulation through the developmental period corresponding to the maturation of echolocation behavior. In my third chapter, I review the evolution of high frequency hearing in mammals, focusing on echolocating bats and whales, which have independently evolved this complex trait. I provide an overview of recent studies that have reported molecular convergence in hearing genes among distantly related echolocators, and assert that the contribution of gene expression to hearing deserves further investigation. Finally, I argue that echolocators provide a unique opportunity to investigate the basis of high frequency amplification, and may possess mechanisms of hearing protection which enable them to prolong the use of echolocation throughout their long lives.

ECHOLOCATION, HIGH FREQUENCY HEARING, AND GENE EXPRESSION IN THE INNER EAR OF BATS

by

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PREFACE

Chapter 2 was previously published in 2016 as "Big brown bats (*Eptesicus fuscus*) reveal diverse strategies for sonar target tracking in clutter" in the Journal of the Acoustical Society of America, volume 140, issue 3, pages 1839–1849. It is reproduced here with the permission of Acoustical Society of America and can be viewed at http://asa.scitation.org/doi/10.1121/1.4962496. See Appendix 12.

Author contributions for Chapter 2 were as follows: conceptualization and design, BM MA CFM; acquisition of data, BM MA; analysis and interpretation of data, BM MA GSW CFM; writing of manuscript, BM; revision and editing of manuscript, BM MA GSW CFM.

Author contributions for Chapter 3 were as follows: conceptualization and design, BM GSW; acquisition of data, BM; analysis and interpretation of data, BM GSW CFM; writing of manuscript, BM; revision and editing of manuscript, BM GSW CFM.

Author contributions for Chapter 4 were as follows: conceptualization, BM GSW CFM; writing of manuscript, BM; revision and editing of manuscript, BM GSW CFM.

DEDICATION

To my father, who fostered my intellectual curiosity, and whose strength in the face of struggle inspired me and gave me perspective; to my mother, whose confidence in me never flagged; to my brother and sister, who keep me grounded; to Dr. Pamela Lanford and Dr. Mercedes Burns, whose friendship has been invaluable; to Cookie, the best dog ever; and most of all, to Caleb Mullins, who has shared the ups and downs, and without whom the completion of this dissertation would not have been possible.

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Regularly attending Ronna's lab meetings and receiving instruction and advice from her lab members (Laura Morrison, Lorna Silipino, and Beatrice Milon) invigorated my interest in studying the cochlea. Pam has been an incredible mentor, and I am forever thankful for her commiseration and perspective. I would not have envisioned the possibility of a post-doc at NIH (under Matt) if she had not taken the initiative of contacting him on my behalf.

Matt invited me to visit his lab several times, and it has been a pleasure to discuss comparative hearing with him during these visits and at Woods Hole. I am grateful for the opportunity to work with him, and very much looking forward to it.

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LIST OF ABBREVIATIONS

ABR – auditory brainstem response
ARHL – age-related hearing loss
BM – basilar membrane
CF – characteristic frequency
CF-FM - constant frequency (of echolocation calls)
CHO – Chinese hamster ovary (of cells)
DFNA – autosomal dominant non-syndromic deafness
DFNB – autosomal recessive non-syndromic deafness
DFNX – X-linked non-syndromic deafness
DNA – deoxyribonucleic acid
FM – frequency modulated (of echolocation calls)
GLM – general linear model
HEK – human embryonic kidney (of cells)
IHC – inner hair cell
MAA – minimum audible angle
MESH – motile eutherian SLC26A helper (of a motif in *prestin*)
MET – mechanoelectrotransduction (of channels)
NIHL – noise-induced hearing loss
NLC – non-linear capacitance
OHC – outer hair cell
PI – pulse interval
RAR – relative amplitude ratio
RARD – relative amplitude ratio difference
RNA – ribonucleic acid
SG – spiral ganglion
SGN – spiral ganglion neurons
SSG – sonar sound group
SWS – short-wavelength-sensitive (of opsins)
TM – tectorial membrane

Chapter 1: Introduction and background

A. Echolocation in bats

Bats are the only mammals capable of true flight and sophisticated laryngeal echolocation. This unique combination of traits allowed them to occupy diverse niches and exploit an unparalleled variety of food sources, including pollen, fruit, flowers, leaves, nectar, blood, insects, arachnids, crustaceans, fish, amphibians, lizards, birds, and mammals, including other bats (Kunz and Pierson 1994). However, not all bats echolocate, and while diversity in echolocation ability has led to interesting comparative studies, it has also posed a challenge for systematists, because the two major groups of echolocating bats are not sister taxa.

Instead, one echolocating clade is most closely related to the Old World fruit bats (e.g. Jones and Teeling 2006), which either do not echolocate, or exhibit a 'primitive' form of echolocation involving tongue-clicking (lingual echolocation) or clicking with the wings, the mechanism of which is not yet understood (Boonman et al. 2014). Some capacity for echolocation therefore seems to be present in all bat lineages, suggesting that the ancestral bat may have been capable of echolocation. A recent study showed that the prenatal cochlear growth rates of non-largyngeally echolocating bats are initially as high as those of echolocating bats, but eventually falls below that of non-echolocating mammals (Wang et al. 2017), supporting the hypothesis that echolocation had a single origin in bats.

Echolocation has also evolved in toothed whales, which use broadband clicks that are shorter and can contain even higher frequencies than the echolocation calls of bats (reviewed in Madsen and Surlykke 2013). Laryngeally echolocating bats exhibit greater

vocal flexibility than echolocating whales, whose sound production mechanism, a derived structure in the nasal passage (Cranford et al. 1996) is not as versatile as the larynx (Madsen and Surlykke 2013). The audiograms of both echolocating bats and whales exhibit much greater sensitivity to high frequencies than other mammals (e.g. Long and Schnitzler 1975; Esser and Daucher 1996; Houser et al. 2008), as would be expected from their echolocation calls.

As high frequency specialists, echolocators provide researchers an opportunity to study how high frequencies are amplified by the cochlea, the mechanisms of which are not clear (e.g. Ashmore et al. 2010). Because echolocators are long-lived, use high-amplitude echolocation calls, and require echolocation for survival, they may also provide insight into protective mechanisms that guard against hearing loss caused by age or noise. Finally, molecular studies of hearing genes in echolocators have focused on convergent sequence substitutions (Li et al. 2008; Li et al. 2010; Liu et al. 2010; Liu et al. 2011; Liu et al. 2012a; Davies et al. 2012; Shen et al. 2012) but have large ignored the potential contribution of gene expression to hearing ability.

B. Adaptive echolocation behavior in the presence of clutter

A key problem facing echolocating bats, particularly insectivores, which need to detect weak echoes returning from small prey, is the avoidance of masking (Schnitzler and Kalko 2001). Bats can experience forward masking when outgoing calls overlap in time with returning echoes. Although call-echo overlap is generally avoided, it can be tolerated (e.g. Siemers and Schnitzler 2000) and is common in the terminal buzz phase (Jones and Holderied 2007). Backward masking occurs when extraneous sounds arrive at

the auditory receiver after the target echo, and poses a challenge for bats that echolocate in cluttered environments (Schnitzler and Kalko 2001).

In Chapter 2, I show how a well-studied FM echolocating bat, *Eptesicus fuscus*, tracks a moving prey object in the presence of symmetrically placed 'distracter' objects that create clutter echoes (Mao et al. 2016). Our previous work showed that *Eptesicus* alters spectrotemporal parameters of its calls, and its call timing, while tracking a moving object in the presence of one distracter placed at different distances and angular offsets from the bat (Aytekin et al. 2010). However, it was unclear whether the bats could move their heads and echolocate off-axis to reduce interference from the distracter. I demonstrate that bats can use alternative strategies to ameliorate the effects of clutter interference, either by changing spectral or temporal features of their calls, or by producing sonar sound groups and turning their heads after the target passes in front of the distracters.

C. Expression of hearing genes in juvenile big brown bats

Evidence for molecular convergence between distantly related echolocating bats, and between echolocating bats and whales, has been documented in several hearing genes (Li et al. 2008; Li et al. 2010; Liu et al. 2010; Liu et al. 2011; Liu et al. 2012a; Davies et al. 2012; Shen et al. 2012). The functional significance of purported convergent and parallel substitution sites is often not clear and has only been explored in the case of one gene, *prestin* (Liu et al. 2014). While comparative differences in gene regulation likely play a role in shaping hearing ability, they not been extensively investigated, and only one study has identified genes that were differentially expressed in echolocating bats vs. non-echolocating bats (Dong et al. 2013). Additionally, most studies of gene expression

in the cochlea have used embryonic or early postnatal tissues, and do not reflect hearing in the adult animal, because high frequency hearing matures relatively late in development (Harris and Dallos 1984; Echteler et al. 1989).

In Chapter 3, I show that several genes implicated in human deafness are differentially expressed between juvenile and adult bats, and/or exhibit sustained upregulation through a key developmental period when juvenile bats' vocalizations (and likely hearing) undergo rapid change. The upregulated genes have been shown to play a key role in sound transduction or the maintenance of hearing, and may reflect a longer growth period in the echolocating bat cochlea corresponding to larger relative cochlea size and expanded high frequency range.

D. High frequency hearing in echolocators

Echolocation is a complex trait, but has evolved independently in multiple lineages, including in shrews, oilbirds and swiftlets, and toothed whales (Brinkløv et al. 2013). In all these cases, vision is of limited use as a sensory cue, because these animals are nocturnal, roost in caves or crevices, or inhabit low-light habitats. Interestingly, some blind humans also use echolocation for orientation (Stroffregen and Pittenger 1995). However, only echolocating bats and whales are capable of high resolution echolocation, which requires high frequency hearing and enables them to catch highly maneuverable prey in the dark. The degree of convergence they exhibit in echolocation call frequencies and behavior are particularly surprising given their vastly different body sizes, evolutionary histories, and properties of sound in the different media they inhabit (Madsen and Surlykke et al. 2013).

In Chapter 4, I review key innovations in the evolution of mammalian high frequency hearing and enumerate selective pressures that may have shaped hearing in mammals. In particular, I focus on echolocating bats and whales, which have independently acquired echolocation and which exhibit convergent adaptations for high frequency hearing. I provide an overview of recent studies on molecular convergence in the sensory genes of echolocators, and call for further investigation into the contribution of gene expression to hearing ability.

Chapter 2: Big brown bats (*Eptesicus fuscus*) reveal diverse strategies for sonar target tracking in clutter

ABSTRACT

Bats actively adjust the acoustic features of their sonar calls to control echo information specific to a given task and environment. A previous study investigated how bats adapted their echolocation behavior when tracking a moving target in the presence of a stationary distracter at different distances and angular offsets. The use of only one distracter, however, left open the possibility that a bat could reduce the interference of the distracter by turning its head. Here, bats tracked a moving target in the presence of one or two symmetrically placed distracters to investigate adaptive echolocation behavior in a situation where vocalizing off-axis would result in increased interference from distracter echoes. Both bats reduced bandwidth and duration but increased sweep rate in more challenging distracter conditions, and surprisingly, made more head turns in the two-distracter condition compared to one, but only when distracters were placed at large angular offsets. However, for most variables examined, subjects showed distinct strategies to reduce clutter interference, either by (1) changing spectral or temporal features of their calls, or (2) producing large numbers of sonar sound groups and consistent head-turning behavior. The results suggest that individual bats can use different strategies for target tracking in cluttered environments.

I. INTRODUCTION

Insectivorous bats show great diversity in echolocation call design (Obrist 1995; Russo and Jones 2002; Obrist et al. 2004) and actively adjust the timing, spectro-temporal structure, and amplitude of their calls to suit the task at hand. As bats approach targets and obstacles, they reduce the duration and pulse interval of their calls to increase localization accuracy, to minimize ambiguity in pulse-echo assignment, and to obtain information at a faster rate (Griffin 1958; Kalko and Schnitzler 1993). Frequency modulated (FM) calls or call components produce echoes that return detailed information about target location and physical characteristics via variation in frequency content (Simmons et al. 1975; Simmons and Stein 1980).

Bats can adjust the frequency content of calls to avoid signal jamming by conspecifics (Gillam et al. 2007; Bates et al. 2008; Chiu et al. 2009) or to resolve pulse-echo assignment ambiguities (Hiryu et al. 2010). Additionally, bats can change the power spectrum of their calls by apportioning more energy to certain frequencies or harmonics (Hartley and Suthers 1989; Jakobsen and Surlykke 2010). This ability to dynamically modify call parameters allows bats to orient in complex environments, which may contain conspecifics, obstacles, and extraneous sounds (Obrist 1995; Moss and Surlykke 2010; Jakobsen et al. 2013) from other bats (Ulanovsky et al. 2004) and/or insects (Fullard et al. 1994; Corcoran et al. 2009).

In addition to adjusting its sonar calls, a bat may employ behavioral strategies, such as head turning, to improve detection or localization of targets. Turning the head directly influences the directional aim of the sonar transmission (Ghose and Moss 2003) and, consequently, echo information carried to the auditory receiver (Aytekin et al.

2004). The width of a bat's sonar beam varies with sound frequency and mouth gape (Jakobsen and Surlykke 2010; Jakobsen et al. 2013; Kounitsky et al. 2015). Centering the sonar beam on a target may assist the bat in defocusing non-target objects that are off-axis (Bates et al. 2011; Simmons 2014). Bats also alternate the direction of their sonar beam, which enables them to simultaneously track objects of interest while planning their flight trajectories through obstacles (Surlykke et al. 2009) or towards the next target (Fujioka et al. 2014).

In a cluttered environment, echoes from non-target objects can mask the target through echo-echo overlap, depending on the number and direction of competing echo sources or maskers (Langendijk et al. 2001; Warnecke et al. 2014) or their angular offset (Sümer et al. 2009). Regions in which echoes from non-target objects interfere with target detection have been experimentally determined to be wider at greater distances (Simmons et al. 1988). Bats that use FM calls respond to clutter by producing groups of echolocation calls, referred to as sonar sound groups (SSGs), which consist of two or more pulses close together, flanked by longer intervals (Moss et al. 2006). The bat's alternation between short and long pulse intervals likely facilitates echo assignment (Moss and Surlykke 2001; Hiryu et al. 2010; Melcón et al. 2011) and may also allow bats to multitask, inspecting close objects while monitoring the greater environment for trajectory planning (Petrites et al. 2009). SSG production is higher when bats attack a moving vs stationary target (Hulgard and Ratcliffe 2016) or when a target moves erratically instead of predictably (Kothari et al. 2014).

While most bats echolocate for spatial orientation in the environment, those that hunt moving prey must be especially adept at processing echoes quickly in order to

inform rapid motor decisions to capture erratically moving targets. The big brown bat (*Eptesicus fuscus*) is an aerial insectivore that hunts in both open areas and edge spaces where it encounters clutter from foliage, making it a model for studying adaptive adjustments to echolocation calls in different environmental contexts. It can detect and localize target echoes in clutter (Simmons et al. 1989; Aytekin et al. 2010) and discriminate between objects using shape (Griffin et al. 1965; Simmons and Chen 1989) and surface texture (Falk et al. 2011). In clutter, Eptesicus apportions more power to higher harmonics relative to the fundamental (Sümer et al. 2009; Aytekin et al. 2010), which may allow better separation of target and clutter echoes.

In a previous experiment, Aytekin et al. (2010) examined how the big brown bat adapts its echolocation signals from a resting position in a target tracking task in the presence of a single "distracter" object (a metal pole positioned vertically to one side of the target motion axis). Because the distracter was placed on only one side of the target motion path, it was unclear whether the bats reduced masking echoes from the distracter by moving their head or ears off-axis.

The present study reports on echolocation behavior in big brown bats tracking a target in the presence of one or two distracters. Specifically, we predicted that the bats would reduce call duration and increase bandwidth, sweep rate, and peak frequency when the distracters were close and the angular offset was small. Due to the increased interference created by the addition of a second distracter, we also predicted adjustments in call intervals, with higher SSG production in the two-distracter condition, as well as when the distracters were placed closer to the bat or at small angular offsets from the target. Because turning the head off-axis from the target in the presence of two

symmetrically placed distracters would increase clutter interference, we predicted that head turns would be more prevalent in the one-distracter condition.

II. METHODS

A. Animals

Big brown bats (*Eptesicus fuscus*) were wild-caught in Maryland under a permit from the Department of Natural Resources. Bats were fed mealworms (*Tenebrio molitor*) only during training and experimental sessions for appetitive motivation, with supplemental feeding provided on non-training days or if daily weight monitoring indicated weight loss beyond minor fluctuations (>5% average weight). Training was initiated with four bats; however, two of these animals became ill or died in the course of the experiment, and complete data sets were obtained for only two animals, Bat 45 and Bat 49. All housing and experiments were conducted with the approval of the University of Maryland Institutional Animal Care and Use Committee.

B. Experimental setup

The experimental setup followed Aytekin et al. (2010). A cable, running along a four-pulley system, was attached to a motorized forcer that slid along a rail to change the position of the target, which the bat tracked from a resting position. An optical sensor (USDIGITAL, EM1-0-200, US Digital, Vancouver, WA) and linear transmissive strip (USDIGITAL, LIN-200-0.5-N) were used to record target distance as the forcer moved. To muffle the sound of the motor, a wooden casement lined with acoustic foam was placed around the rail. The forcer's motion was controlled and recorded by a computer using custom software written in MATLAB-2007b. Two microphones used to record

echolocation calls were placed at a distance of 2.8m from the bat, behind, and on either side of, the pulley apparatus.

The target was attached to the cable with fishing line and stabilized with an extra loop tethered on both sides of the line to prevent excessive swinging at the beginning and end of programmed movements. For distracter conditions, either one or two distracters were placed at different distances (45 and 70 cm) and angular offsets (10, 20, 30, and 40) from the platform (Figure 1). Distracter positions were changed between but not within test days. Each distracter object consisted of a 1.27 cm wide metal rod attached to a baseboard by a flange. Distracters were always placed at the same location on either side of the target motion axis, resulting in a symmetrical arrangement. In the baseline condition, no distracter objects were present. Two sets of baseline data were obtained, one before each clutter experiment. They will be referred to as "baseline 1" and "baseline 2," respectively.

C. Animal training

Bats were trained to sit on a platform in an anechoic room illuminated with low-level, long wavelength light. They were conditioned to associate the initiation of a trial with a sound produced by a clicker. After the presentation of a click, the motor-driven pulley system was used to deliver the target to the bat. During training, the target's initial distance from the platform and its delivery speed were gradually increased until it reached 170 cm and 1.27 m/s, respectively. The two-distracter data presented here were gathered with no additional training beyond that reported in Aytekin et al. (2010). As in the one-distracter experiment, probe trials consisting of a change in the normal pattern of

target delivery were interspersed randomly each day of data collection to check for and maintain active engagement in the task.

Data from the one-distracter experiment were obtained in the fall of 2009, after which the bats were given an 8 week break due to metabolic and behavioral changes related to hibernation. We ceased testing during this period to avoid collecting data that could potentially be affected by physiological state, and also to avoid stressing the animals. Testing resumed with the two-distracter experiment in February 2010. A total of 48 768 calls from 936 trials from the one- and two-distracter experiments were examined (Appendix 1), although only one call per trial was used in statistical tests on acoustic parameters (see section F, "Statistical Analysis").

D. Audio recordings and sonar call analysis

Each bat's echolocation calls were recorded with two microphones (Ultrasound Advice), amplified (Ultrasound Advice, SM3), bandpass filtered from 10 to 100 kHz (Krone- Hite 3550), and converted from analog to digital (National Instruments, PCI-6071E). Identification, measurement, and analysis of calls were performed using custom programs written in MATLAB, versions 2007b–2015a (see Aytekin et al. 2010 for details). Trials in which the bats did not appear to be engaged in the task (e.g., trials in which the pulse interval pattern did not decrease monotonically, or in which the bat emitted only a few echolocation calls) were excluded from analyses. Additionally, because the bats would sometimes anticipate the arrival of the target by jumping or lunging forward, causing their close-range echolocation calls to differ from the typical pre-capture pattern, we excluded calls that occurred in the final 15 cm of the target's approach to the bat.

For the analysis of temporal variables, calls that were extreme outliers (duration >5ms or pulse interval >150 ms) were excluded from analysis. Temporal measurements were manually checked and corrected if necessary. Peak frequencies for the fundamental and first harmonic were measured from power spectra of each component. Calls that had durations of less than 1.33 ms were excluded from spectral analyses, because the signal-to-noise ratio of these signals was low and frequency measurements were less reliable. While call intensity was likely adjusted by the bats as part of their strategy to ameliorate echo clutter, we do not report on absolute or relative intensity levels, because microphones were not calibrated, and sensitivity settings changed day-to-day, along with changes in distracter positions.

We characterized SSGs as call clusters with surrounding pulse intervals at least 20% longer than those within the SSG. If three or more calls occurred in a group, an additional criterion of stable pulse intervals (65%) was applied (see Moss and Surlykke 2001; Moss et al. 2006). We counted the number of SSGs (doublets, triplets, and quadruplets) in each trial for all conditions.

E. Head turns

To measure head turns as the bats tracked the approaching target, we compared the relative amplitude of echolocation signals picked up by the two microphones positioned to the left and right of the bat. First, the relative amplitude ratio (RAR) was calculated as the ratio between the raw amplitudes of channels 1 and 2, corresponding to right and left floor microphones, respectively. We then subtracted each call's RAR from the close mean ratio for that trial, which was calculated as the average RAR from calls in that trial occurring in the last 5 cm (at target distances of 15–20 cm), when the influence

of the distracter(s) was predicted to be minimal and the bat was expected to be vocalizing straight ahead. Calls that were overloaded on both floor microphones were excluded from analysis.

Head turns were counted when consecutive relative amplitude ratio difference (RARD) values in the reduced data set changed from negative to positive or positive to negative, indicating a switch in head direction across the target motion axis. While we do not have video to validate this method, we set a conservative threshold RARD value of 60.3 to ensure that small deviations of head direction across the target approach axis would not be counted, although this may have resulted in under-counting of head turns. Calls with RARD values below this threshold were eliminated, as were calls with RARD values exceeding 61, which were not considered reliable.

F. Statistical analysis

To assess the relative importance of the number and location of distracters on bat echolocation behavior we measured eight response variables: two behavioral counts (number of SSGs and number of head turns) from each trial and six acoustic parameters (call duration, pulse interval, bandwidth, and sweep rate of the fundamental, fundamental peak frequency, first harmonic peak frequency) for a single call from each trial when the target distance was at 7062.5 cm. We adjusted duration and pulse interval measurements by subtracting means for each bat in the absence of any distracters to control for slight differences in baseline values across the one- and two-distracter experiments. SSG and head turn counts were similarly adjusted by subtracting mean baseline values from each trial.

All response variables were then fit to general linear models (GLMs) through a backward stepwise procedure using least squares. Distracter distance (45 or 70 cm), distracter number (one or two), and bat identity (#45 or #49) were categorized as nominal effects while angular offset, which was measured in degrees, was classified as a continuous covariate. We used the minimum Bayesian Information Criterion to select the best model among all possible models. All statistical analyses were performed in JMP 12.1.0.

III. RESULTS

A. Call duration and pulse interval

Both bats decreased call duration as the target approached in all conditions (Figure 2). At target distances beyond 20–30 cm both bats produced shorter duration calls when the distracters were present, when they were placed at the closer distance of 45 cm, and when they were placed at smaller angular offsets (Figure 2). Once the target had passed the distracters and was within 20–30 cm of the platform, all duration vs target distance profiles for the two-distracter condition converged on baseline levels. For the 45 cm distracter distance, convergence of call duration profiles occurred when the target was at the distracter distance or just after it passed the distracters, while for the 70 cm distracter distance, call duration did not converge to baseline levels until the target was in front of the distracters (25 cm in front of the bat). Both bats also tended to use shorter pulse intervals beyond target distances of 40–45 cm when the distracters were present (Appendix 2).

The two bats differed in the range of call durations and pulse intervals they used in the baseline condition, with Bat 45 using shorter duration calls than Bat 49, from 4ms

at a target distance of 100 cm down to 1ms at 15 cm, compared to 2.8 ms down to 0.5 ms for Bat 49 (Figure 2). Bat 45 also used shorter pulse intervals than Bat 49, ranging from 60 ms at 100 cm down to 10 ms at 15 cm, compared to 50 ms down to 7ms for Bat 49 (Appendix 2). Scatter plots of call duration against previous pulse interval show that Bat 49 had less variability in its calls than Bat 45 regardless of the number of distracters presented, and differences in pulse interval were smaller than differences in call duration between the bats in the two-distracter condition (Figure 3).

The analysis of baseline-adjusted durations for calls produced when the target was near 70 cm revealed that both bats used shorter calls when distracters were present (Figure 4). The GLM explained nearly half of the variance in call duration, and included every experimental factor examined (angular offset, distracter distance, and number of distracters; Table 1). The largest source of variation in call duration was bat identity, with Bat 45 reducing its call duration more across distracter conditions than Bat 49 (Figure 4). Angular offset was the second largest source of variation, with both bats using shorter durations as angular offset decreased, but the bat identity by angular offset interaction was significant, reflecting the steeper slope of adjustment exhibited by Bat 45 (Figure 4a). Both bats reduced their call durations more when the distracter distance was 45 cm as compared to 70 cm (Figure 4b). The effect of the number of distracters also significantly differed between the bats: Bat 49 used similar call durations regardless of the number of distracters present, while Bat 45 used shorter calls when two distracters were presented (Figure 4c).

In contrast to call duration, pulse interval changed very little across experimental conditions (Appendix 2). Notably, the GLM for pulse intervals of calls made near a target

distance of 70 cm included just two factors, did not include bat identity, and explained only 4% of the variation in pulse interval (Table 1). Both bats used shorter pulse intervals when angular offset was small (Appendix 3a) and produced shorter pulse intervals when the distracter was at 45 cm, but the difference between distracter distances was more pronounced for Bat 45 (Appendix 3b).

B. Peak call frequencies

Bat identity explained the most variation in fundamental peak frequency and contributed heavily to the model's explanatory power (R^2=0.67, Table 1). Bat 45 exhibited more variability in fundamental peak frequency than Bat 49 in response to clutter (Figure 5). Bat 45 also used higher fundamental peak frequencies in distracter conditions relative to baseline while Bat 49 made only slight changes to fundamental peak frequency in distracter conditions relative to baseline (Figure 6a) and across angular offsets (Figure 6b; Appendix 4a). These differences are reflected in the significance of two interaction effects (bat identity by number of distracters, and bat identity by angular offset) in the GLM for fundamental peak frequency.

As with peak frequency of the fundamental, bat identity explained the most variation in first harmonic peak frequency, followed by the number of distracters. But by contrast, Bat 49 lowered its first harmonic peak frequency in distracter conditions, especially when two distracters were present, while Bat 45 did not change its first harmonic frequency relative to baseline in the two-distracter condition (Appendix 4b), although it did slightly increase it in the one-distracter condition (Figure 7a). Accordingly, the bat identity by the number of distracters interaction effect was significant in the model (Table 1). Bat 49 used lower first harmonic peak frequencies at

small angular offsets, and Bat 45 showed little difference from baseline unless angular offset was large (Figure 7b).

C. Bandwidth and sweep rate

Both bats lowered bandwidth when distracters were present, particularly at low angular offsets (Figure 8a) and when two distracters were present (Figure 8b). These were the two largest sources of variation in the GLM (R^2=0.22, Table 1). As with peak frequency, Bat 45 exhibited more variability in bandwidth than Bat 49 in response to clutter (Figure 5). Changes to bandwidth were generally larger for Bat 45, and while differences between bats were clear at the 70 cm distracter distance, they virtually disappeared when the distracter distance was 45 cm (Figure 8c). Accordingly, the number of distracters by distracter distance interaction effect was the third largest source of variation in bandwidth (Table 1).

Sweep rate increased when angular offset was low (Figure 9a) and when the distracter distance was 45 cm (Figure 9b). As with bandwidth, angular offset explained the most variation in the model for sweep rate (R^2=0.38), followed by distracter distance, bat identity, and the bat identity by angular offset interaction effect. Interestingly, the difference between bats was less pronounced in the two-distracter condition than the one-distracter condition (Appendix 5a) and the bats appeared to more drastically increase sweep rate with decreasing angular offset when the distracter distance was 45cm (Appendix 5b), as reflected in the significance of these two-way interactions in the GLM (Table 1).

D. SSGs

Both bats produced more SSGs with decreasing angular offset (Figure 10a) and when the distracter distance was 45 cm as opposed to 70 cm (Figure 10c). The number of distracters also influenced adjusted SSGs per trial but this effect differed by bat, with Bat 49 using fewer SSGs in the one distracter condition and Bat 45 using more SSGs regardless of how many distracters were present (Figure 10b). This was reflected in the significance of the bat identity by the number of distracters interaction term in the model (Table 2). While Bat 45's adjusted mean SSGs per trial were higher than Bat 49's in all conditions, Bat 49 produced more SSGs overall and in every experimental condition compared to Bat 45 (Appendix 6). This was due at least in part to Bat 49's abnormally high use of SSGs in baseline 1, which exceeded all conditions except the 10angular offset (Appendix 6a).

The bats differed most markedly in their use of doublets (SSGs consisting of two calls), and, as with total SSGs, all three types generally increased at low angular offsets (Appendix 7). Overall, it appears that the bats differed in their overall use of SSGs, but both bats changed their production of SSGs similarly with angular offset and distracter distance. However, the effect of number of distracters on SSG production is unclear, and the GLM explained relatively little of the variation in SSGs (R^2=0.17).

E. Head turns

Head turns, as measured by our criteria, were generally infrequent—the highest average adjusted to baseline was fewer than one per trial—and the GLM explained only 10% of the variance in head turns (Table 2). Nevertheless, the presence of distracters clearly influenced head movements given that both bats used more head turns in the two

distracter condition than in the one-distracter condition. Bat 49 showed a larger increase in head turns between the one and two-distracter conditions relative to baseline than Bat 45 (Figure 11a). It is noteworthy that Bat 45's mean head turns in the one-distracter condition were inflated by an abnormally high number produced at the 20angular offset (Appendix 8a). The GLM accordingly showed that the bat by number of distracters term was significant (Table 2). Both bats also turned their heads more frequently when the distracter was at 45 cm (Figure 11b). The interaction between angular offset and number of distracters was significant, and showed that more head turns occurred when the angular offset was high, but only in the two-distracter condition (Figure 11c). RARDs plotted against target distance showed that head turns were prominent and consistent in most trials for Bat 49, but less so for Bat 45, and appeared to be initiated just after the target passed the distracter(s) (Appendix 9). The number of distracters, distracter distance, and the interaction between the bat and number of distracters accounted for most of the variation in adjusted head turns while the interaction between angular offset and number of distracters was less influential (Table 2).

IV. DISCUSSION

This study investigated how big brown bats adjust their echolocation behavior when tracking a moving target in a cluttered environment, with differing levels of clutter interference created by distracter objects placed at different distances and angular offsets from the bat. Analysis of the temporal and spectral variation in the calls and head movements with a series of GLMs provides compelling evidence that these bats used different strategies for target tracking in clutter.

A. Effect of distracters on call duration and pulse interval

When the target was close (30 cm from the platform), both bats produced calls that were very similar in duration between two-distracter and baseline conditions (Figure 2), illustrating that the distracters no longer influenced call duration, despite the distracters' large reflecting surfaces compared with the target (1.27 cm compared to 0.38 cm). Both bats used shorter calls when acoustic interference from the distracter echoes was greatest, i.e., the distracter distance was close or angular offset was small. Additionally, the bats showed less change in call duration as the target approached when the distracters were at small angular offsets (Figure 2). These findings are consistent with earlier reports that bats adjust call duration primarily in response to the nearest object (Aytekin et al. 2010). By using shorter call durations, bats reduce the potential for echo-echo overlap. If target and non-target objects are sufficiently close, such that returning echoes overlap in time, the neural representations of the objects may merge, causing clutter interference (Simmons et al. 1989).

Consistent with a previous study (Aytekin et al. 2010), call durations were influenced by the distracters for a period after the target had passed the distracters, and this zone was larger when the distracter was at 70 cm than at 45cm (Figure 2). This may reflect range-dependence in the size of clutter interference zones. In a phantom target echo detection task, Simmons et al. (1988) reported that the size of the clutter interference zone in *Eptesicus fuscus* increases with range, suggesting that the spatial region over which clutter and target echoes interfere scales with distance. These zones are created by forward, simultaneous, and backward masking, as the target is first behind, then near, then in front of, the distracter object(s) (Simmons et al. 1988).

Both bats systematically shortened pulse interval with target distance, regardless of distracter condition, suggesting that they could track the moving target even when it was behind the distracter(s). For all angular offsets, in both the one- and two-distracter conditions, pulse interval changed with target distance until the target was very close to the bat (20 cm), suggesting that the distracters influenced the timing of calls, even well after the target had passed the distracter(s).

The bats exhibited consistent differences in call duration and pulse interval (Figure 3). Bat 45 used shorter duration calls at small angular offsets, at the distracter distance of 45 cm, and when two distracters were present, while Bat 49 changed its call durations relatively little across distracter conditions (Figure 4). Similarly, Bat 49 changed its pulse intervals very little across angular offsets and between distracter distances, while Bat 45 clearly reduced its pulse intervals in these conditions (Appendix 3). That the bats differed in the temporal parameters of their calls under different distracter conditions reveals that adjustments in sonar call duration and pulse interval differ among individual bats.

B. Effect of distracters on peak frequency

We predicted that the bats would increase the peak frequency of echolocation calls when two distracters were present and at small angular offsets, to sharpen the sonar images created by more directional sonar information carried by higher frequencies. The bats showed opposite patterns of adjustment in peak frequency, with one subject changing only the peak frequency of the fundamental, and the other changing only the peak frequency of the first harmonic (Appendix 4).

Counter to our prediction, the bat that made its calls more directional by increasing peak frequency did so in the one-distracter condition and not the two-distracter condition (Appendix 4a). Even at the largest angular distracter offsets, neither bat could have avoided ensonifying the distracters entirely by narrowing their sonar beams (Hartley and Suthers 1989), and it is therefore likely that additional strategies allowed the bat to disambiguate echo streams from objects in a cluttered environment (Bates et al. 2011; Simmons 2014; Wohlgemuth et al. 2016).

C. Effect of distracters on bandwidth and sweep rate

We expected that the bats would produce calls with higher bandwidth and sweep rate as the distracter number and position created more echo clutter, to sharpen the target image and improve localization accuracy. However, the fundamental bandwidth of calls made when the target was near 70 cm was consistently lower in distracter conditions than in baseline. Although adjusted bandwidth decreased at small angular offsets, adjusted sweep rate (calculated as bandwidth divided by call duration) increased, indicating that higher sweep rates were achieved through reductions in call duration.

Interestingly, the second-largest source of variation identified in the GLM for adjusted bandwidth was the number of distracters, which was not significant in the GLM for adjusted sweep rate. Similarly, the second-largest source of variation in adjusted sweep rate was distracter distance, which was not significant in the GLM for adjusted bandwidth. This result lends support to the assertion made by Boonman and Ostwald (2007) that broader bandwidth calls helps bats correctly count the number of echoes (which would change depending on the number of distracters present) while higher sweep

rates help with accuracy of distance estimates based on echo delays (which would change depending on distracter distance).

Additionally, while bat identity was the third largest source of variation in adjusted sweep rate, sweep rates of both bats converged as the angular offset became smaller when there were two distracters present or when the distracter distance was 45 cm (Appendix 5), suggesting that under challenging conditions there may indeed be an optimal sweep rate which balances resolution of echoes in a cascade with echo delay acuity (Boonman and Ostwald 2007).

D. Effect of distracters on use of SSGs

We hypothesized that under more challenging conditions (e.g., when two distracters were present, distracter distance was 45 cm, and angular offset was small), the bats would produce more SSGs to improve spatial resolution and counteract ambiguity in echo assignment. The GLM fit to adjusted SSGs generally supports this hypothesis (Table 2), although the R2 was low (0.17). Contrary to our prediction, however, both bats used fewer SSGs in the two-distracter condition than the one-distracter condition (Appendix 6). The considerable difference between bats in unadjusted SSG totals per trial, and the significance of bat identity in the model, also suggests that individual bats may rely more heavily on other acoustic or behavioral adjustments in response to a challenging task.

E. Effect of distracters on head turns

We hypothesized that bats would employ more head turns in the one-distracter condition when the distracter distance was 45 cm and angular offset was small, and fewer head turns in the two-distracter condition. As predicted, both bats used more head turns

when the distracter was closer (Table 2; Figure 11b). Inspection of RARDs by target distance appeared to show more evidence of head turning at the 45 cm distracter distance when two distracters were present (Appendix 9). That the head turns seemed to occur more consistently after the target passed the distracters at 45 cm (relative to at 70 cm) may reflect a greater need or ability of the bats to track the target when it passed in front of the distracters at closer range.

Surprisingly, both bats used fewer head turns in the one distracter condition and more in the two-distracter condition, relative to baseline (Figure 11a). This effect contrasts with our prediction, as the echoes returning from the distracter toward which the bat turned its head would be strengthened in amplitude and bandwidth, while echoes from the target would be weakened in amplitude and low-pass filtered. However, examination of the other significant interaction term, angular offset by number of distracters, revealed that adjusted head turns were higher at large angular offsets in the two-distracter condition, while in the one-distracter condition they remained the same across angular offsets (Figure 11c).

Differences in head turning between the one- and two distracter conditions suggests that bats may not need to employ head turns to disambiguate echo sources when clutter is low or confined to one side of the bat, but head turning might help when clutter is high (e.g., on both sides of the bat), as long as the clutter objects are separated from the target by a sufficiently large azimuthal angle. At high angular offsets, the bats may have been able to turn their heads or move their pinna to more accurately represent the location of the distracters using interaural difference cues, while maintaining the distracters sufficiently off-axis to result in low-pass filtered, "defocused" clutter echoes (Bates et al.

2011). Adjusted head turns were low in both one- and two distracter conditions at small angular offsets (Figure 11c), presumably because head turning would result in directly ensonifying the distracters and increasing the strength of clutter echoes. Alternatively, the head turn counts may be biased toward more exaggerated head turns due to our use of a conservative RARD threshold, and larger head turns might only benefit the bats when the distracters were placed at large angular offsets.

V. CONCLUSIONS

In this experiment, we showed that bats make adjustments to their echolocation calls and head movements in response to clutter, which we created by introducing one or two distracters at two distances and four angular offsets from an approaching target. Although the bats were stationary rather than free-flying, this design allowed us to systematically investigate the effect of clutter distance and angular offset on echolocation behavior. As hypothesized, call durations shortened as clutter interference increased. Pulse interval was not strongly influenced by clutter, indicating that the bats could still track the target even when it was beyond the distracter(s). Consistent with other studies, the bats used higher sweep rates and more SSGs when clutter was increased. Head turns were used more frequently in the two distracter condition, but mostly at large angular offsets.

Notably, individual bats used different strategies to track a moving target in the presence of distracter objects. One bat primarily changed the spectro-temporal features of its calls, shortening duration, and increasing peak frequency, while the other used more SSGs and exhibited consistent head turning in high-clutter conditions. While limited to two subjects, this study suggests that call duration, peak frequency, SSGs, and head

movements can all be dynamically adjusted to ameliorate clutter interference at different ranges and angular offsets, and that individual bats may use different combinations of vocal and behavioral adjustments to track targets in the presence of other objects.

Table 1. Best general linear models for five parameters of echolocation calls emitted when the target distance was near 70 cm, adjusted by baseline means. Interaction effects are denoted by an asterisk between factors. F values are given for all factors included in each model, with significance level indicated by asterisks (* = p≤0.01, ** = p≤0.001, *** = p≤0.0001). Factors not included in the final model for a given parameter are denoted with a dash (-), and overall model statistics are given at the bottom.

Source	Call Duration	Pulse Interval	Peak Frequency (Fundamental)	Peak Frequency (First Harmonic)	Bandwidth	Sweep Rate
Bat	314.3***	-	1535.0***	169.0***	13.5**	116.3***
Angular offset	161.1***	10.2*	59.0***	24.5***	73.4***	192.5***
Distracter distance	66.8***	18.2***	-	14.8***	3.2	124.4***
Number of distracters	31.2***	-	213.5***	115.3***	51.1***	3.4
Bat*Angular offset	19.2***	-	19.3***	0	17.5***	61.7***
Bat*Distracter distance	-	-	-	5.8	18.1***	3.9
Bat*Number of distracters	11.1**	-	32.7***	20.7***	-	26.6***
Angular offset*Distracter distance	-	-	-	4.8	0.0	17.9***
Angular offset*Number of distracters	15.0***	-	-	-	23.0***	11.2**
Distracter distance*Number of distracters	9.9*	-	-	7.9*	34.1***	-
Bat*Angular offset*Distracter distance	-	-	-	24.7***	16.5***	12.5**
Bat*Angular offset*Number of distracters	-	-	-	-	-	-
Bat*Distracter distance*Number of distracters	-	-	-	-	-	-
Angular offset*Distracter distance*Number of distracters	-	-	-	-	-	-
Model n	664	664	879	879	879	879
Model R^2	0.47	0.04	0.67	0.29	0.22	0.38
Model F	73.6***	13.7***	356.1***	37.8***	25.3***	53.8***

Table 2. Best general linear models for per-trial totals of two behavioral parameters, adjusted by baseline means. Interaction effects are denoted by an asterisk between factors. F values are given for all factors included in each model, with significance level indicated by asterisks (* = p≤0.01, ** = p≤0.001, *** = p≤0.0001). Factors not included in the final model for a given parameter are denoted with a dash (-), and overall model statistics are given at the bottom.

Source	Sonar sound groups	Head turns
Bat	46.0***	4.0
Angular offset	59.4***	6.0
Distracter distance	8.6*	20.0***
Number of distracters	47.2***	37.83***
Bat*Angular offset	9.0*	-
Bat*Distracter distance	-	-
Bat*Number of distracters	34.8***	19.2***
Angular offset*Distracter distance	-	-
Angular offset*Number of distracters	-	7.9*
Distracter distance*Number of distracters	11.8**	-
Bat*Angular offset*Distracter distance	-	-
Bat*Angular offset*Number of distracters	-	-
Model n	857	857
Model R^2	0.17	0.10
Model F	26.0***	16.3***

Figure 1. Overhead view of experimental set-up showing distracter distances and angular offsets (not to scale). Inset diagrams show a call (C), distracter echo (D), and target echo (T) when the target is behind or in front of the distracters at two example distracter positions. When calls are short (top), no overlap of echoes occurs, whereas distracter and target echoes do overlap when the call is long (bottom).

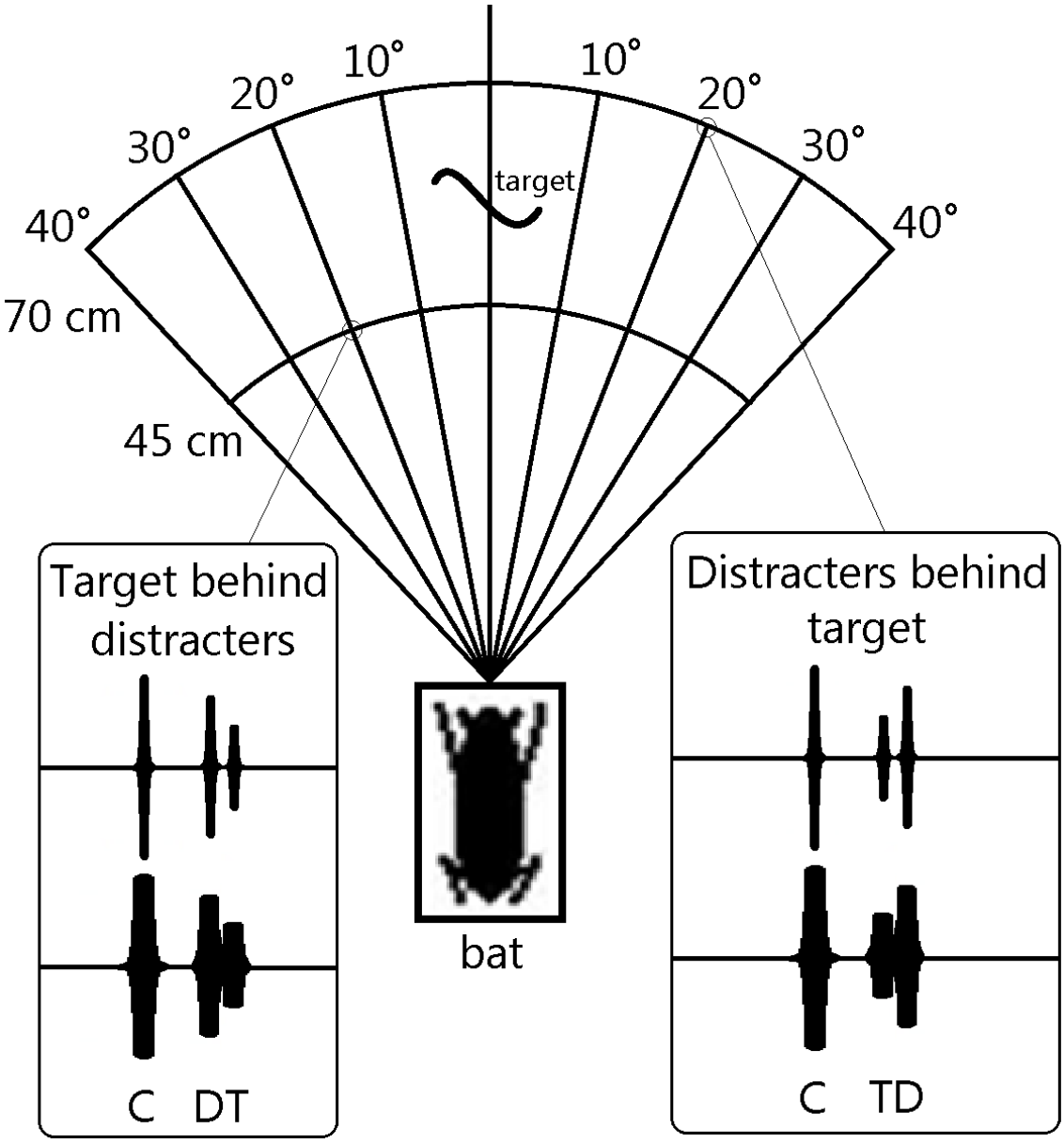


Figure 2. Average call duration plotted against target distance for Bat 45 (a, b) and Bat 49 (c-d) in the two-distracter condition when the distracters were located at 45 cm (a-c) and 70 cm (b-d). Averages were calculated across trials within 5 cm bins. Baseline 2 is reproduced across both distracter distances for comparison. Distracter distance is shown as a vertical black line.

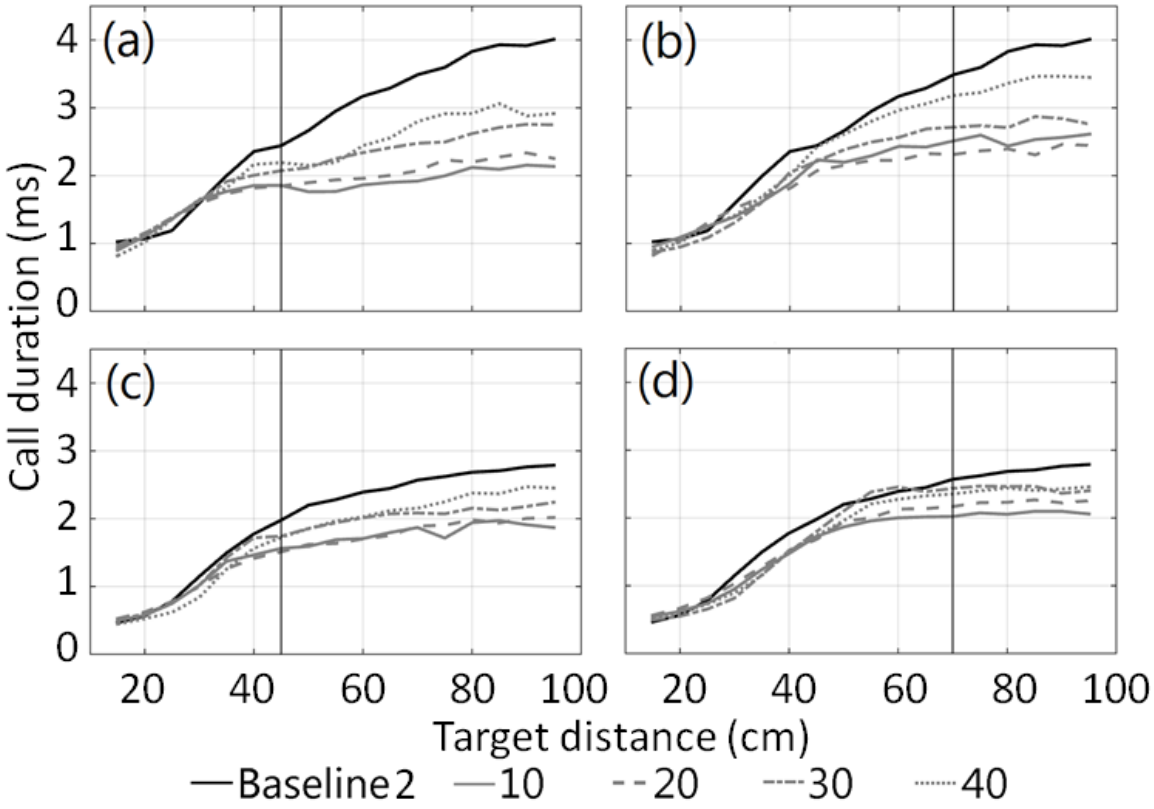


Figure 3. Pulse interval plotted against duration for all calls except outliers (duration > 5 ms or pulse interval > 150 ms) recorded in the baseline (a), one-distracter (b), and two-distracter (c) conditions.

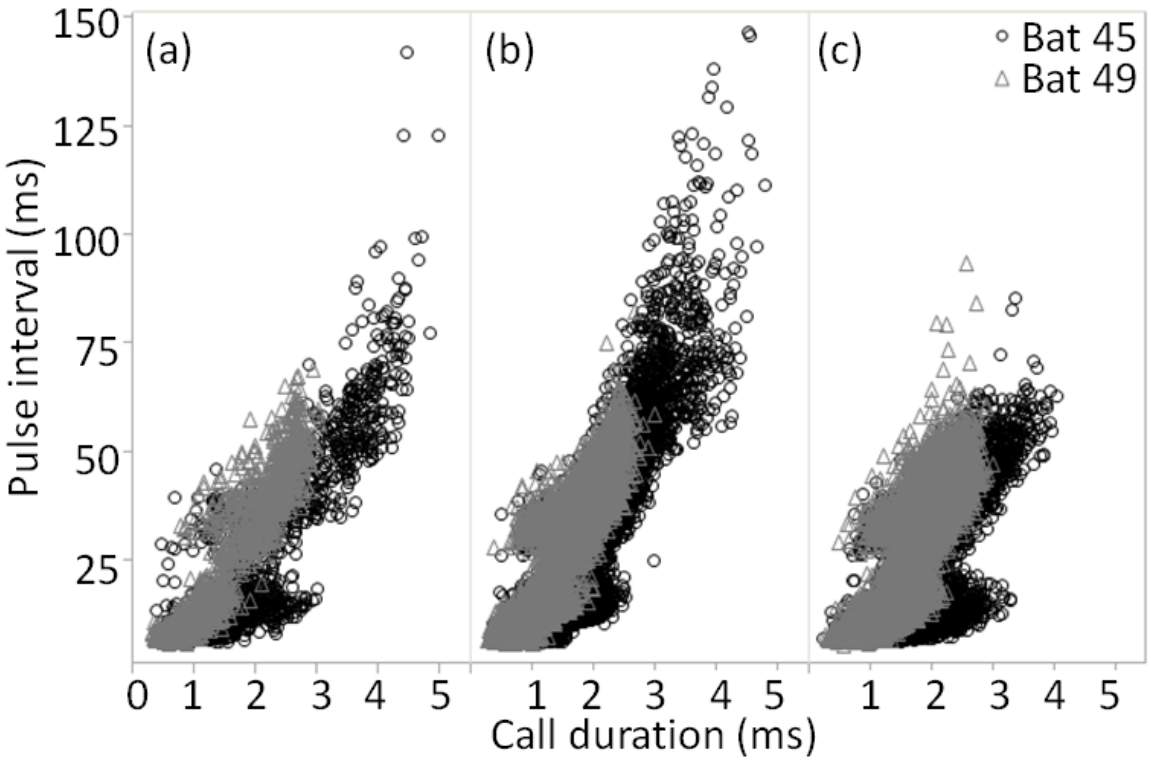


Figure 4. (a) Lines of fit (with confidence intervals) through adjusted duration for one call per trial made when the target distance was 70 cm (±2.5 cm), relative to baseline, across angular offsets. (b) Means and standard errors of adjusted duration for one call per trial made when the target distance was 70 cm (±2.5 cm) from the platform when the distracter distance for was 45 cm or 70 cm, and (c) in the one- and two-distracter conditions.

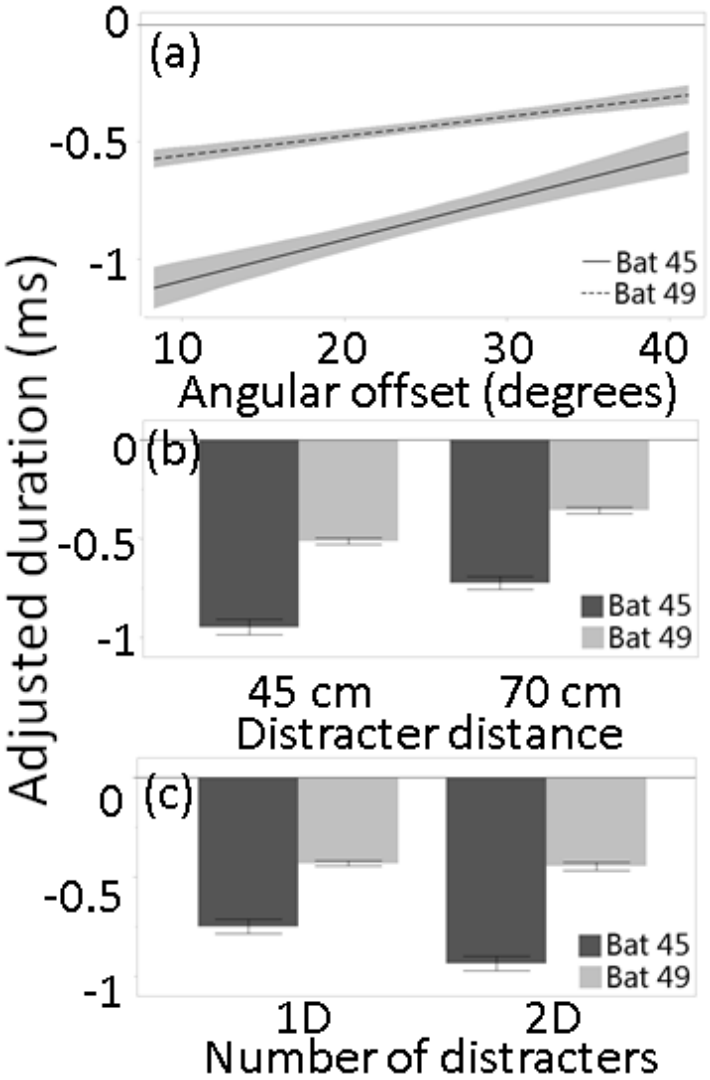


Figure 5. Fundamental peak frequency plotted against bandwidth for all calls for which frequency could be reliably estimated (duration<1.33 ms) recorded in the baseline (a), one-distracter (b), and two-distracter (c) conditions.

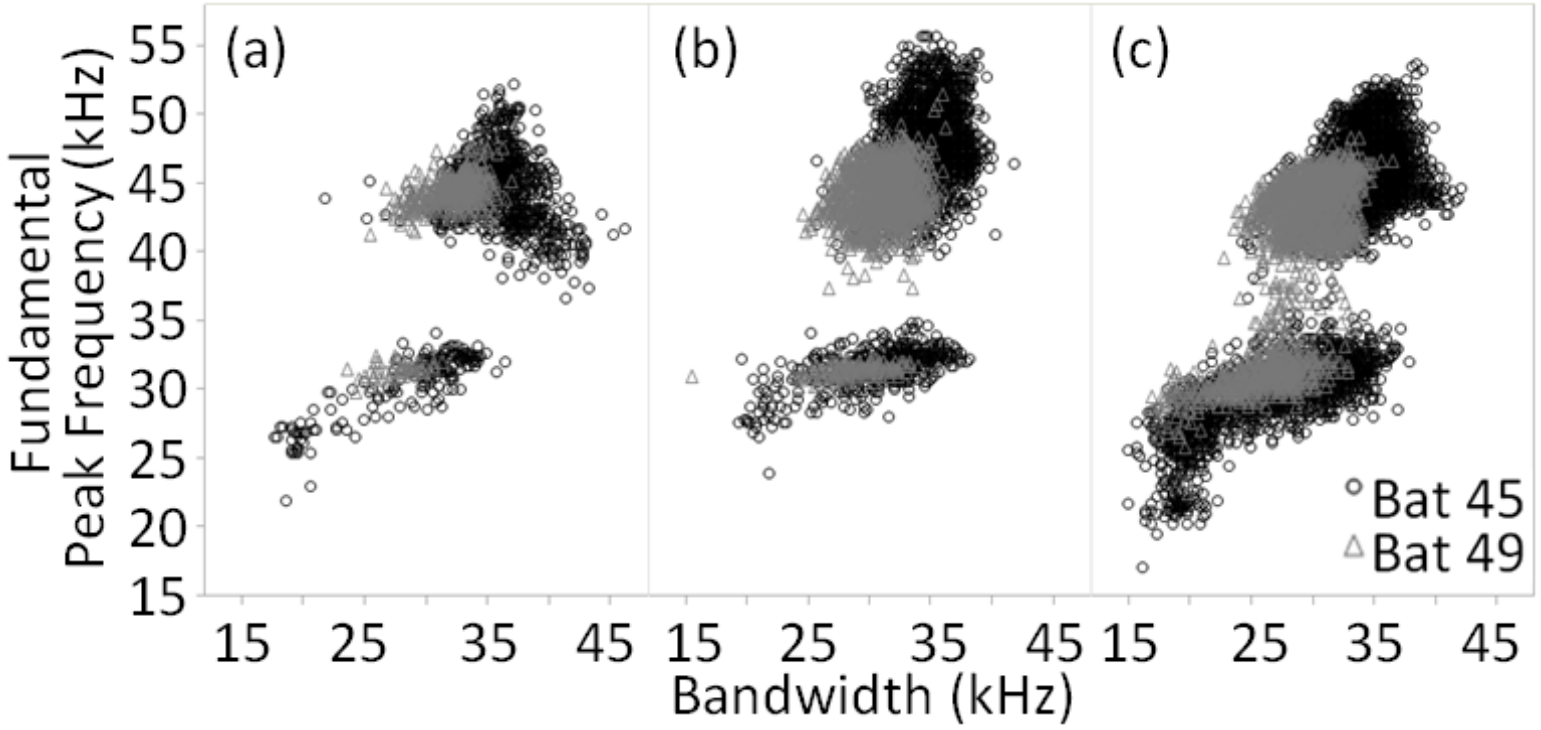


Figure 6. (a) Means and standard errors of adjusted fundamental peak frequency for one call per trial made when the target distance was 70 cm (±2.5 cm) by number of distracters. (b) Lines of fit (with confidence intervals) to adjusted fundamental peak frequency of one call per trial made when the target distance was 70 cm (±2.5 cm), relative to baseline, across angular offsets.

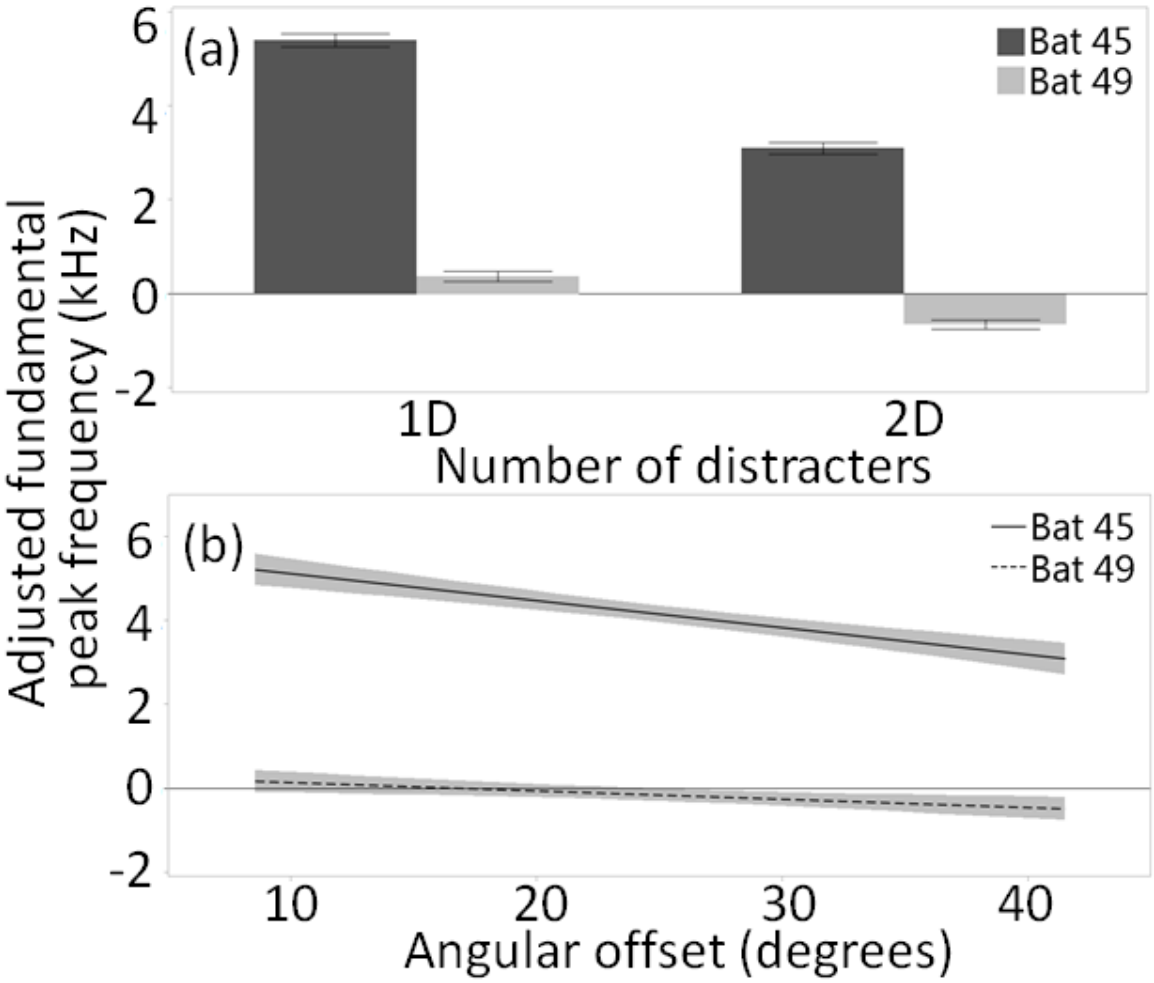


Figure 7. (a) Means and standard errors of adjusted first harmonic peak for one call per trial made when the target distance was 70 cm (±2.5 cm) by number of distracters. (b) Lines of fit (with confidence intervals) to adjusted first harmonic peak frequency of one call per trial made when the target distance was 70 cm (±2.5 cm), relative to baseline, across angular offsets.

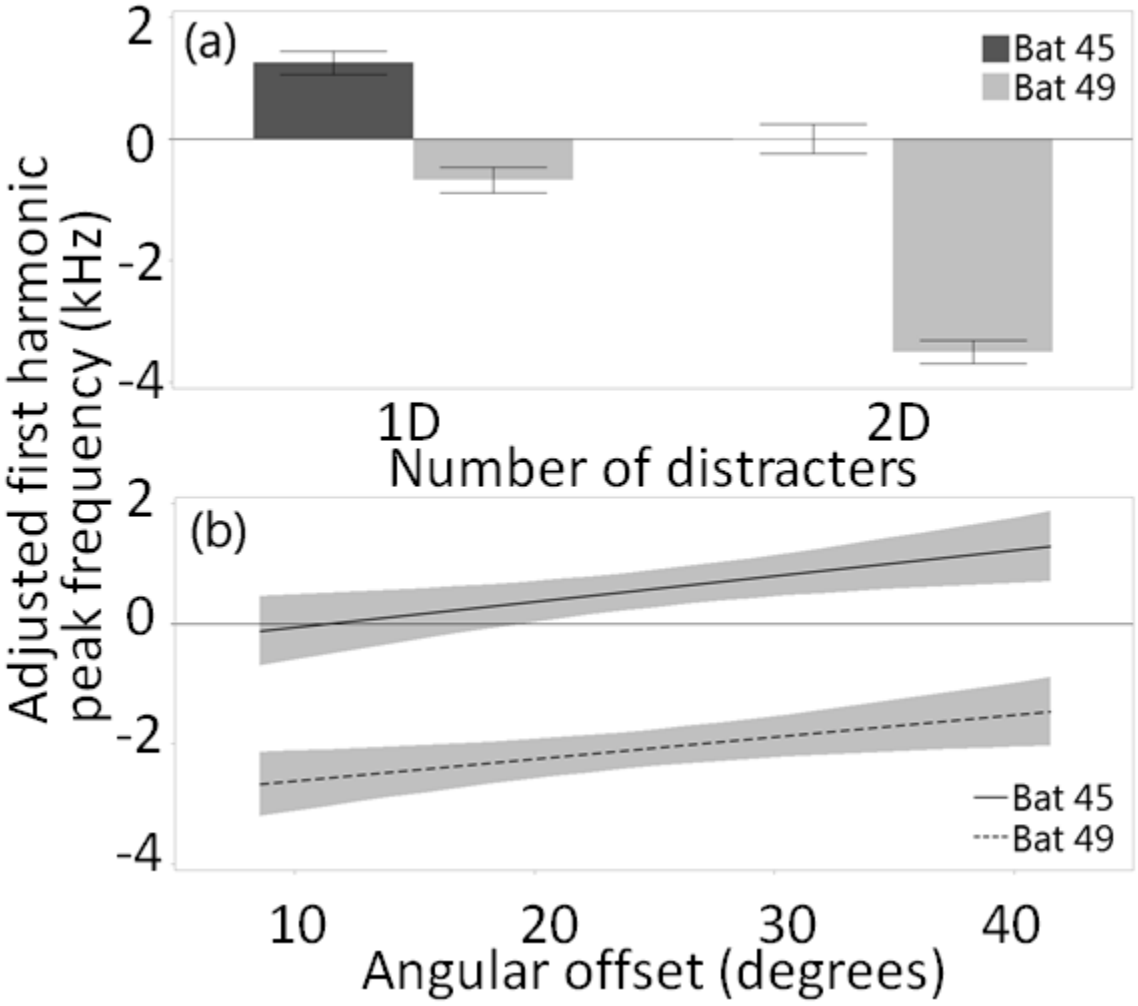


Figure 8. (a) Lines of fit (with confidence intervals) to adjusted fundamental bandwidth of one call per trial made when the target distance was 70 cm (±2.5 cm), relative to baseline, by number of distracters and across angular offsets. (b) Means and standard errors of adjusted fundamental bandwidth for one call per trial made when the target distance was 70 cm (±2.5 cm) by number of distracters and (c) by distracter distance and number of distracters.

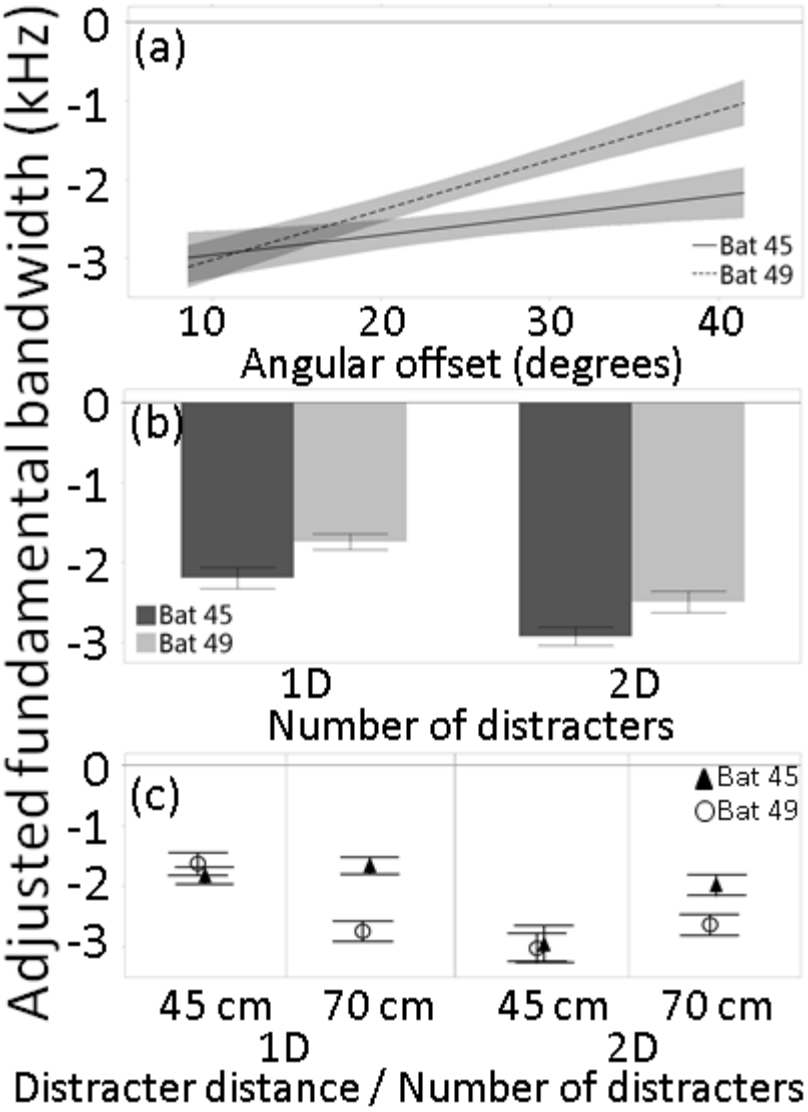


Figure 9. (a) Lines of fit (with confidence intervals) to adjusted fundamental sweep rate of one call per trial made when the target distance was 70 cm (±2.5 cm), relative to baseline, across angular offsets. (b) Means and standard errors of adjusted fundamental sweep rate for one call per trial made when the target distance was 70 cm (±2.5 cm) by distracter distance.

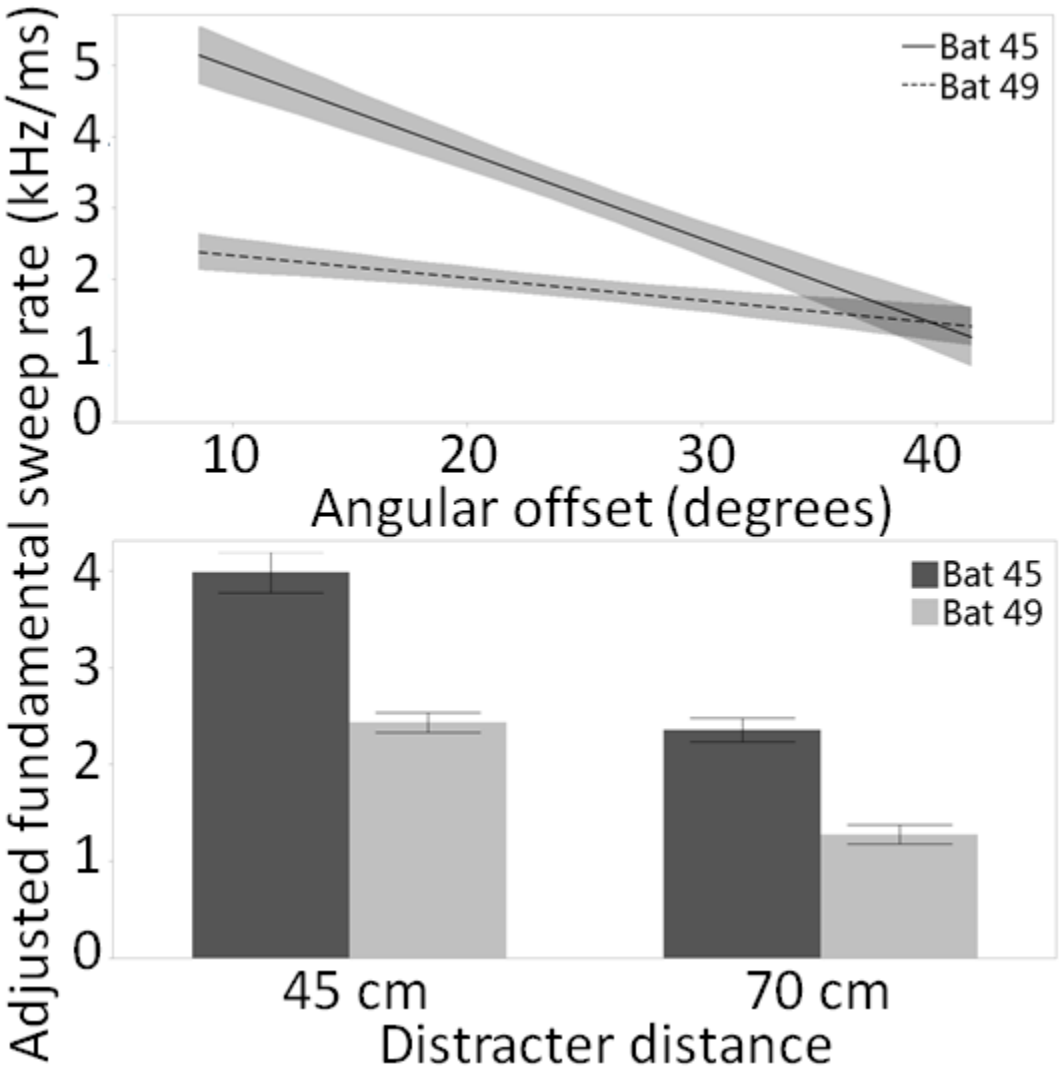


Figure 10. (a) Lines of fit (with confidence intervals) through number of sonar sound groups (SSG) per trial across angular offsets, adjusted by mean baseline SSGs per trial. (b) Means and standard errors of adjusted SSGs per trial by number of distracters present, and (c) distracter distance.

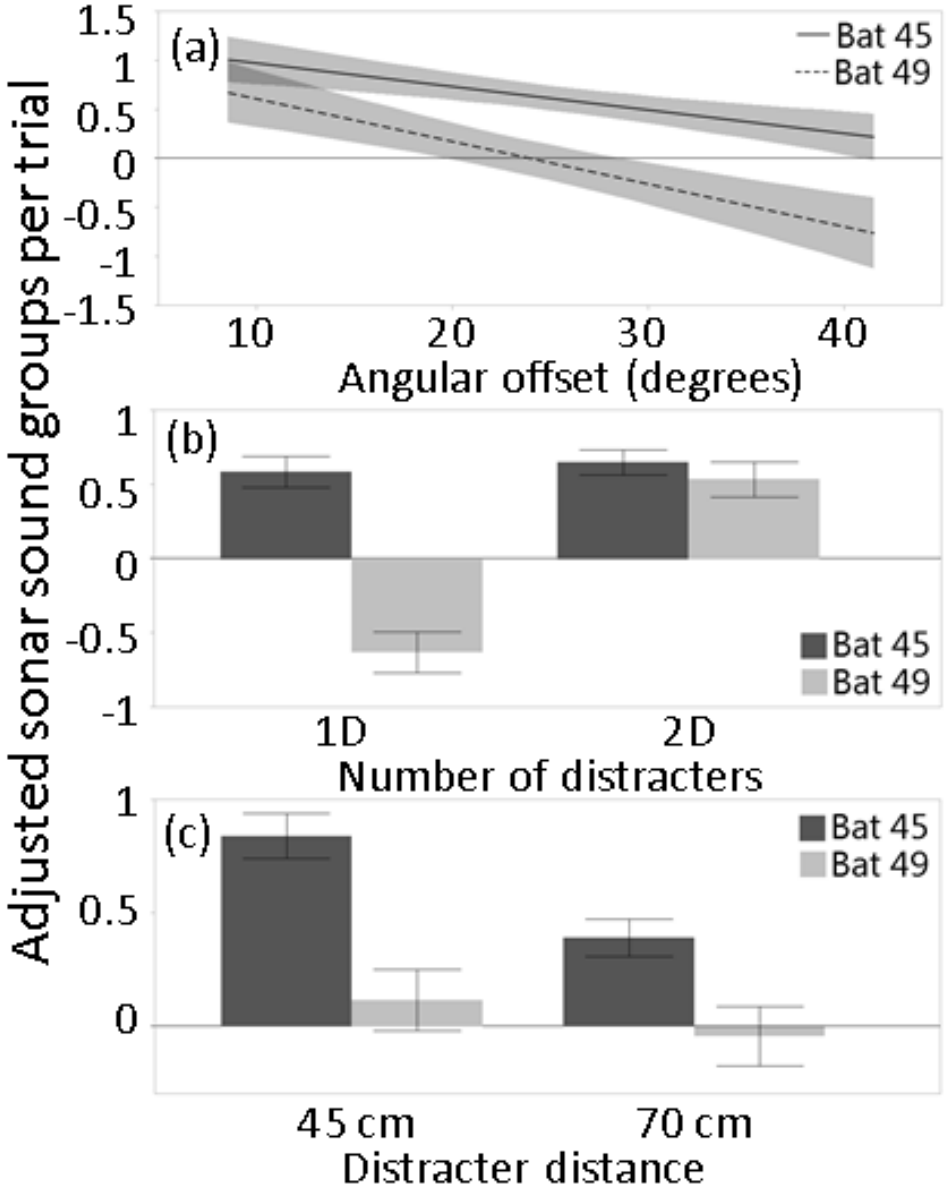
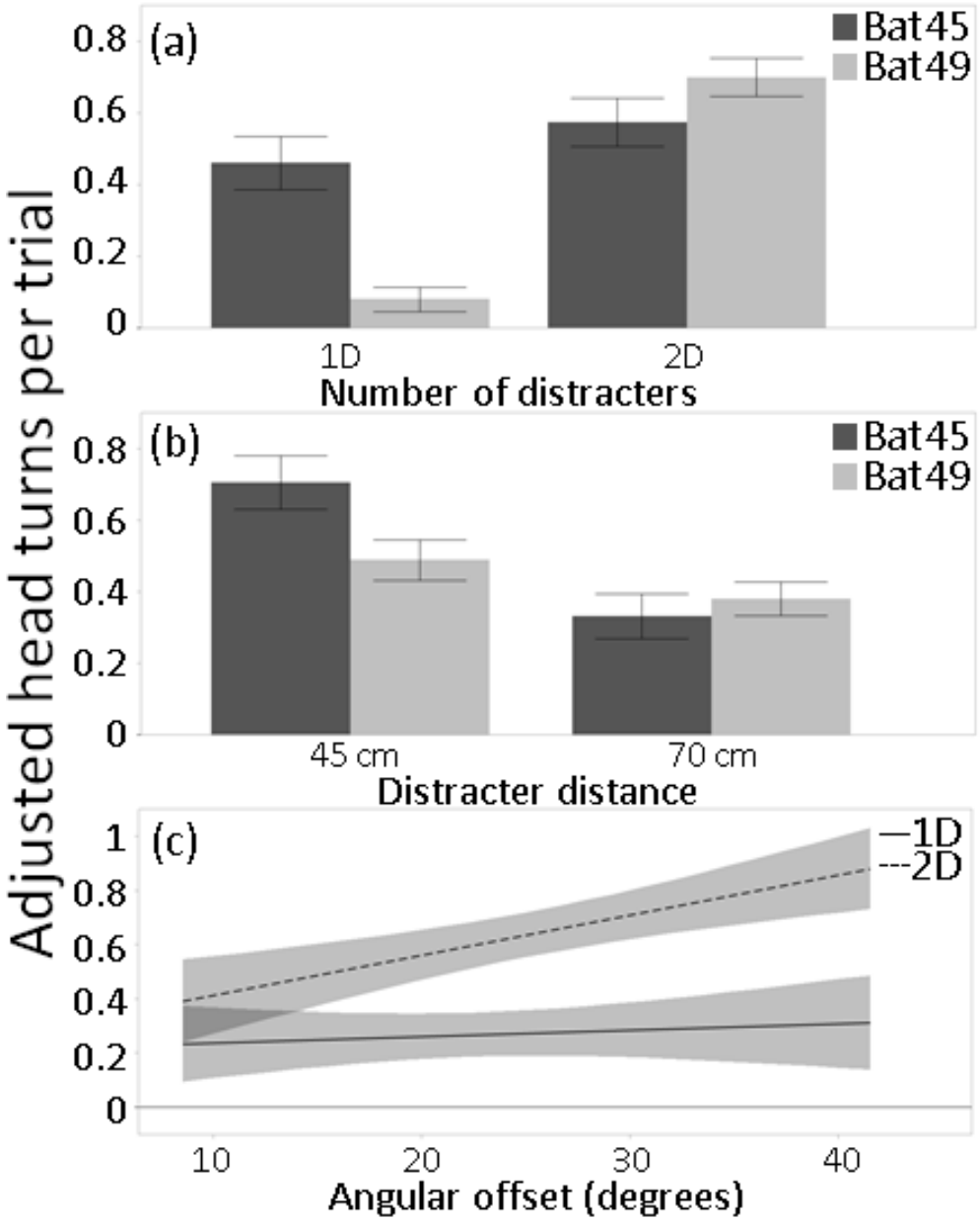


Figure 11. Means and standard errors of head turns, adjusted to baseline, by (a) number of distracters present and (b) distracter distance. (c) Lines of fit (with confidence intervals) to adjusted head turns relative to baseline by angular offset and number of distracters.



Chapter 3: Age-dependent gene expression in the inner ears of big brown bats

ABSTRACT

For echolocating bats, hearing is essential for survival. Specializations for detecting and processing high frequency echoes are apparent throughout their auditory systems. Recent studies on echolocating mammals have reported evidence of parallel evolution in some hearing-related genes in which distantly related groups of echolocating bats, or even echolocating bats and whales, cluster together in gene trees due to apparent amino acid convergence. However, molecular adaptations can occur not only in coding sequences, but also in the regulation of gene expression. The aim of this study was to examine the expression of hearing-related genes in the inner ear of the developing big brown bat, *Eptesicus fuscus,* during the period in which young bat vocalizations increase dramatically in frequency. We found that seven genes were significantly upregulated in juveniles relative to adults, and that the expression of four genes through development correlated with estimated age. Compared to available data in the developing mouse, it appears that expression of some hearing genes is prolonged in the juvenile bat. These results are consistent with a larger cochlea relative to body size, a later maturation of high frequency hearing, and a greater dependence on high frequency hearing in echolocating bats.

I. INTRODUCTION

Echolocating bats have among the highest frequency hearing in the animal kingdom (Heffner and Heffner 2008). Additionally, because echolocating bats emit calls in air, they must be able to detect, and extract information from, echoes which return greatly weakened relative to outgoing calls due to spreading and interference. While good high frequency hearing confers a survival benefit to many animals, it is absolutely essential for the survival of bats that rely on echolocation to avoid obstacles, obtain food, and find roosts and conspecifics. Furthermore, bats are exceptionally long-lived for their size, with individuals of some 10 g or smaller species living more than 30 years (Wilkinson and South 2002). The need for echolocation throughout life suggests that the ability to hear high frequencies without severe age-related difficulties may have been under strong positive selection in echolocating bats. This stands in contrast with the occurrence of age-related hearing loss (presbycusis) in humans, which has been estimated to be 40% among those over 70 (Collins 1997).

The importance of hearing to echolocators has been illustrated by a number of recent studies examining the molecular evolution of genes involved in hearing in bats. Many of the genes known from human and house mouse (*Mus musculus*, heretofore referred to as 'mouse') studies to be crucial for normal hearing, such as transmembrane channel-like 1 (*Tmc1*) and *Prestin/SLC26A5*, exhibit convergence between the two distantly related groups of echolocating bats, or even between echolocating bats and whales, such that gene trees sometimes group echolocators together to the exclusion of non-echolocators (Li et al. 2008; Li et al. 2010; Liu et al. 2010; Liu et al. 2011; Liu et al. 2012a; Davies et al. 2012; Shen et al. 2012). While the results of these studies are compelling, they may underestimate the degree to which genetic change is involved in

high-frequency hearing given that the expression of hearing genes in bats has received little attention, despite the potential for differences in the amount or timing of gene expression to explain different phenotypes without requiring changes in coding sequence. Additionally, predicting the functional effect of up- or down-regulating a gene may be more straightforward than predicting the effect of an amino acid change, which will depend on the location of the substitution and the degree to which the substituted residue differs in its physicochemical properties from the original. Recent studies have shown that changes in gene regulation can influence the physical differences between bats and other mammals: transgenic mice possessing bat limb enhancers exhibit prolonged expression of limb elongation genes (Booker et al. 2016) and develop significantly longer limbs than control mice (Cretekos et al. 2008).

Some studies aimed at establishing the functional role of hearing genes in mice have examined gene expression in embryonic or early postnatal animals (e.g. Verpy et al. 2000; Kurima et al. 2002, Kawashima et al. 2011). Cochleae are easier to dissect during this period, because the otic capsule has not fully ossified, and the sensory epithelium degrades more slowly than in older animals, making extraction of RNA and estimation of gene expression easier at younger ages (e.g. Kawashima et al. 2011). However, in mammals high frequency hearing matures last, both peripherally (Harris and Dallos 1984; Echteler et al. 1989) and centrally (reviewed in Rübsamen 1992). High frequencies are particularly important to bats, who use them to control directionality of calls (Surlykke et al. 2009), determine distance to targets (Simmons 1973), reject non-target echo clutter (Simmons and Stein 1980), and resolve fine spatial details such as shape, size, and texture (Ostwald et al. 1988).

The big brown bat (*Eptesicus fuscus*) is an insectivore that hunts in edge spaces between open and cluttered environments. This behavior requires the disambiguation of cascades of echoes from multiple objects into separate percepts, which must occur quickly enough to inform motor decisions in flight (e.g. to avoid collision with a conspecific or an obstacle, or to catch an insect making erratic avoidance maneuvers). Because echolocation and flight are interrelated and critical for a young bat's survival, development of hearing occurs concurrently with that of motor skills involved in flight and echolocation calls (Moss 1988; Mayberry and Faure 2015). Juvenile big brown bats undergo significant changes in their call characteristics between birth and three weeks of age (Mayberry and Faure 2015). Their frequency-modulated (pre-echolocation) calls become shorter (from approximately 7 ms to 2-4 ms) and higher in frequency, with the fundamental sweeping from 24-14 kHz in the first week to 42-20 kHz by the third week (Moss 1988; Monroy et al. 2011). These changes in echolocation call frequencies likely coincide with changes in their hearing, because the frequency place map of the cochlea changes as it matures, with higher frequency hearing developing later (Harris and Dallos 1984; Echteler et al. 1989).

The mouse, which has been used as a model for hearing research, also hears ultrasonic frequencies, is of similar mass to the big brown bat, and has a similar head size (Heffner and Heffner 2008). However, behavioral audiograms indicate that its upper frequency limit is about 20 kHz lower than that of the big brown bat (88 kHz vs. 104 kHz at a threshold of 60 dB, as reported in Koay et al. 1997 and Koay et al. 2002 using the same paradigm) and neurons in the mouse inferior colliculus have thresholds below 20 dB SPL only in the 4-50 kHz range (Egorova et al. 2001) in comparison to 10-80 kHz

range in the big brown bat (Jen and Schlegel 1982). While mice emit high frequency sounds (Sales 1972), some of which appear to be important for male mating success (reviewed in Asaba et al. 2014), hearing does not appear to be critical to the development of normal vocalizations (Hammerschmidt et al. 2012; Mahrt et al. 2013). Similarly, juvenile big brown bats (*Eptesicus fuscus*), which use frequency modulated calls, develop adult-like echolocation calls even after deafening (Woolf 1974). By contrast, deafened juvenile horseshoe bats (*Rhinolophus rouxi*) and adult mustached bats (*Pteronotus parnelli*), both of which use constant-frequency calls, produced echolocation pulses that differed by 2-14 kHz from controls (Rübsamen and Schafer 1990; Kössl and Vater 2000). However, the call frequencies of five species of bats using both types of call designs were lower in the first year of life than in later adulthood, suggesting that fine tuning of echolocation calls may occur well after the development of hearing is complete (summarized in Jones 2000).

Because of their dependence on hearing for survival and their relatively well-developed auditory systems, echolocating bats provide a unique opportunity to examine postnatal hearing development in an auditory specialist. Bats using both frequency-modulated and constant-frequency calls possess larger cochlea (Hsiao et al. 2015) relative to basicranial width than non-echolocating or non-laryngeally echolocating bats (Kössl and Vater 1995). Bats using constant-frequency calls also exhibit overrepresentation of dominant call frequencies in basilar membrane dimensions and spiral ganglion density (Kössl and Vater 1985), and extremely short hair cells and stereocilia (Vater and Lenoir 1992). Wang et al. (2017) recently showed that echolocating bats sustain a high cochlear growth rate throughout development compared to non-echolocating bats and other

mammals, but which genes change expression during bat cochlear development is unknown. Here, we examine the temporal expression patterns of genes known to be upregulated in the cochlea of echolocating bats relative to non-echolocating bats, or which exhibit signs of parallel or convergent evolution among echolocators, in the inner ears of young big brown bats over a two-week period when pronounced changes in their vocalizations (and likely their hearing) occur.

II. METHODS

A. Subjects and sample preparation

Pregnant female *Eptesicus fuscus* were captured in the wild under a permit from the Maryland Department of Natural Resources. All twelve juvenile subjects were born in captivity. Because they were group-housed and often cluster together, exact dates of birth could not be directly recorded. Instead, forearm length was measured with calipers and used to estimate age following Burnett and Kunz (1982). Forearm length is a more accurate age estimator than mass for big brown bats, and results from formulae relating forearm length to age do not differ between wild and captive bats (Mayberry and Faure 2015). Estimated ages ranged from postnatal day (PND) 9 to 19. Juveniles were weighed, anesthetized with isoflurane and euthanized. All procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. Samples were also obtained from two adult individuals under a protocol approved by the University of Maryland Institutional Animal Care and Use Committee.

Inner ear samples, consisting of the entire otic capsule and its contents, were collected immediately post-mortem and placed into liquid nitrogen prior to storage at -80˚C until extraction. Samples were homogenized with a mortar and pestle while

submerged in liquid nitrogen. RNA extraction was performed using a mirVana kit (Ambion), with added proteinase K (Sigma Aldrich) to improve yield (Egyházi et al. 2004). All samples were treated with TURBO DNA-free DNAse (Ambion) and cleaned with isopropanol and ethanol. Sample quality was checked on a Nanodrop spectrophotometer and reverse transcribed with M-MLV (Thermo Fisher) using a 50/50 mix of oligo-dT and random primers to lower the risk of bias or truncated transcripts associated with a single priming method (Nam et al. 2002; Ståhlberg et al. 2004).

B. Gene selection and primer design

Candidate genes were selected based on one or more of the following criteria: upregulated in an echolocating bat vs. a non-echolocating bat; upregulated in an adult mouse relative to juvenile mouse; expressed in mid- to late- development; exhibits signs of parallel or convergent evolution between echolocating bats and whales; exhibits signs of parallel or convergent evolution between distantly related echolocating bats; or participates in forming essential cochlear structures (Table 3). For each gene, all available mRNA transcripts from *Eptesicus fuscus* and all bats of the genus *Myotis* (another genus in the same family, Vespertilionidae), were downloaded from GenBank (NCBI) and aligned using Clustal Omega (EMBL-EBI). Sequences from *Myotis* spp. were included in order to reduce the risk of designing primers in regions with polymorphic sites. All primer pairs were designed within the same exon to permit preliminary testing on genomic DNA.

To identify exons in an *Eptesicus fuscus* transcript, exonic regions of the *Myotis lucifugus* transcript, as identified in Ensembl, were blasted against the transcript for *Eptesicus fuscus*. If the *Myotis* transcript was not available in Ensembl, the mouse (*Mus*

musculus) transcript was used instead. If the exonic region was well conserved among *Eptesicus* and *Myotis* spp., it was entered into Primer-BLAST (NCBI). Potential primer pairs were checked for specificity against *Eptesicus fuscus* RefSeq data, potential for cross- and self-dimerization, and potential to form hairpins using Beacon Designer (Premier Biosoft). Only primers that were 100% conserved across all known transcripts from *Eptesicus* and *Myotis* spp. were used for quantitative PCR. Primer sequences are given in Appendix 10.

Five-point dilution series (1:3 or 1:4) were performed for each gene and only primer pairs with efficiencies greater than 90% after exclusion of non-linear dilutions (typically at the highest or lowest concentration of template) were selected for use. Post-amplification melt curves were checked to ensure each product consisted of a single, narrow peak, and gel electrophoresis was performed for each amplicon to ensure a single product of correct size was produced during amplification.

C. qPCR and data analysis

For each primer pair, 20 µL reactions were prepared for each of the samples in triplicate using SYBR Select Master Mix (Thermo Fisher). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was included as a reference gene on each 96-well plate. Fluorescence was measured using a Roche 480 Lightcycler and melt curves were measured immediately after the completion of all amplification cycles. Technical replicates which reached threshold two or more cycles earlier or later than the other two replicates were excluded from analyses.

For each sample-primer combination on a given plate, the comparative C_T method (Pfaffl 2001) was used to calculate relative expression. Briefly, delta C_T was calculated as

the average threshold cycle of replicates from the gene of interest minus the average threshold cycle of the *GAPDH* replicates. To control for any batch effects, delta C_T values were adjusted by the difference in mean delta C_T between batches for each gene. Delta C_T values were then normalized by subtracting the average delta C_T for all juvenile samples for a given gene (yielding delta-delta $C_{T)}$. Fold expression was calculated as the efficiency-adjusted amplification factor raised to the negative delta-delta $C_{T.}$

We performed t-tests to determine whether the mean adjusted fold expression values of juveniles differed from adults for 13 genes. We also fitted least squares regression lines between estimated age and adjusted fold change to identify genes that exhibited age-dependent expression. All statistical analyses were performed in JMP 13.0.0 (SAS Institute). Figures were generated in JMP and MATLAB R2015a (The Mathworks).

III. RESULTS

A. Adult vs. juvenile expression

Of the 13 genes tested, eight exhibited differential expression between juveniles and adults (Table 4; Figure 12). Expression was higher in adults for six genes, bone morphogenic protein 7 (*Bmp7*), carcinoembryonic antigen-related cell adhesion molecule 16 (*Ceacam16*), collagen type XI alpha 2 chain (*Col11A2*), POU class 4 transcription factor 3 (*Pou4f3*), transmembrane channel-like 2 (*Tmc2*), and USH1 protein network component harmonin (*Ush1C*), and higher in juveniles for the remaining two genes, gap junction protein beta 2 (*Gjb2*) and POU class 3 transcription factor 4 (*Pou3f4*).

B. Age-related gene expression

Linear fits of adjusted fold change to estimated age revealed that juvenile age over a two-week period predicted expression for four genes: POU class 3 transcription factor 4 (*Pou3f4*), transmembrane channel-like 1 (*Tmc1*), and gap junction protein beta 2 (*Gjb2*) and 6 (*Gjb6*; Table 4; Figure 13).

IV. DISCUSSION

In this study, we found significant differences in the expression of seven hearing-related genes between the inner ears of juvenile and adult bats. We further observed positive age-related changes in the expression of four genes. Below, we discuss these findings in the context of the roles that these genes play in hearing, as reported in human and mouse studies.

A. Adult vs. juvenile expression

Among the genes significantly upregulated in adult samples was *Tmc2* (Figure 12). Together with *Tmc1*, which did not show significant differences in expression between adults and juveniles (Table 4; Figure 12), these genes encode components of the mechanoelectrotransduction (MET) channels of hair cells (Pan et al. 2013). Both genes are expressed in the cochlea as well as the vestibular system (Kawashima et al. 2011; Kurima et al. 2015), and their protein products may form heteromeric assemblies that could result in hair cells with different electrophysiological properties along the basilar membrane (Pan et al. 2013).

In mice, *Tmc2* expression in the inner ear is initially high in the cochlea but falls during the first postnatal week, while *Tmc1* expression is initially low but increases over the same period. Both *Tmc1* and *Tmc2* expression continues through the first few

postnatal weeks in the mouse utricle, although the level of *Tmc2* appears to fall off after the first week (Kawashima et al. 2011). This restricted expression pattern after the first postnatal week results in *Tmc2* not being able to compensate for *Tmc1* despite their functional redundancy. While exogenous expression of either *Tmc1* or *Tmc2* was sufficient to restore mechanotransduction in hair cells, deletion of *Tmc1* results in deafness because *Tmc2* expression does not persist in the cochlea (Kawashima et al. 2011). The patterns of expression observed in postnatal mice suggest that continued *Tmc2* expression into adulthood in bats may be restricted to the balance organs.

Bmp7, *Ceacam16*, *Col11A2*, and *Ush1C* were also upregulated in adults and showed similar expression differences between juvenile and adult samples (Figure 12). *Bmp7* is expressed in a gradient along the basilar papilla (the avian equivalent to the cochlea), and disruption of this gradient results in morphological changes in sensory cells that are consistent with a loss of tonotopy (Mann et al. 2014). While we found that it was upregulated in adult bats relative to juveniles, Lou et al. (2014) reported that *Bmp7* is downregulated in postnatal day 60 mice relative to postnatal day 1 mice. Mutations in *Ush1C* are associated with Usher syndrome type 1 in humans, which involves profound deafness, balance problems, and blindness (Verpy et al. 2000). The protein product of *Ush1C*, harmonin, is a component of the upper tip-link densities of stereociliary bundles (Grillet et al. 2009b), and mouse mutants exhibit splayed bundles and progressive loss of hair cells and spiral ganglion neurons (Johnson et al. 2003). Interestingly, while expression of *Ush1C* drops prior to birth and then increases into adulthood in mice (Sanchez-Calderon et al. 2010), *Ush1C* appears to be downregulated through juvenile

development, (albeit not significantly so; see Table 4 and Appendix 11), but was expressed at significantly higher levels in adults than in juveniles (Table 4; Figure 12).

Both *Ceacam16* and *Col11A2* encode proteins that are expressed in the tectorial membrane (TM), and deletion of these genes disrupts TM structure (Masaki et al. 2009; Cheatham et al. 2014), resulting in hearing loss (McGuirt et al. 1999; Kammerer et al. 2012). The TM is an acellular structure consisting of several types of collagen and glycoproteins, which lays over the organ of Corti and into which the stereocilia of the OHCs are embedded. As is true for the basilar membrane, the cross sectional area and stiffness of the TM change along the longitudinal axis of the cochlea (reviewed in Richardson et al. 2008). The TM acts as an inertial mass which allows the OHCs to respond to, and appropriately amplify, basilar membrane motion (Legan et al. 2000). Reducing the acting mass of the TM by deleting *Tectb* has been shown to improve frequency selectivity of the basilar membrane and neural response at high frequencies (Russell et al. 2007).

Seventy percent of *Ceacam16* null mutant mice exhibited spontaneous otoacoustic emissions compared to three percent of wildtype mice, potentially because Ceacam16 stabilizes interactions between TM glycoproteins, such that cochlear amplification becomes unstable without it (Cheatham et al. 2014). While the TM is typically stiffer in the radial direction than in the longitudinal direction, and this anisotropy is considered important for normal hearing, deletion of *Col11A2* resulted in a TM with equivalent radial and longitudinal shear impedance, suggesting that *Col11A2* is responsible for TM anisotropy (Masaki et al. 2009). Therefore, while expression data

from adult mice is not available for comparison, the upregulation of these TM genes in adult bats may reflect ongoing development or maintenance of the TM.

The remaining gene to exhibit higher expression in adults than juveniles, *Pou4f3*, had the greatest difference in expression between groups, but also between adult samples (Figure 12). *Pou4f3* is a transcription factor that has been implicated in progressive nonsyndromic hearing loss in humans (Vahava et al 1998). The hair cells of mice lacking *Pou4f3* fail to develop stereociliary bundles (Xiang et al. 1998), resulting in the loss of hair cell and spiral ganglion neurons (Xiang et al. 1997). In mice, *Pou4f3* is expressed into adulthood (Erkman et al. 1996; Xiang et al. 1997; Xiang et al. 1998) but Lou et al. (2014) reported that it is downregulated at the mRNA level in postnatal day 60 mice compared to postnatal day 1 mice. Taken together, the upregulation of *Tmc2*, *Bmp7*, *Ush1C*, *Ceacam16*, *Col11A2*, and *Pou4f3* in adult vs. juvenile big brown bats suggests continued growth or maintenance of essential inner ear architecture. The two genes, *Gjb2* and *Pou3f4*, that were significantly upregulated in juveniles relative to adult bats are discussed in further detail in the next section, as their expression also correlated with juvenile age.

B. Age-related gene expression

Our examination of gene expression in the inner ear from a series of young big brown bats from PND 9 to PND 19 revealed significant up-regulation of four genes: *Gjb2*, *Gjb6*, *Pou3f4* and *Tmc1* (Table 4; Figure 13). All four of these genes have been implicated in non-syndromic deafness in humans (Kelsell et al. 1997, Grifa et al. 1999, de Kok et al. 1995, Kurima et al. 2002). We examine the functional role of each of these genes below and compare our results to similar data on developing mice.

Gjb2 and *Gjb6* encode gap junction proteins that are expressed in some non-sensory cells of the inner ear, and may participate in potassium recycling, which is important for hearing because potassium is the major charge carrier in transduction (reviewed in Jagger and Forge 2015). *Gjb2* expression appears critical to cochlear function and is implicated in the most common form of congenital deafness in humans (Kelsell et al. 1997; Green et al. 1999). *Gjb6* has also been linked to human deafness (Grifa et al. 1999), although mutations in *Gjb6* can co-occur with mutations in *Gjb2*, which is located at the same locus (del Castillo et al. 2002). The amount of Cx26 and Cx30 (the proteins encoded by *Gjb2* and *Gjb6*, respectively) in the cochlea levels off and begins to decline at postnatal day 14 (Qu et al. 2012). By contrast, we found that the expression of *Gjb2* and *Gjb6* mRNA continued to rise through the third postnatal week in the inner ears of bats.

The upregulation of *Gjb2* and *Gjb6* may also correspond to a larger number of gap junctions in the bat cochlea, perhaps due to a higher concentration of gap junctions per supporting cell, or as a result of continued growth of their relatively large cochleae (Kössl and Vater 1995; Hsiao et al. 2015). A recent paper by Wang et al. (2017) showed that the median growth rate of echolocating bats' cochleae relative to basicranial width was approximately two and four times larger, respectively, than that of non-echolocating mammals and non-laryngeally echolocating bats. Gap junctions may also provide a path for current flow which enables outer hair cell amplification to operate at high frequencies (Mistrík and Ashmore 2009; Mistrík et al. 2009; Mistrík and Ashmore 2010), which could be particularly important for echolocating bats, who must hear high-frequency echoes despite severe atmospheric attenuation in air.

Conditional knockdown of *Gjb2* in early postnatal development in mice caused impairment of the active amplification process carried out by outer hair cells and most severely affected high frequency hearing (Zhu et al. 2015). Active amplification by outer hair cells improves frequency selectivity (Mellado Lagarde et al. 2008), and both frequency selectivity and high frequency hearing are critical for echolocation because template matching between the calls and returning echoes yields information about distance, position, and physical characteristics of the ensonified object (Simmons 1973; Ostwald et al. 1988). Depolarization of sensory cells at high frequencies likely requires a higher rate of potassium uptake from the extracellular space, and *Gjb2* mutants suffer sensory cell loss as a result of accumulated potassium (Cohen-Salmon et al. 2002; Kudo et al. 2003). The deleterious effects of *Gjb6* knockdown are less severe and likely due in large part to associated downregulation of *Gjb2* (Ortolano et al. 2008; Boulay et al. 2013).

The continued upregulation of both gap junction proteins observed in *E. fuscus* may also reflect a greater need for protection against hearing loss due to their high amplitude calls and long lives: Martínez et al. (2009) suggested that Cx26 and Cx30 are targets of oxidative damage which may lead to age-related and noise-induced hearing loss. Deletion of *Gjb6* in mice resulted in reduced intercellular communication between supporting cells, and abnormal epithelial repair processes carried out by the supporting cells after the deletion-induced loss of hair cells (Forge et al. 2013). Additionally, conditional knockdown of *Gjb2* in mice during late postnatal development (postnatal day 18) was associated with greater susceptibility to noise-induced hearing loss in P30 and P45 adult mice (Zhou et al. 2016).

The increase of *Gjb2* and *Gjb6* expression during juvenile development may therefore reflect a robust system of gap junctions in bats which facilitates cochlear protection or repair. Big brown bats show no significant threshold shifts after an hour of broadband noise exposure at 152 dB SPL (Simmons et al. 2015; Simmons et al. 2016). Additionally, big brown bats exposed to broadband noise at 152 dB for one hour successfully navigated through a cluttered corridor afterwards without increased errors or significant changes to their echolocation behavior (Hom et al. 2016). Bat echolocation calls have been shown to be as intense as 140 dB, although they are short in duration, lasting only milliseconds (Surlykke and Kalko 2008). Echolocating bats possess specialized hypertrophied tensor tympani muscles (Henson 1961) and arrays of highly developed smooth muscle surrounding the tympanum (Henson and Henson 2000) which may prevent hearing damage by dampening conductive transfer to the inner ear during vocalization (Henson 1965). However, it is unclear whether wild bats encounter sounds that are sufficiently intense and/or long enough to be damaging, or to what degree molecular processes may play a role in protection against noise-induced hearing loss.

Tmc1 encodes a MET channel protein (Pan et al. 2013), which localizes to the tip-links of stereocilia (Kurima et al. 2015) and which is essential for mechanotransduction in cochlear hair cells (Kawashima et al. 2011). Reports of its postnatal expression pattern conflict. Kurima et al. (2002) found a slight increase, then decrease in *Tmc1* expression in the inner ear of mice from P9 to P19, with a net change of approximately -8% over the period. By contrast, Kawashima et al. (2011) reported a 2-fold increase between P9 and P19 in the utricle and a much greater increase over the same time period in the apex of the cochlea, although other sections were not tested. The increase in expression of *Tmc1*

with age we observed in big brown bats is consistent with the latter study, although our samples consisted of the entire otic capsule and were not subdivided, similar to those used by Kurima et al. (2002).

In a transcriptomic comparison of inner ear genes in bats, Dong et al. (2013) reported that *Tmc1* was upregulated in echolocating bats relative to non-echolocating bats and other mammals. Together with the short stereocilia typically found in the high frequency regions of vertebrate hearing organs, including bats (Yao et al. 2007), the upregulation of *Tmc1* in echolocating bats could reflect a greater number of MET channels per hair cell, which might increase sensitivity to high frequencies by strengthening the influx of calcium and reducing the adaptation time of hair cells (reviewed in Fettiplace and Fuchs 1999). In midshipman fish (*Porichthys notatus*), fluctuations in the expression of a calcium-activated potassium (BK) channel conferred greater hearing sensitivity during the breeding season (Rohmann et al. 2013), and knockdown of BK channel genes produced increased thresholds in zebrafish larvae (Rohmann et al. 2014). Interestingly, 'thickened' tip links have been observed in the basal inner hair cells of the horseshoe bat *Rhinolophus rouxi*, which may suggest the presence of multiple MET channels, but these cells also possessed fewer stereocilia than hair cells in other regions (Vater and Lenoir 1992), and no data on the number of MET channels is currently available for hair cells of any bat. Alternatively, the number of MET channels may be unaffected, but bat MET channels may contain more Tmc1 subunits. Because mouse hair cells expressing only wildtype *Tmc1* had faster adaptation times than those expressing only *Tmc2* or only a *Tmc1* mutant (Pan et al. 2013), MET channels

incorporating more Tmc1 subunits might be better suited to responding at high frequencies.

Pou3f4 is a transcription factor that has been implicated in X-linked non-syndromic deafness (de Kok et al. 1995). *Pou3f4* mouse mutants exhibit a number of audiological and balance impairments, and exhibit reduced coiling of the cochlea (Phippard et al. 1999) as well as defects in gap junctions (Kidokoro et al. 2014). Deletion of *Pou3f4* from otic mesenchyme causes defasciculation of spiral ganglion neurons (Coate et al. 2012), which could disrupt coordination of hair cell and neuronal frequencies (Rubel and Fritzsch 2002). These studies suggest that the continued upregulation of *Pou3f4* in the developing bat inner ear may be linked to cochlear elongation and functional organization. Kirwan et al. (2013) did not find support for positive selection on *Pou3f4* among echolocating bats, perhaps because *Pou3f4* is a transcription factor and mutations could have widespread pleiotropic consequences on cochlear development or other traits.

Both echolocation and flight might require a more highly developed inner ear than is found in non-echolocating terrestrial mammals. Without separation of the cochlea from the vestibular organs, it is not possible to ascribe expression differences to one section of the inner ear or the other. However, while mutation or deletion of the transcription factors *Pou3f4* and *Pou4f3* affect both auditory and vestibular function (Phippard et al. 1999; Xiang et al. 1997) *Tmc1* and *Gjb2* mouse mutants exhibited hearing loss without vestibular dysfunction, suggesting these genes are particularly important for audition (Kurima 2002; Cohen-Salmon et al. 2002). Furthermore, echolocating bats and whales exhibit varying degrees of convergence in key hearing genes that have been implicated in

human deafness including *otoferlin*, *cadherin 23*, *protocadherin 15* (Shen et al. 2012), *prestin* (Li et al. 2008, Li et al. 2010, Liu et al. 2010), *KCNQ4* (Liu et al. 2011; Liu et al. 2012a), *pejvakin*, and *Tmc1* (Davies et al. 2012) consistent with positive selection acting to confer improved hearing in echolocators. Interestingly, we found that *Tmc1* was upregulated in three-week old bats, which conflicts with some reports in mouse (Kurima et al. 2002), but is consistent with upregulation of *Tmc1* in echolocating bats relative to non-echolocating bats and other mammals (Dong et al. 2013), illustrating that sequence evolution and expression may both be under selection in echolocators.

V. CONCLUSIONS

Here we provide the first time series of gene expression in bat cochleae and illustrate differences in expression of hearing-related genes between juvenile and adult bats. That most of the nine genes exhibiting significant upregulation with age have been linked to human deafness underscores their essential roles in the development of normal hearing. Upregulation of *Gjb2*, *Gjb6*, *Pou3f4* and *Tmc1* through development, and of *Gjb6*, *Pou3f4*, and *Ush1C* in adults, is consistent with the findings of Dong et al. (2013) who reported that these genes were upregulated in an echolocating bat relative to a non-echolocating bat. Our results are also consistent with the sustained cochlear growth rate exhibited by echolocating bats compared to non-echolocating bats and other mammals (Wang et al. 2017). The heightened expression of these genes may therefore reflect evolutionary pressures on echolocators to hear higher frequencies and to protect against noise- and age-related hearing loss in accordance with echolocating bats' dependence on hearing for survival.

Table 3. Criteria for inclusion and other relevant information for genes included in this study, and references. In the "Criteria for inclusion column," letter codes mean the following: A, upregulated in an echolocating bat vs. a non-echolocating bat; B, upregulated in an adult mouse relative to juvenile mouse; C, expressed in mid- to late-development; D, exhibits signs of parallel or convergent evolution between echolocating bats and whales; E, exhibits signs of parallel or convergent evolution between distantly related echolocating bats; F, participates in forming essential cochlear structures. Abbreviations: OHC, outer hair cell; MET, mechanoelectrotransdution; SG, spiral ganglion; SGN, spiral ganglion neurons; TM, tectorial membrane; DFNA, autosomal dominant non-syndromic deafness; DFNB, autosomal recessive non-syndromic deafness; DFNX, X-linked non-syndromic deafness. [a]Mutations in *Gjb6* may cause hearing loss by inducing a downregulation of *Gjb2*. *Gjb6* appears not be critical for hearing, unlike *Gjb2* (see Boulay et al. 2013).

Gene symbol	Full name of gene	Criteria for inclusion	Location of gene product	Morphological effects of deletion or mutation in mouse models	Associated with human deafness (and loci if applicable)	Sources
Bmp7	Bone morphogenic protein 7	F	throughout cochlear duct	loss of position-specific sensory cell morphology consistent with loss of tonotopy	yes	Wyatt et al. 2010; Mann et al. 2014
Ceacam16	Carcinoembryonic antigen-related cell adhesion molecule 16	A, F	tallest OHC stereocilia tips; TM	disruption of normal striated-sheet matrix of TM, Hensen's stripe absent	DFNA4	Kammerer and Zimmerman 2010; Zheng et al. 2011; Kammerer et al. 2012; Dong et al. 2013; Cheatham et al. 2014
Col11A2	Collagen type XI alpha 2 chain	A	TM, cartilaginous otic capsule, spiral limbus, lateral wall, cristae ampullaris	enlarged TM containing disorganized collagen fibrils; reduced density of radial collagen fibers in the TM	DFNA13; DFNB53	McGuirt et al. 1999; Chen et al. 2005; Masaki et al. 2009; Dong et al. 2013
GFAP	Glial fibrillary acidic protein	B	supporting cells, Schwann cells in SG and osseous spiral lamina	greater loss of OHCs after noise exposure		Rio et al. 2002; Masuda et al. 2008; Smeti et al. 2012
Gjb2	Gap junction protein beta 2	AF	gap junctions of supporting cells	severe degeneration of the organ of Corti and SGN loss	DFNB1	Kelsell et al. 1997; Cohen-Salmon et al. 2002; Dong et al. 2013; Takada et al. 2014
Gjb6 [a]	Gap junction beta protein 6	A, F	gap junctions of supporting cells	missing OHCs	DFNB1; DFNA3	Grifa et al. 1999; Teubner et al. 2003; Boulay et al. 2013; Dong et al. 2013; Miwa et al. 2013
LOXHD1	Lipoxygenase homology domains 1	A, B	cochlear and vestibular hair cell stereocilia	fused stereocilia and ruffled apical cell surface at cochlear base, leading to eventual hair cell and SGN loss	DFNB77	Grillet et al. 2009a; Dong et al. 2013
Pou3F4	POU class 3 transcription factor 4	A	throughout otic capsule	radial bundle defasciculation; abnormal gap junctions;	DFNX2	de Kok et al. 1995; Phippard et al. 1999; Coate et al. 2012; Dong et al.

				malformed stapes footplate; reduced cochlear coiling; other abnormalities		2013
Pou4f3	POU class 4 transcription factor 3	C	nuclei of cochlear and vestibular hair cells	loss of auditory and vestibular hair cells; failure of differentiated hair cells to develop stereociliary bundles; loss of spiral and vestibular ganglion neurons	DFNA15	Xiang et al. 1997; Xiang et al. 1998; Vahava et al. 1998
Tmc1	Transmembrane channel-like 1	A, D, E, F	MET channels of hair cells	none	DFNA36; DFNB7; DFNB11	Kurima et al. 2002; Kawashima et al. 2011; Davies et al. 2012; Dong et al. 2013; Pan et al. 2013
Tmc2	Transmembrane channel-like 2	F	MET channels of hair cells	none		Kawashima et al. 2011; Pan et al. 2013
Tspan1	Tetraspanin 1	B	in zebrafish, rostral mantle cells within neuromasts			Smeti et al. 2012; Steiner et al. 2014
Ush1C	USH1 protein network component harmonin	A, B, C, F	Upper tip link density of stereocilia bundles; cochlear and vestibular neurosensory epithelia	splayed hair cell bundles; progressive degeneration of hair cells	DFNB18	Verpy et al. 2000; Johnson et al. 2003; Grillet et al. 2009b; Sanchez-Calderon et al. 2010; Tian et al. 2010; Dong et al. 2013

Table 4. Results of t-tests performed on adjusted fold change between adults and juveniles (top) and bivariate fits of adjusted fold change by estimated age (bottom). Fold change values were adjusted to the mean of all juvenile samples and also to differences in mean juvenile expression between batches (see Methods). Asterisks denote level of significance (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$)

	Adult vs. juvenile t-test				Age vs. adjusted fold change bivariate fit	
	t Ratio	**Adjusted R^2**	**Mean ± SE, adult**	**Mean ± SE, juvenile**	**F ratio**	**Adjusted R^2**
Bmp7	-3.25**	0.47	6.60 ± 5.44	1.04 ± 0.09	1.75	0.06
Ceacam16	-3.22**	0.46	6.79 ± 5.61	1.08 ± 0.12	1.88	0.07
Col11A2	-2.92*	0.42	7.70 ± 6.98	1.17 ± 0.20	0.84	-0.02
GFAP	-1.90	0.23	5.16 ± 4.68	1.55 ± 0.48	0.39	-0.06
Gjb2	2.21*	0.29	0.30 ± 0.18	1.12 ± 0.14	14.85***	0.56
Gjb6	1.89	0.23	0.27 ± 0.23	1.25 ± 0.20	18.62***	0.62
LoxHD1	-1.93	0.24	5.24 ± 4.76	1.56 ± 0.48	0.32	-0.07
Pou3f4	2.31*	0.31	0.28 ± 0.03	1.11 ± 0.14	7.32*	0.37
Pou4f3	-3.21**	0.46	49.44 ± 48.20	1.15 ± 0.19	1.02	0
Tmc1	-1.88	0.23	3.03 ± 2.18	1.30 ± 0.24	5.82*	0.31
Tmc2	-3.97***	0.57	6.18 ± 4.04	1.06 ± 0.11	1.14	0.01
Tspan1	1.98	0.25	0.41 ± 0.23	1.12 ± 0.14	3.75	0.2
Ush1C	-3.01*	0.43	7.98 ± 7.27	1.08 ± 0.14	1.49	0.04

Figure 12. Log_2-scaled means and standard errors of adult and juvenile expression relative to *GAPDH*. Values were adjusted to remove the effect of batch and normalized to average juvenile expression (see Methods). Juvenile data are shown in light grey, and adult data are shown in dark grey. Asterisks denote level of significance of associated t-tests (see Table 4; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$).

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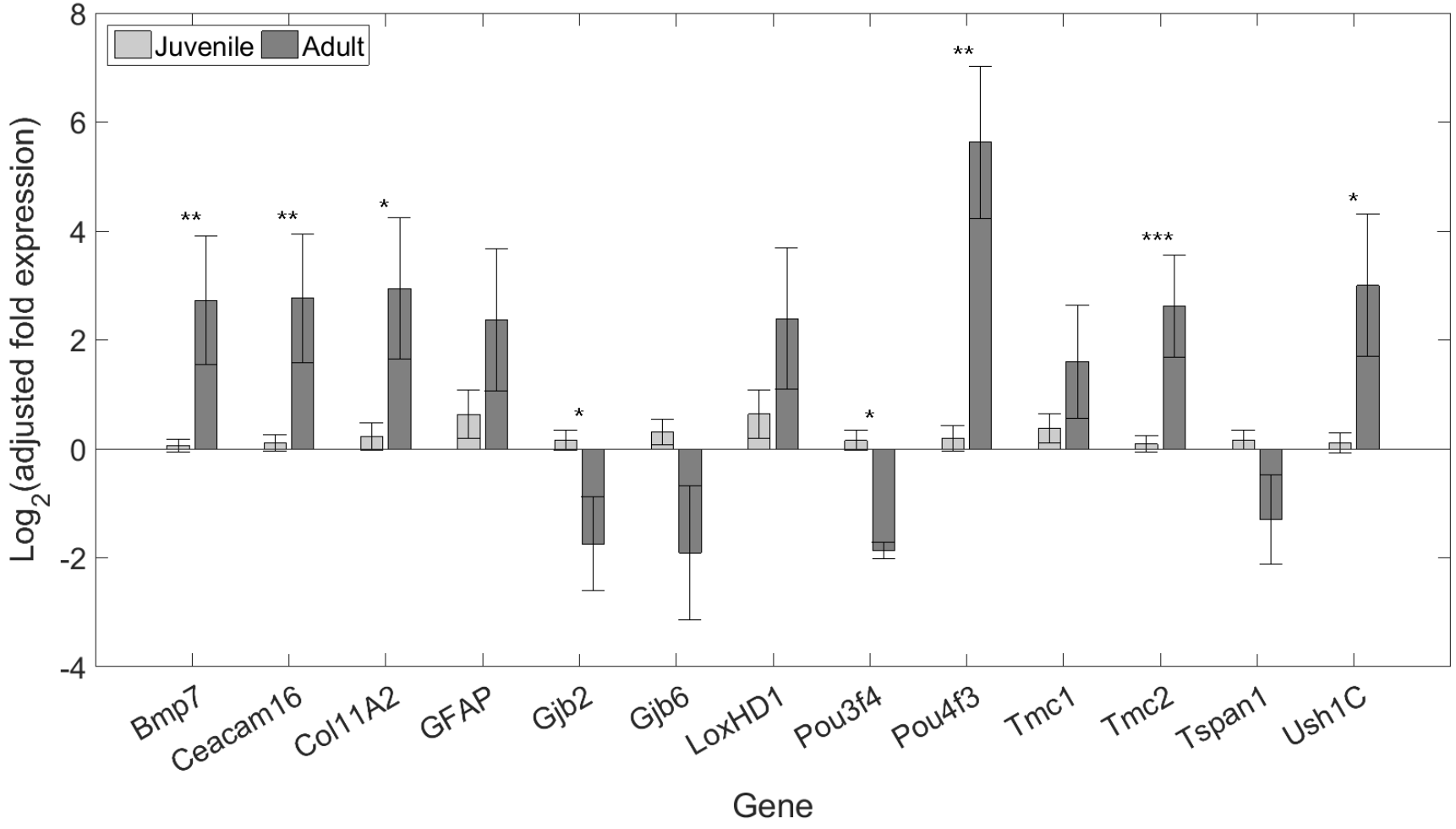
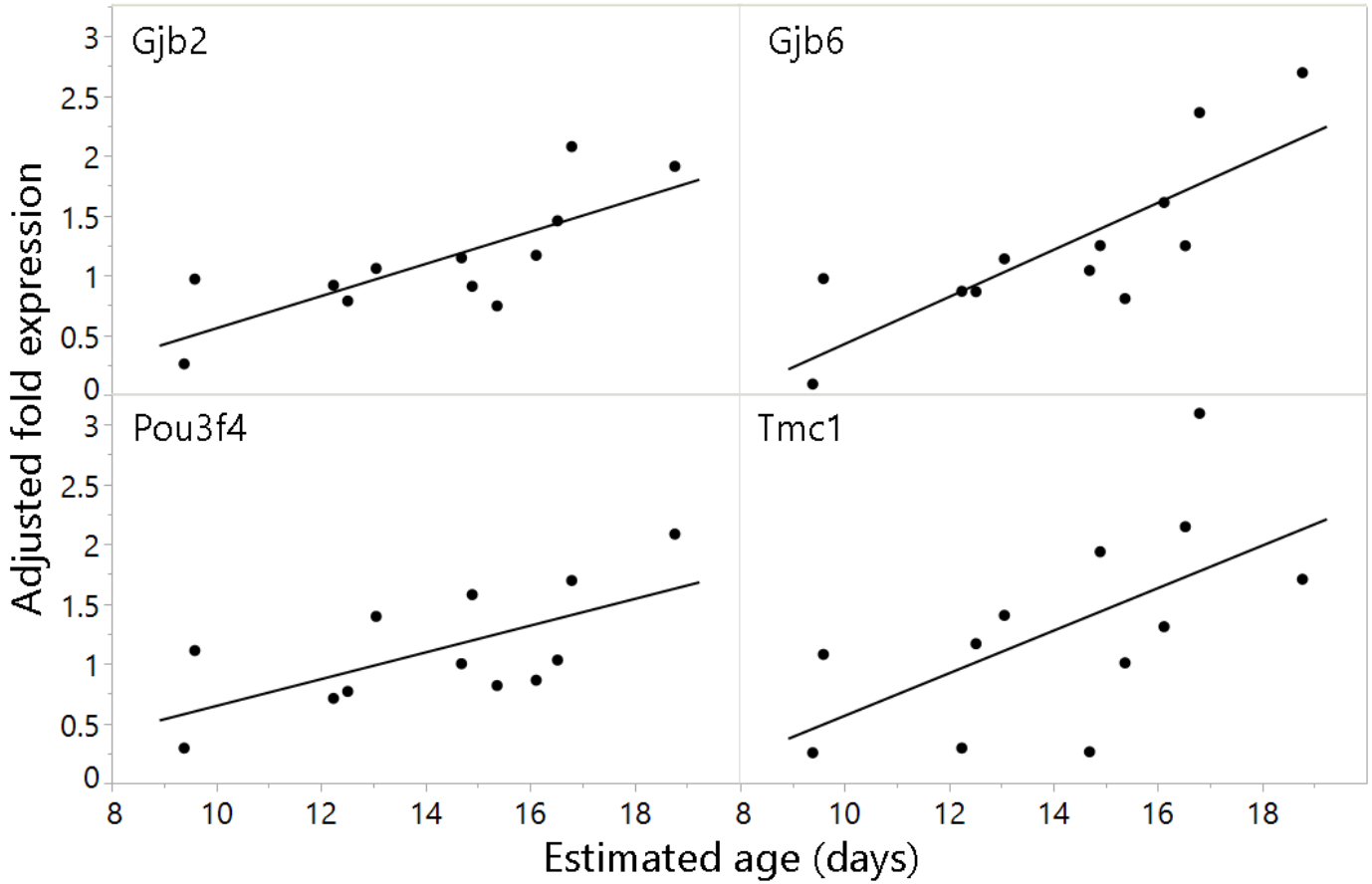

Juvenile
Adult
Log2(adjusted fold expression)
8
6
4
2
0
-2
-4
**
**
*
*
*
**
***
*
Bmp7
Ceacam16
Col11A2
GFAP
Gjb2
Gjb6
LoxHD1
Pou3f4
Pou4f3
Tmc1
Tmc2
Tspan1
Ush1C
Gene

Figure 13. Genes for which the relationship between adjusted fold change and estimated age was significant for juvenile bats. Values were normalized to average juvenile expression and adjusted to remove the effect of batch (see Methods). Asterisks denote level of significance of associated t-tests (see Table 4; *p≤0.05, **p≤0.01, ***p≤0.005).



Chapter 4: High frequency hearing in mammals: biological importance, unresolved questions, and potential insights from echolocators

ABSTRACT

High frequency hearing is important for many mammals, including humans. But unlike vision, where the effect of a single amino acid change in an opsin protein can be used to predict the wavelength of maximum sensitivity, no single gene has been identified which conveys frequency selectivity for hearing. The peripheral auditory systems of mammals exhibit several features that improve high frequency hearing, but how the cochlea amplifies high frequencies is not well understood. Here we provide an overview of high frequency hearing and expand on why it remains challenging to study, with particular attention to one group of mammals—echolocators—whose combination of very high frequency hearing, dependence on hearing for survival, long life span, and potential resistance to noise-induced hearing loss, make them a unique model for studying the molecular bases of high frequency hearing and its maintenance.

1. INTRODUCTION

A. Scope

The aim of this review is to describe the importance of high frequency hearing in mammals and to identify key challenges that must be overcome to understand how it is achieved. We also provide a general overview of the possible genetic underpinnings of high frequency hearing, drawing from both biomedical literature and studies on non-model organisms with very high frequency sensitivity, echolocating mammals. Attention will primarily be paid to terrestrial mammals, but information from cetaceans will also be provided where relevant. It should be noted that perception of sound occurs after processing by the central auditory system, and that post-cochlear processes, such as disproportionate neural representation of biologically-relevant sound frequencies, may influence hearing perception (e.g. Suga and Jen 1976; Schuller and Pollak 1979). However, our discussion is limited to sound transduction in the cochlea. Several excellent reviews of cochlear mechanics and the amplification provided by the outer hair cell (OHC) protein, prestin, have been published (e.g. Robles and Ruggero 2001; Ashmore 2008, Dallos 2008), as have discussions of unanswered questions pertaining to OHC amplification at high frequencies (e.g. Ashmore et al. 2010).

B. The peripheral auditory system of mammals

After their separation from monotremes ~140 million years ago (MYA), therian mammals developed several specialized structures to aid in hearing (Manley 2010). Many of these features improve in the detection or localization of sound in air, which is more severely and quickly attenuated than is sound in water. In terrestrial therian mammals, the pinnae, or external ears, collect and amplify sound while also providing cues for

localizing sound sources (e.g. Batteau 1967; Rice et al. 1992). In the middle ear, the ossicles, which had previously formed part of the jaw apparatus in proto-mammalian ancestors (reviewed in Maier 1989), became miniaturized and suspended into a 'chain' which transfers the vibrations arriving at the tympanum to the inner ear. The ossicles perform impedance matching such that sounds are successfully transduced from air to the fluid-filled cochlea, without which much of the sound energy would be reflected due to the greater impedance of the fluids relative to air. That the mammalian ossicular chain comprises three bones, and not a single one as in the case of the columella in other vertebrates, likely allowed for more efficient transduction of sound energy, particularly at high frequencies (Manley 2010).

In whales, which evolved from terrestrial mammal mammals ~50 MYA, the external and middle ears have changed considerably: the pinna has been lost and the ear canal is occluded. Instead, sounds are transmitted through a specialized fat pad in the jaw and up to the tympanic plate, a bony structure that functionally replaces the tympanum. Transduction through the ossicles to the inner ear still occurs, although the manubrium of the malleus, which contacts the tympanum in terrestrial mammals, has been lost. These changes reflect the different requirements of hearing in water, where impedance mismatch between the inner ear and the medium is much reduced compared to that in air. (reviewed in Nummela et al. 2007). Interestingly, despite these differences in external and middle ear anatomy, both echolocating bats and the dolphin exhibit structural specializations in the inner ear, including a particularly thick and narrow basilar membrane (BM) at the cochlear base, an ossified secondary spiral lamina, and relatively short OHCs and stereocilia (reviewed in Vater and Kössl 2011).

The inner ear consists of several mechanosensory organs in addition to the cochlea—the vestibular organs (saccule and utricle) and semicircular canals—which sense motion, acceleration, and position of the head. All inner ear organs are part of a membranous labyrinth which is encapsulated within bone, with the exception of the oval and round windows at the base of the cochlea. The oval window is the point of contact between the stapes and the cochlea, while the round window accommodates sound-induced pressure changes passing through cochlear fluids by bulging out into the middle ear space. Unlike the hearing organs of other vertebrates, the mammalian cochlea is lengthened and coiled, accommodating their greater frequency range within a confined space. The cochlea consists of three tubular compartments, of which two—the scala vestibuli and scala tympani—are connected at the apical extreme and filled with perilymph, which is similar to extracellular fluids and continuous with cerebrospinal fluid via the perilymphatic duct. The middle compartment, known as the scala media or cochlear duct, is continuous with the other inner ear organs and is filled with a potassium-rich fluid, endolymph.

The sensory cells are found in the organ of Corti, which consists of a single row of inner hair cells (IHCs) and three to five rows of outer hair cells, surrounded by non-sensory, or supporting, cells. The organ of Corti rests on the BM at interface between the cochlear duct and the scala tympani. These compartments contain fluids of different ionic composition that create an electrical potential (called the endocochlear potential) which drives both the amplification provided by OHCs and transduction by the IHCs. Both types of hair cells have stereocilia, or 'hair,' bundles at their apical surfaces. Within each bundle, several rows of stereocilia are embedded and arranged by height. The stereocilia

are connected by tip links that extend from the lateral surface of one sterocilium to the tip of its shorter neighbor, where mechanoelectrotransduction (MET) channels are located (Denk et al. 1995). The tectorial membrane (TM) rests on top of the organ of Corti, and is in contact with the stereociliary bundles of the OHCs, but not the IHCs (Lim 1980). How sound waves travel through this elaborate organ and result in transduction is described below.

C. Cochlear mechanics and transduction

Sounds transduced into the cochlea travel upward from the base towards the apex, displacing the BM at increasing amplitude until a maximum is reached. The location of maximum displacement corresponds to the frequency of the sound, such that each location on the BM has a characteristic frequency (CF). This tonotopy, or place code, results in part from morphological variance along the length of the BM: the base is narrow but thick and relatively stiff, and becomes wider, thinner, and more pliant towards the apex. Movement of the BM extinguishes sharply after reaching the location of the CF, such that the section of BM basal to a sound's characteristic location is always displaced, but apical regions experience less BM movement (von Békésy 1960). The vibration of the BM causes a shearing motion between it and the TM, which causes OHC hair bundles to be deflected (Rhode and Geisler 1967). This deflection opens the MET channels, allowing an influx of potassium from the endolymph.

Depolarization causes the OHCs to lengthen and contract as a result of the motor protein prestin, which evolved from an ancestral anion transporter (Dallos and Fakler 2002). In non-mammalian vertebrates, prestin can still act as an anion transporter but may also exhibit some motor function, albeit with less force generation than in modern

mammals (Franchini and Elgoyhen 2006; Elgoyhen and Franchini 2011). The length changes of OHCs provide additional energy, which may improve frequency selectivity of BM movement (see section 4). Both the shearing motion between the BM and TM and the forces generated by the OHCs contribute to fluid motion around the stereocilia of IHCs. Hair bundle deflection results in depolarization, and the IHC receptor potentials then cause action potentials in spiral ganglion fibers, sending sensory information to the brain.

II. IMPORTANCE OF HEARING IN MAMMALS

A. Detection of biologically important sounds

Detecting and localizing animal-generated sounds—whether produced by prey, predators, or conspecifics—is important for the survival and reproduction of most animals. In addition to environmental sounds that might signal the presence of another animal (e.g. rustling grass, branches snapping), communication signals may have been particularly important to detect, as they convey additional information such as predator category or location (e.g. Schel et al. 2010, Murphy et al. 2012), or food availability or type (e.g. Clay and Zuberbühler 2009; Kitzmann and Caine 2009). Acoustic signals can also possess information which listeners use to inform mating decisions (e.g. Charlton et al. 2007; Voigt et al. 2008). In many species, neonatal vocalizations facilitate recognition or caregiving by adults (reviewed in Lingle et al. 2012). Communication signals might be particularly important for social species, in which individuals must live and move together (e.g. King and Sueur 2011), engage in group antipredator behaviors (e.g. Graw and Manser 2007; Kern and Radford 2016), or participate in group foraging or hunting (e.g. Wilkinson and Boughman 1998; Gersick et al. 2015).

Consequently, signals and sensory receptors should coevolve to maximize the probability of detection of biologically important signals (Endler 1992; Bradbury and Vehrencamp 1998). In human audiograms, the lowest threshold occurs near the frequency which mothers find most distressing in infant cries (4 kHz; Gustafson and Harris 1990) and hearing is sensitive across the frequencies corresponding to human speech, approximately 150 Hz – 8 kHz (reviewed in Coleman 2009). Similarly, many bat audiograms exhibit maximal sensitivity to the dominant frequencies of echolocation and social calls (Esser and Daucher 1996; Koay et al. 2003; Bohn et al. 2004; Bohn et al. 2006). However, the range of frequencies that a terrestrial animal can produce or hear may be partly constrained by physical limitations, since sound production and reception depends on the size of anatomical characteristics that tend to scale with overall size (e.g. Heffner 1983; Reby and McComb 2003).

B. High frequency hearing and localization

In non-mammalian vertebrates, the ears are connected through the head, allowing them to use directional cues generated by interaction between tympana (reviewed in Christensen-Dalsgaard 2011). However, in animals with these 'pressure gradient' or 'pressure difference' ears, the interference between sound pressures from the external and internal sides of the eardrum can also result in cancellation of the signal, lowering sensitivity. For mammals, whose ears are effectively isolated, all directional information must instead be computed in the central nervous system (Christensen-Dalsgaard and Carr 2008). Without sound pressure difference cues generated by connected middle ears, terrestrial mammals may have experienced selective pressure to hear higher frequencies in order to provide stronger cues for localization. For whales, sounds of a given

frequency will have a longer wavelength because the speed of sound is five times higher in water. While this could impact their ability to use interaural time differences for localization, the size of toothed whales' heads compensates for these longer wavelengths (Nummela et al. 2007).

The relationship between upper frequency limit at 60 dB SPL and functional head size (calculated as the time it takes a sound to reach one ear from the other) is robust across mammals (Heffner and Heffner 2008). Figure 14 shows behavioral audiograms of four species from three orders (bats, rodents, primates). Sensitivity to higher frequencies (and, conversely, to low frequencies) clearly varies with functional head size (given in parentheses) both within and across orders (Figure 14). This relationship between high frequency limit and head size may be an adaptation for sound localization: the smaller an animal's interaural difference, the higher frequencies it will need to hear in order to use binaural intensity cues. Wavelengths larger than an animal's head will bend around it without attenuation, resulting in little or no difference in amplitude at the two ears. Similarly, an animal will only be able to utilize pinna cues if a sound contains frequencies which are attenuated by the pinnae.

However, functional head size alone is not sufficient to explain frequency range. In such cases, discrepancy between predicted hearing range based on head size and audiograms may reflect selective pressures (as discussed above) which have shaped mammalian hearing. For example, upper frequency limit does not vary between small and large dogs, suggesting that hearing range is species-specific (Heffner 1983). In primates, auditory sensitivity and high frequency limit are correlated with group size but not interaural distance, perhaps due to increased diversity of vocal signals in socially

complex groups (Ramsier et al. 2012). Many echolocators also live in groups (Bradbury 1977; reviewed in Connor 2000) and must detect a variety of signals, including social calls, the echolocation calls of conspecifics, and echoes returning from objects in the environment. In cetaceans, social complexity correlates not only with whistle complexity (May-Collado et al. 2007), but also with brain size (Marino et al. 2007), which may be due in part to a greater need for sound processing in echolocating whales.

III. ECHOLOCATING MAMMALS' DEPENDENCE ON HEARING

A. Convergence between echolocating bats and toothed whales

Audition is a particularly important sensory modality for echolocating mammals, because they rely on hearing to obtain food, locate conspecifics, and orient in and through environments. Both echolocating bats and whales operate in relatively low-light conditions and exhibit evidence of relaxed selection in some visual genes. In some echolocating bats, including species using both CF-FM and FM call designs, the vision-related genes *Gja10* (gap junction protein alpha 10) and *Rbp3* (interphotoreceptor retinoid-binding protein) exhibit insertions, deletions, and premature stop codons (Shen et al. 2013), consistent with pseudogenization of retinal genes in species that operate in low-light conditions, such as subterranean mammals (Emerling and Springer 2014). Both the absence of short-wavelength-sensitive (SWS) cones and pseudogenization of SWS cone pigment genes have been reported in whales (Peichl et al. 2001; Levenson and Dizon 2003), and their visual and olfactory senses are relatively poor even when compared to other marine mammals (reviewed in Ketten 1992).

Hearing therefore not only provides echolocating bats and whales with cues to direct vision (as is the case in other mammals), but may be the dominant sense for scene

analysis (Griffin 1958; Ketten 1992), and its maintenance is likely crucial for survival. Behavioral experiments carried out by the Italian scientist Lazzaro Spallenzani in the eighteenth century established that bats could fly, land, avoid obstacles, and successfully hunt even after being blinded. Griffin (1958) showed that bats were unable to learn a food association task when high contrast visual cues (stripes) were used, and remarked that their brains exhibit enlarged auditory centers with a concomitant decrease in visual centers.

In an early study, Langworthy (1931) noted that the auditory nerves and brain centers of the bottle-nosed dolphin (*Tursiops truncatus*) were highly developed, in contrast to those associated with other senses. He hypothesized it was an adaptation for hearing and sound processing in the same way that the large cerebral cortex of primates was thought to have evolved, in part, to process and integrate visual signals (as a result of their binocular, stereoscopic vision). Brill et al. (1988) demonstrated that the performance of a dolphin on an echolocation task was significantly hindered when it wore a neoprene hood on its jaw that partially blocked sound, compared to one that allowed sound to pass through, validating the hypothesis that toothed whales hear through their jaws (Norris 1964). Additionally, the fractured ear bones of non-echolocating whales obtained through commercial fishing showed extensive remodeling, but those of beached echolocating whales found dead were less remodeled, perhaps reflecting that hearing loss can be lethal to echolocators (Yamato et al. 2016). Perhaps most convincingly, Mann et al. (2010) demonstrated that 57% of stranded bottlenose dolphins and 36% of stranded rough-toothed dolphins (*Steno bredanensis*) exhibited severe hearing loss as measured by

evoked potentials, and André et al. (2003) found that a stranded striped dolphin had auditory thresholds greater than 115 dB, and was effectively deaf.

Despite having evolved in different media (air and water) and being several orders of magnitude apart in terms of size, echolocating bats and whales use a similar range of echolocation frequencies and vocalization rate prior to prey capture (Madsen and Surlykke 2013). Echolocators can change their call features in order to direct their attention to particular features in the environment. This active sensing may have given them a competitive advantage over other low-light predators, which must rely on prey-generated cues for detection. Both groups have undergone extensive adaptive radiation, and are far more speciose than their non-echolocating sister lineages. Bats alone account for approximately a quarter of all extant mammalian species, and most are echolocators. Recent molecular work has also claimed that parallel and convergent amino acid substitutions have occurred in sensory genes, and even throughout the genomes, of echolocating bat and whale lineages due to convergent selection pressures acting on echolocators (see Section 5).

B. Localization in echolocators

Insectivorous bats hunt small, mobile prey, some of which are capable of predator evasion movements that can be complex and difficult to predict (Roeder 1962, Yager et al. 1990). These bats fly up to four times faster than whales swim, and must capture prey on much smaller time scales, requiring more tightly coordinated sensorimotor operation (Madsen and Surlykke 2013). Field experiments in which prey items were removed just before capture have shown that bats' reaction time can be as short as two-hundredths of one second, or 20 milliseconds (Geberl et al. 2015). Insectivorous bats and those which

catch prey by trawling over water produce echolocation calls at a rate of up to 200 times per second just prior to prey capture, called the 'terminal buzz' phase. The terminal buzz provides high temporal and spatial resolution, and is not exhibited by bats which listen passively to catch prey (some carnivorous bats) or which subsist on plant-based foods (reviewed in Moss et al. 2011). Laryngeal muscles capable of extremely fast contraction have been documented in echolocating bats and are likely operating at their physiological limit during the terminal buzz (Elemans et al. 2011).

Echolocating whales also produce terminal buzzes when they are within one body length of a prey item (e.g. Madsen et al. 2005b), at rates exceeding those exhibited by bats (at least 300 calls per second, Johnson et al. 2006; DeRuiter et al. 2009). The mechanism of sonar click production in whales involves a derived nasal structure (Cranford et al. 1996), although the larynx may also participate (Huggenberger et al. 2008). The rate of call emission in whales' terminal buzz phase scales with body size but not speed of movement, which suggests that successful capture of highly maneuverable prey requires rapid updating of location information (Madsen and Surlykke 2013).

The bottlenose dolphin has a minimum audible angle (MAA)—a measure of sound localization acuity—of less than one degree when listening to broadband clicks (Renaud and Popper 1975).While the MAA in echolocating bats is 9-15 degrees, about average among mammals (Heffner and Heffner 2016), bats which hunt moving prey may not require high acuity based on single clicks due to the rapid rate of call emission at the terminal buzz. The MAA approach may not accurately reflect localization ability (Hartmann and Rakerd 1989), and measures only passive localization acuity, which may not be the most appropriate assessment method for echolocators that mostly rely on

active sensing. In fact, behavioral experiments have shown that different species of echolocating bats are capable of detecting and avoiding wires as thin as 0.06-0.1 mm while flying (reviewed in Neuweiler 2000). Additionally, echolocating bats that hunt prey must localize and track moving targets in three dimensions, integrating information about the elevation, azimuth, and range of objects (Wohlgemuth et al. 2016a). The big brown bat (*Eptesicus fuscus*) adaptively controls its vocalizations during behavioral tasks while also moving its head and/or ears to maximize auditory cues for localization (Kothari et al. 2014; Mao et al. 2016; Wohlgemuth et al. 2016b).

C. High frequency calls and hearing in echolocators

Both echolocating bats and whales hear higher frequencies than expected based on their interaural distance, suggesting that high frequency hearing is particularly important for echolocation (Heffner and Heffner 2008). Bat echolocation calls can be either frequency modulated (FM), broadband downward sweeps, or mostly constant frequency, tonal emissions with brief FM regions at the start or end of the call (CF-FM). The calls of echolocating bats range from as high as 212-183 kHz in Percival's trident bat (*Cloeotis percivali*) to as low as 14.5-8.6 kHz in the spotted bat (*Euderma maculatum*) although many bats echolocate at intermediate frequencies between 25 to 65 kHz (Fenton and Bell 1981). Echolocating cetaceans produce broadband clicks with peak frequencies ranging from 135 kHz in the harbor porpoise (*Phocoena phocoena*, Madsen et al. 2005a) to approximately 12.5 kHz in the spermwhale (*Phyester microcephalus*, Madsen et al. 2002).

Echolocating bats and whales can gain valuable information about their environments by producing ultrasound signals and processing information carried by

echoes from objects in the path of the sound beam (Moss et al. 2014). However, using high frequency echolocation calls may limit operating range because high frequency sounds are subject to more severe atmospheric attenuation than low frequency sounds (Hartley and Suthers 1989). This attenuation is greater for bats than for whales, because the rate of energy loss increases exponentially with the viscosity of the medium (Stokes' law of sound attenuation). As a result, despite using a similar range of echolocation frequencies, toothed whales can detect prey up to several hundred meters away, whereas the maximum prey detection distance is about 10 meters in bats (Madsen and Surlykke 2013).

Due to the inherent difference in transmission loss between frequencies, echoes returning to both echolocating bats and whales will be low-pass filtered relative to the outgoing calls, with less sound energy at higher frequencies (e.g. Lawrence and Simmons 1982). This characteristic may initially seem sub-optimal: why expend energy to emit high frequency components when they almost never return in echoes because of attenuation? In fact, the absence of these high frequency components is itself informative. Because high frequencies are more directional, echoes that are strongly low-pass filtered are likely returning from objects that are distant or off-axis relative to the direction of call emission. Some echolocating bats and whales inhabit noisy or cluttered environments which can result in the return of multiple and overlapping echoes, potentially making perception of individual objects more difficult. In the presence of noise, both echolocating bats and whales have been reported to use higher frequency calls (e.g. Au et al. 1985, Lesage et al. 1999; Gillam et al. 2007; Hage et al. 2013). High frequencies may aid in the 'defocusing' of these non-target echoes because the intensity difference

between low and high frequency components will translate into a time delay at the auditory receiver, effectively blurring the acoustic images of clutter objects (Bates et al. 2011).

In addition to beam directionality, high frequencies also benefit echolocators by providing more detailed acoustic images. The short wavelengths of high frequencies can create interference patterns from objects with non-uniform surfaces, enabling bats to obtain information about fine physical characteristics, such as texture (Falk et al. 2011). The hearing sensitivities of CF-FM bats are closely matched to the dominant frequencies of their calls (e.g., Long and Schnitzler 1975) and, remarkably, these bats lower their outgoing call frequencies to compensate for Doppler shifts introduced by their relative velocity to the target (Schnitzler 1968; Schnitzler 1970; Habersetzer et al. 1984). This Doppler shift compensation allows CF-FM bats to separate frequency shifts in their echoes caused by their own locomotion from those created by fluttering insect wings (reviewed in Moss and Schnitzler 1995). 'Acoustic glints' of amplitude and frequency caused by fluttering wings can only occur in high frequencies, because their wavelengths are small enough to reflect off the wing surface (Kober and Schnitzler 1990). This may explain why CF-FM bats, whose long-duration, narrowband calls are otherwise relatively poor for localization (Schnitzler and Kalko 2001), tend to use higher frequency calls than FM bats (Fenton and Bell 1981). Finally, high frequency echolocation calls in some bats may also reflect selective pressure to avoid detection by insect prey (Fullard and Dawson 1997, Bogdanowicz et al. 1999; Schoeman and Jacobs 2003). Similarly, high frequency echolocation calls by cetaceans may avoid detection by fish prey (Mann et al. 1998;

Wilson and Dill 2002) or killer whale predators (e.g. Madsen et al. 2005a; Curé et al. 2013).

Echolocators must produce calls in the range of their best hearing. With one known exception (Woolf 1974), both echolocating bats and whales produce altered calls after hearing damage, indicating that they actively match their vocalizations to hearing during development (Rubsamen and Schafer 1990) and even in adulthood (Kössl and Vater 2000; Kloepper et al. 2010a). In mice, however, normal vocalizations develop in animals that have been deafened at birth or in early postnatal stages (Hammerschmidt et al. 2012; Mahrt et al. 2013), and even when the hippocampus and parts of the cortex are missing (Hammerschmidt et al. 2015). Consequently, while hearing loss would likely impact the survival of any mammal, it is likely fatal for echolocators, which rely heavily on hearing to perceive the environment. Hearing loss due to age and noise appear to be nearly universal in mammals, and tends to affect high frequencies most severely (see below).

IV. HEARING LOSS AND DAMAGE

A. Comparative differences in the maintenance of hearing

Hearing loss caused by damage to cochlear sensorineural tissues is almost always irreversible in the mature mammalian cochlea, which exhibits only limited repair capabilities. However, some regenerative capacity remains up to the first postnatal week in mice, and may persist in adult vestibular organs (reviewed in Walters and Zuo 2013). Sensory cell loss is the most common histopathological finding associated with sudden deafness, but damage to other cochlear tissues can also result in hearing loss, including components of the transduction process, such as the tectorial membrane, or elements that

are critical for maintenance of the endocochlear potential, such as the stria vascularis or supporting cells (Schuknecht et al. 1973; Merchant et al. 2005). This damage results in part from oxidative stress, which appears to be a major contributor to hearing loss generally (reviewed in Poirrier et al. 2010). Pathologies in the central auditory system also contribute to hearing loss (Ouda et al. 2015; Moser and Starr 2016).

Much biomedical hearing research has focused on identifying mechanisms of hair cell regeneration, but a major goal of such research is to develop treatments in adults with hearing loss. The loss of regeneration ability in the mature cochlea, which occurs during the aging process, likely involves multiple changes in gene expression and biochemical pathways (Walters and Zuo 2013; Wong and Ryan 2015). Unlike mammals, non-mammalian vertebrates can regenerate both auditory and vestibular sensory cells into adulthood (reviewed in Meyers and Corwin 2008) and exhibit functional recovery in measures of auditory function and behavior (reviewed in Saunders and Salvi 2008; Dooling et al. 2008; Rubel et al. 2013). While using non-mammalian systems to investigate mechanisms of repair and regeneration has proven valuable (reviewed in Burns and Corwin 2013), significant changes to the cochlea have occurred since mammals diverged from other vertebrates. The specialization of cell types in the organ of Corti may have enabled high frequency hearing (Warchol 2011), but the differentiation and maturation of different cellular morphologies restricts re-entry into the cell cycle (Groves 2010), and a terminally mitotic state may be necessary for the survival and function of mammalian hair cells (Kelley 2007).

Echolocating bats and whales provide an opportunity to study the maintenance of hearing in mammals which are long-lived and dependent on hearing for survival. Both

groups encounter and presumably tolerate very high sound levels produced by conspecifics. Furthermore, high frequency hearing is typically the first sign of age-related hearing loss (Gates and Mills 2005; Agrawal et al. 2008), and the base of the cochlea where high frequencies are represented is more susceptible to damage than apical regions (see below). The inclusion of echolocating mammals—whose longevity, frequent exposure to noise, expanded high frequency range, and reliance on sensitive hearing—in future work may provide new insights to researchers interested in the prevention and treatment of hearing loss.

B. Aging and age-related hearing loss

Age-related hearing loss (ARHL), or presbycusis, encompasses all age-related processes which contribute to hearing loss, including sensory, strial, and neural degeneration (Gates and Mills 2005). ARHL has been documented in both laboratory and companion animals (e.g. Henry and Chole 1980; Knowles et al. 1988; Shimada et al. 1998), suggesting that the mechanisms of ARHL may be common to all mammals. In humans, two-thirds of adults over 70 years of age suffer from ARHL (Lin et al. 2011). ARHL tends to be most severe at high frequencies, which makes consonant recognition difficult for those afflicted, as consonants contain high frequency components (Bilger and Wang 1976). Loss of high frequency hearing may additionally harm temporal processing (Leigh-Paffenroth and Elangovan 2011), and detriments in both can reduce the intelligibility of speech in noise (Frisina and Frisina 1997; Baer et al. 2002). The resulting difficulty in communicating can lead to a decline in mental health and lower quality of life (Ciorba et al. 2012). However, for patients with mild to moderate ARHL (Hogan and

Turner 1998) and those without cochlear dead regions (Vickers et al. 2001), speech recognition improves with increasing high frequency information.

Studies examining the effect of caloric restriction on ARHL have found that calorically restricted mice, rats, and rhesus monkeys exhibited delayed onset of hearing loss (Seidman 2000; Someya et al. 2007; Someya et al. 2010) and longer lifespan (McCay et al. 1935; Colman et al. 2009). Bats can live more than three times longer than other mammals of similar size (Austad and Fischer 1991), making them an attractive model for studying how hearing is maintained despite aging. Their longevity correlates with hibernation (Wilkinson and South 2002; Turbill et al. 2011), suggesting it is related to lowered metabolism. Recent studies have revealed that genes implicated in metabolic suppression, oxidative stress, the cell cycle and apoptosis, and neuroprotection are differentially expressed in hibernating bats (Chen et al. 2008; Lei et al. 2014). Because they typically inhabit temperate regions where food availability is seasonally variable, hibernation may therefore not only increase chances of surviving through winter, but also temporarily spare the cochlea from metabolism-related damage.

However, while hibernation extends lifespan by an estimated 6 years on average (Wilkinson and South 2002), non-hibernating bats still live much longer than other mammals of their body size (Brunet-Rossinni and Austad 2004), suggesting some adaptations related to longevity may not be strictly related to hibernation or caloric restriction. A recent transcriptomic analysis revealed that the greater mouse-eared bat (*Myotis myotis*) exhibited an up-regulation of micro RNAs that suppress tumors and genes that participate in DNA repair, while tumorigenesis promoters and genes involved in mitochondrial activity were down-regulated (Huang et al. 2016).

Unfortunately, estimates of hearing in aged bats are not available, so the degree to which they experience ARHL is unknown. Whales are also long-lived (summarized in Garde et al. 2007), and data from captive or stranded animals suggests a similar progression of ARHL as seen in other mammals, with greatest sensitivity losses at high frequencies (Brill et al. 2001; Houser and Finneran 2006; Houser et al. 2008; Kloepper et al. 2010a, Li et al. 2013). Kloepper et al. (2010b) showed that a false killer whale (*Pseudorca crassidens*) with significant high-frequency hearing loss performed much worse (up to a 36% reduction in performance) on a target discrimination task than it did sixteen years previously. However, due to the high sound pressures they encounter, noise-related damage may also impact echocators.

C. Noise and noise-induced hearing loss

In humans, noise-induced hearing loss (NIHL) is estimated to affect less than 10% of adults in the general population (Dobie 2008) but is a significant occupational risk for military personnel (Ylikoski and Ylikoski 1994; Henselman et al. 1995) and those who work in noisy environments (Nelson et al. 2005; Jansen et al. 2009). As with ARHL, oxidative damage plays a major role in NIHL (Henderson et al. 2006). Noise-induced damage may continue even after exposure (Patterson and Hamernik 1997; Yamashita et al. 2004) and treatment with antioxidant compounds before or after noise can limit the extent of noise-related damage (Ohinata et al. 2000; McFadden et al. 2005; Yamashita et al. 2005; Campbell et al. 2007; Coleman et al. 2007).

For echolocators, noise can interfere with the audibility of echoes and perception of the environment. Both bats and whales appear to avoid noise (Richardson et al. 1990; Schaub et al. 2008; Bunkley et al. 2015) alter their echolocation calls and/or behavior in

noisy environments (Habersetzer 1981; Foote et al. 2004; Bates et al. 2008; Chiu et al. 2009; Aytekin et al. 2010; Hiryu et al. 2010; Pirotta et al. 2012). However, the nature of these changes is not uniform. For example, bats may fall silent and orient using another bat's calls and echoes (Chiu et al. 2008), increase spectral separation by shifting call frequency away from a conspecific's dominant frequency (Ulanovsky et al. 2004; Gillam et al. 2007; Chiu et al. 2009), or change primarily temporal features such as duration and pulse interval (Götze et al. 2016). The variation between species, or even conspecifics (Tressler and Smotherman 2009; Mao et al. 2016) suggests that the effect of noise on perception by echolocation may be ameliorated by altering echolocation calls or timing in a number of ways.

Interestingly, bat echolocation calls can be as intense as 140 dB (Surlykke and Kalko 2008). The use of such high amplitude echolocation calls necessitates some mechanism to protect hearing during sound production. Echolocating bats have well-developed tensor tympani muscles (Henson 1961) and smooth muscle arrays surrounding the tympanum (Henson and Henson 2000). Their stapedius muscles engage shortly before the onset of vocalizations. These middle ear specializations presumably protect bats from damaging their own hearing while echolocating (Henson 1965). It is unlikely that this mechanism could protect against all high-amplitude sounds a bat encounters, since bats commonly fly near, and are exposed to the vocalizations of, other bats. However, bat echolocation calls are relatively short (lasting only milliseconds), are strongly attenuated while traveling in air, and are highly directional (Jakobsen et al. 2013), so the extent to which bats encounter high-intensity sounds in the wild is not clear. Additionally, hearing

sensitivity must be restored in the silent periods to enable detection and analysis of returning echoes (Griffin 1958).

Whales also produce very high amplitude echolocation clicks of over 225 dB re: 1 μPa (Au 1980; Møhl et al. 2003; Madsen et al. 2004), although direct comparison of sound pressure levels in air and water are complicated by different reference pressures and medium properties. The strength of these sounds was previously hypothesized to stun prey prior to capture (Norris and Møhl 1983), but no behavioral changes were observed in fish exposed to high-amplitude clicks (Benoit-Bird et al. 2006). Whales appear to protect their hearing from their own clicks during echolocation, although little is known about underlying mechanisms (Nachtigall and Supin 2008, 2013, 2014). In addition to their own calls, whales (like bats) are presumably exposed to others' echolocation clicks, although whale echolocation sounds are much shorter than those of bats, sometimes lasting only tens or hundreds of microseconds (Madsen et al. 2004; Madsen et al. 2005; Johnson et al. 2006). Whales may also encounter anthropogenic noise, such as that produced by sonar, shipping, pile-driving, and explosions (Weilgart 2007), which can damage the ears and other organs at high exposure levels (Fernández et al. 2005). Notably, temporary threshold shifts of only 5 dB were observed in harbor porpoises (*Phocoena phocoena*) exposed to pile-driving sounds at a rate of 2760 strikes per hour at 145 dB re: 1 μPa^2s, even after 6 hours (Kastelein et al. 2016). The authors attributed this low threshold shift to the short duration of the pile-driving sound (124 ms), suggesting that the durations of bat and whale echolocation sounds are so short that they may not be likely to damage hearing, even at high intensities.

Evidence that 'sound conditioning' (long term exposure to an acoustic stimulus at a sub-traumatic amplitude) can protect against NIHL (Canlon and Fransson 1995; Canlon 1997) suggests that echolocators may develop some noise protection naturally by frequent exposure to noisy environments. Noise-induced temporary threshold shifts have been reported in several species of echolocating whales, and tends to maximally affect frequencies about one-half octave above exposure frequencies, similar to observations in terrestrial mammals (reviewed in Finneran 2015). In noise exposure experiments, both Yangtze finless porpoise (*Neophocaena phocaenoides asiaeorientalis*) and beluga whale (*Delphinapterus leucas*) exhibited much smaller temporary threshold shifts to noise with high center frequencies (128 kHz and 90 kHz, respectively) than to noise at lower frequencies (Popov et al. 2011; Popov et al. 2013). By contrast, no significant changes in auditory brainstem response (ABR) were observed after 30 minutes of broadband noise exposure at 90 dB in Japanese house bats (*Pipistrellus abramus*, Simmons et al. 2015), and exposure to broadband noise at a level of 152 dB for an hour did not result in temporary threshold shifts in big brown bats (*Eptesicus fuscus*, Simmons et al. 2016).

Even in cases where auditory sensitivity does not appear to be affected by noise, changes in auditory system activity are sometimes documented, such as a decrease in transient-evoked otoacoustic emissions (Pawlaczyk-Luszczyńska et al. 2004) or primary auditory cortex activity (Pienkowski and Eggermont 2010). However, Hom et al. (2016) demonstrated that big brown bats exposed to noise (152 dB broadband noise for one hour) could still successfully navigate through a cluttered corridor without increased errors or significant changes to their echolocation behavior (number or timing of calls, or call amplitude). That both echolocating whales and bats appear to be less susceptible to

noise at frequencies corresponding to their echolocation calls is consistent with the existence of an evolved protective mechanism that operates by sound conditioning. This protection of high frequency hearing warrants further research, and is notable because the cochlear base, where high frequencies are represented, is generally more susceptible to injury and damage.

D. Greater susceptibility of the cochlear base to damage

Basal hair cells and outer hair cells (OHCs) are generally more affected than apical hair cells and IHCs in various forms of hearing loss, including ARHL (Spongr et al. 1997; reviewed in Lee 2013), NIHL (Bohne and Harding 2000; Chen and Fechter 2003; Jensen et al. 2015), and aminoglycoside ototoxicity (reviewed in Selimoglu 2007). This greater susceptibility of the base may contribute to the fact that high frequency hearing loss occurs in an estimated 32% of the U.S. adult population (Agrawal et al. 2008). Basal and OHCs can also be more susceptible to damage and apoptosis induced by genetic mutations (e.g. Griffiths et al. 2001; Johnson et al. 2003; Dallos et al. 2008; Grillet et al. 2009; Forge et al. 2013) even when the mutated gene is not expressed in hair cells (Zhu et al. 2015; Zhou et al. 2016) or when the change is minor (a single amino acid change rather than entire deletion) and cell death is not an expected outcome (Cheatham et al. 2015).

The greater susceptibility of the cochlear base may be linked to greater oxidative stress. Sha and colleagues (2001) found that basal OHCs expressed less of the antioxidant glutathione than apical OHCs and may therefore be more intrinsically susceptible to damage. Mice with mutations in superoxide dismutase 1 (*SOD1*) exhibited accelerated hearing loss with age (McFadden et al. 1999), and glutathione peroxidase (*Gpx1*) mouse

mutants showed greater hearing loss caused by noise exposure (Ohlemiller et al. 2000). In both studies, damage and loss of cells in mutants was more pronounced in OHCs and the hair cells in the basal region. Overexpression of some antioxidant genes has been shown to protect cochlear hair cells from aminoglycoside ototoxicity (Kawamoto et al. 2004), and aging dogs fed a high antioxidant diet exhibited less cochlear degeneration with age (Le and Keithley 2007).

This intrinsic sensitivity presents a challenge for researchers interested in high frequency hearing because otherwise healthy cochlear explants degrade more quickly at the base than other portions of the basilar membrane (BM). OHCs are particularly susceptible: more than 70% of explanted basal OHCs died after 5 hours relative to only 10% in the apex, while both basal inner hair cells (IHCs) and supporting cells survived (Sha et al. 2001). This makes examination of the basal cochlea more difficult and can lead to missing data from this region (e.g. Kawashima et al. 2011). Researchers interested in high frequency hearing in adults face the additional problem of more rapid degeneration of the adult sensory epithelia (e.g. Malgrange et al. 2002, Kawashima et al. 2011). Because high frequency hearing matures late in development (Harris and Dallos 1984; Echteler et al. 1989), due at least in part to the gradual detachment of the tectorial membrane from the hair cells (Lenoir et al. 1987; Roth and Bruns 1992; Rueda et al. 1996), examination of cochleae from young animals may not be representative of adults.

V. CELLULAR MECHANISMS OF HIGH FREQUENCY HEARING

Given that key innovations in hearing occurred separately over the course of mammalian evolution, it is unlikely that high frequency hearing (as exhibited by modern mammals) arose prior to 100 MYA (Manley 2012). The mammalian frequency range is

unparalleled among vertebrates, with hearing above 10 kHz being nearly universal (Heffner and Heffner 2008). For decades, researchers have been interested in what factors set the high frequency hearing limit. That mammals exhibited several derived features (e.g. pinnae in therians, a three-bone ossicular chain, and a long, coiled cochlea) together with high cochlear resistance led many auditory researchers to attribute high frequency hearing to external and middle ear structures (reviewed in Ruggero and Temchin 2002). However, by comparing audiograms to measurements of stapes and columella velocity, Ruggero and Temchin (2002) showed that sensitivity of ossicles at high frequencies exceeded that of behavioral thresholds measured in the turtle (*Chrysemys scripta*), pigeon (*Columba livia*), guinea pig (*Cavia porcellus*), and horseshoe bat (*Rhinolophus ferrumequinum*). They also established a correlation between the high frequency cutoffs of cochlear CFs to behavioral thresholds, and concluded that the range and shape of audiograms was set at the inner ear, with the external and middle ears passing broadband information to the cochlea for frequency analysis.

Because the cochlea is filled with viscous fluids, additional energy must be supplied to overcome the damping that incoming sounds encounter (Gold 1948). The OHCs in the organ of Corti actively amplify BM vibrations (Ruggero and Rich 1991) created by incoming pressure waves through the action of *prestin/SLC26A5*, a membrane protein which causes OHCs to lengthen and contract when depolarized (Brownell et al. 1985; Zheng et al. 2000). Mellado Lagarde et al. (2008) showed that prestin also contributes to frequency selectivity by imparting passive stiffness to the BM. *Prestin* knockout mice exhibit BM thresholds as sensitive as wildtype mice, but BM vibrations are broadly tuned, shifted down half an octave, and do not differ from movements

observed in dead *prestin* knockout mice. Furthermore, *prestin* knockouts do not have impaired mechanotransduction or synaptic transmission, but their neural responses can be attenuated by about 40-60 dB compared to BM sensitivity (Liberman et al. 2002; Cheatham et al. 2004; Mellado Lagarde et al. 2008). While knocking out *prestin* also resulted in shorter OHCs (Liberman et al. 2002) and disruption of prestin targeting to the plasma membrane (Zheng et al. 2005), experiments using a *prestin* mutant that did not exhibit shorter OHC length or reduced plasma membrane expression had similar hearing deficits (Dallos et al. 2008).

OHC amplification is nonlinear, which ensures that sounds are not uniformly amplified, but that amplification is inversely proportional to sound pressure level (Rhode 1971). This property might be particularly helpful for mammals that require high frequency hearing, since high frequencies are more directional and attenuated by atmospheric spreading than are lower frequency sounds (Hartley and Suthers 1989). However, many questions remain about how the cochlea is able to respond to high frequencies. First, in order to amplify BM movement at the CF, OHCs must undergo electromechanical length changes cycle-by-cycle. OHC conductance is voltage-dependent (Santos-Sacchi and Dilger 1988), but the resistor-capacitor (RC) time constant of OHC membranes—a measure of the time needed to re-charge the capacitor of an RC circuit, or in this case, to replenish the voltage across the membrane—should limit OHC amplification to frequencies of only several hundred hertz (Housley and Ashmore 1992). Yet, OHC oscillations have been recorded up to 79 kHz (Frank et al. 1999), and Johnson et al. (2011) recently showed that half of the OHC mechanotransduction channels may be open at rest, resulting in a membrane potential that is relatively depolarized (-40 mV). As

a result, the membrane time constant may be much smaller than previously estimated, such that OHC frequency response may be effectively unlimited.

Second, it is still unclear how OHCs produce sufficient force to amplify pressure waves at the basal part of the cochlea, where the BM and TM are stiffest (Ghaffari et al. 2007). Housley and Ashmore (1992) measured higher currents from basal OHCs in guinea pig and inferred that they have 15-20 times as many ion channels as apical OHCs. However, less force may be generated by smaller cells (Iwasa and Adachi 1997) and the amplitude of OHC movement declines with frequency (Frank et al. 1999). It was also recently demonstrated that the density of prestin protein is similar in basal and apical OHCs (Mahendrasingam et al. 2010), confirming that intrinsic force production is likely not different between the apex and base.

Finally, OHCs only amplify the CF, but how they manage to respond so selectively is unknown. BM movements are too broad to explain the observed frequency selectivity by tonotopy. This has led to the invocation of another filtering process to sharpen OHC response, such as amplification by OHC hair bundles, which can generate force in the hair cells of non-mammalian vertebrates (Crawford and Fettiplace 1985; Howard and Hudspeth 1987) as well as those of mammals (Kennedy et al. 2005). Another potential source of additional filtering is the TM which, as previously described, is coupled to the tips of the OHC stereocilia (Lim 1980). Experiments designed to clarify the contributions of different structures to cochlear amplification have been suggested, but one significant methodological challenge is that the cochlea possesses many parts that may contribute to its kinetic and electrophysiological properties, and removal or

interference with any component potentially affects the entire feedback system involved in amplification (Ashmore et al. 2010).

Genetic manipulations that disrupt or abolish the function of a single gene—such as deletions/knockouts, mutations at critical codons, or silencing of expression—is arguably the gold standard for establishing gene function, especially when rescue can be demonstrated by effective reversal of the manipulation. However, the difficulty of controlling for unanticipated side effects, or potential pleiotropy of the gene in question, may make such manipulations an inefficient tool for identifying candidate genes. To this end, studies examining the evolution of hearing-related genes in echolocating bats and whales may provide a basis for identifying candidate genes or potentially important substitutions. Many such studies have emerged in recent years, as discussed below.

VI. MOLECULAR EVOLUTION IN ECHOLOCATORS

A. Conceptual basis

Phenotype can be influenced by sequence evolution of a coding gene, which alters the properties of its protein product, or by changes in gene regulation, which can manifest as differences in the timing, location, or amount of protein expression. Substitutions are more likely to be detrimental than adaptive, such that substitutions may be less risky when a gene has been duplicated, which ensures functional redundancy. Changes in gene expression, on the other hand, require no change to the coding sequence, but may have pleiotropic effects if other genes are co-regulated. Both sequence evolution and gene expression can influence a single phenotype. For example, Hofmann et al. (2009) found that the *SWS1* (ultraviolet) and *LWS* (red) opsins of African cichlids exhibit more amino acid substitutions than opsins that are maximally sensitive to intermediate wavelengths.

They proposed that these opsins, which produce pigments at the extremes of the visual spectrum of cichlids, were more likely to evolve by sequence substitution because expression of existing opsins could not broaden spectral sensitivity. Additionally, they found that differential expression of opsins was the main driver of visual pigment sensitivity, giving rise to expression profiles that differed according to foraging habits in Lake Malawi (which has clear water), or availability of ambient light in Lake Victoria (where the water is turbid).

As DNA and RNA sequence information has become increasingly abundant, accessible, and less expensive to obtain, it has become easier to conduct large-scale molecular analyses across taxa. Such studies necessarily focus solely on sequence substitutions to infer selection pressures acting on a gene or genome. This approach has recently been applied to the sensory genes of echolocating bats and whales, which exhibit a number of similarities, presumably due to convergent evolutionary pressures acting on echolocators. Two lines of evidence for positive selection on protein-coding genes have been considered. One involves finding convergent or parallel amino acid substitutions between the gene sequences of two groups of distantly related echolocating bats, or between those of echolocating bats and whales. Convergent substitutions occur when two taxa exhibit the same, derived amino acid substitution from two different ancestral states, while parallel substitutions occur when the same, derived amino acid substitution occurs from the same ancestral state. The other potential signature of positive selection uses the dN/dS, or omega (ω) value, which is a measure of the average number of non-synonymous nucleotide substitutions (resulting in a change of amino acid identity) per non-synonymous site, divided by the average number of synonymous nucleotide

substitutions (not resulting in a change of amino acid identity) per synonymous site. If dN/dS is greater than 1, relatively more non-synonymous substitutions have occurred than synonymous substitutions, which is taken as evidence of positive selection pressure on that coding region since divergence from some recent common ancestor.

B. Recent work showing convergence between echolocators

Coding sequences of some hearing-related genes—prestin/SLC26A5 (Li et al. 2008; Li et al. 2010; Liu et al. 2010), transmembrane channel-like protein 1 (*Tmc1*) and pejvakin (*pjvk*, Davies et al. 2012), KQT member 4 (*KCNQ4*, Liu et al. 2011; Liu et al. 2012a), cadherin 23 (*cdh23*), protocadherin 15 (*pcdh15*), and otoferlin (*otof*, Shen et al. 2012)—exhibit varying degrees of convergent evolution among distantly related echolocating bats, or even echolocating bats and whales. The most extensively studied is *prestin*, which encodes the protein that confers electromotility to OHCs (Zheng et al. 2000). The extent of convergent substitutions was such that distantly related echolocating bats or whales erroneously grouped together on the gene tree. Liu et al. (2010) attempted to correlate amino acid substitutions in prestin with the frequency of best hearing (where the auditory threshold is lowest). They found a significant correlation between the number of nonsynonymous substitutions per species and the frequency of best hearing among echolocating whales. This relationship remained significant after phylogenetic correction (which was not the case for bats), but its strength appeared to be due in large part to one paired comparison consisting of two toothed whales, the sperm whale (*Physeter microcephalus*) and the pygmy sperm whale (*Kogia breviceps*) which had frequencies of best hearing separated by an order of magnitude. By contrast, Okoruwa et al. (2008) found no evidence for a correlation between *prestin* sequence and high

frequency hearing, although their sample included only the mouse (*Mus musculus*) and the little brown bat (*Myotis lucifugus*) as high-frequency specialists, and did not include whales.

After a later study purported to have found evidence of genome-wide convergence between echolocating bats and whales (Parker et al. 2013), the evidence for widespread molecular convergence was examined by other groups and found to have resulted from inadequate null hypotheses, reliance on an indirect measure of convergence, and failure to conduct similar comparisons using other non-echolocating mammals (Thomas and Hahn 2015; Zou and Zhang 2015). These studies also re-examined sensory genes, including the aforementioned hearing-related genes (*Tmc1, pjvk, KCNQ4, cdh23, pcdh15,* and *otof*). One group found that the majority (12 out of 14) of convergent sites shared among echolocating bats and whales occurred in six out of the seven hearing-related genes, although the comparison of convergent vs. divergent sites between all echolocators and an appropriate null model set (all echolocating bats plus cow instead of dolphin) did not reach significance (Zou and Zhang 2015). This analysis excluded *prestin* due to missing data.

The other group compared bat (*Myotis lucifugus*) and dolphin vs. bat and cow, and found that both sets had the same number of sensory genes that contained convergent substitutions. If convergent evolution had occurred in all echolocators, one would expect to observe convergence in many more sensory genes between bat and dolphin than bat and cow (*Bos torus*). Many of these genes (9 out of 22) contained convergent substitutions in both sets, including *prestin*, which contained four convergent sites in the bat-dolphin comparison and one in the bat-cow comparison (Thomas and Hahn 2015). In

sum, while convergence at several sites in the sensory genes of echolocators may contribute to high frequency hearing, these studies emphasize the need for functional assessment of particular amino acid substitutions to infer biological importance.

C. Functional studies

Prior to direct examination through genetic manipulation, inspecting where amino acid substitutions occur may inform hypotheses about possible functional effects. Many of the substitutions responsible for the erroneous grouping of echolocating bats and whales together on the *prestin* gene tree occur in the intracellular C terminus of the prestin protein (Li et al. 2008; Li et al. 2010; Liu et al. 2010), the region that is least conserved in the SLC26A family (Zheng et al. 2005), and which exhibits relatively high variability among mammals (Okoruwa et al. 2008) and vertebrates (Liu et al. 2012b). Despite sequence variation in this region, these substitutions could potentially alter prestin function, because the C terminus contains the sulfate transporters and antisigma factor antagonists (STAS) domain and two charged residues. The STAS domain is highly conserved among mammals (Okoruwa et al. 2008) but exhibits a high dN/dS ratio in the ancestral branch leading to mammals (Franchini and Elgoyhen 2006), suggesting it may have been important for OHC electromotility in the mammalian cochlea.

Prestin will confer electromotility even when transfected into human embryonic kidney (HEK) cells or Chinese hamster ovary (CHO) cells, and researchers have used this technique to examine properties of different prestin proteins corresponding to different species, or harboring different mutations or truncations in various regions. Studies employing this technique have established fundamental differences among different forms of prestin, such as weaker non-linear capacitance (NLC, a measurement of charge

movement) exhibited by the prestins of lower vertebrates compared to mammals (Tan et al. 2011; Tang et al. 2013). In experiments which examined the effect of different C-terminus truncations on prestin activity, certain residues or portions of the termini were found to affect both targeting of prestin to the OHC membrane and NLC (Navaratnam et al. 2005; Zheng et al. 2005). Reversing the polarity of charged residues in the C terminus, however, did not affect NLC (Bai et al. 2006).

On the other hand, neutralizing charged residues within the prestin transmembrane region resulted in significantly reduced unitary charge movement in transfected CHO cells (Bai et al. 2009). Furthermore, experiments using chimeric prestin proteins consisting of the sulfate transporter (SulTP/SulpTP/SUL1) domain of rat prestin combined with the N and C termini of zebrafish prestin, or the SulTP domain of zebrafish prestin coupled with the N and C termini of rat prestin, showed that voltage sensing and anion transport was conferred by the SulTP domain (part of the hydrophobic transmembrane core), not the intracellular termini (Schaechinger et al. 2011). Tan et al. (2012) identified a segment of 11 amino acids in the SulTP domain, which they called the Motile Eutherian SLC26A Helper (MESH) motif, that had previously been reported as variable in non-mammalian vertebrates but highly conserved in mammals (Okoruwa et al. 2008). They then made chimeras with zebrafish and chicken, replacing the corresponding residues with the gerbil MESH motif, and observed a gain of motor function.

In the only functional assay of *prestin* sequences from echolocators, Liu et al. (2014) identified parallel substitutions between echolocating bats and whales and mutated one amino acid at a time in HEK cells transfected with *prestin* from non-echolocating bats and whales. They found that several parallel substitutions shared by echolocators

significantly changed three functional parameters derived from the NLC curves of the transfected cells, although some substitutions had opposite effects in the non-echolocating bat and non-echolocating whale mutants. When they generated a mutant cell containing a non-echolocating bat sequence with four parallel substitutions, they found that all electrophysiological parameters shifted in the direction exhibited by echolocators, demonstrating that functional studies must also account for potential epistatic interactions among substitution sites. These studies of protein function are valuable for demonstrating whether a sequence difference results in a phenotypic difference, but do not account for the potential contribution of gene expression to phenotype.

D. Gene expression

Molecular studies of adaptation for high frequency hearing have been dominated by sequence comparisons, but as discussed above, predicting the effect of amino acid substitutions remains challenging, especially when three-dimensional models of the protein of interest are not available (as is the case with prestin). Differential expression may be more straightforward to interpret, particularly when the gene of interest encodes a protein which has been shown to play a specific role in cochlear function. Gene expression is an obvious contributor to phenotype—for example, all the cells in an animal's body have the same genetic sequence, but differences in tissue and cell types are created by differential expression. This is also the cause in the developing cochlea, where the onset and location of gene expression correlates with the specification of the prosensory domain, the determination of distinct cell types that will arise from it, and the highly organized architecture of the organ of Corti (e.g. Bermingham et al. 1999; Lanford et al. 1999; Woods et al. 2004; Kiernan et al. 2006; Dabdoub et al. 2008).

While developmental gene expression in the cochlea has been an active area of research, in part due to the aforementioned interested in hair cell regeneration, there are very few comparative studies of cochlear gene expression to date. In one such study, Dong et al. (2013) used transcriptomic data to show that a number of genes were up-regulated in the inner ear of an echolocating bat (*Myotis ricketti*) relative to a non-echolocating bat (*Cynopterus sphinx*), including *Tmc1* and gap junction proteins beta 2 and beta 6 (*Gjb2* and *Gjb6*). All three of these genes have been implicated in non-syndromic deafness in humans, with mutations in *Gjb2* being the most common cause of congenital deafness (Kelsell et al. 1997; Green et al. 1999; del Castillo et al. 2002). Interestingly, despite the fact that its gene product (connexin 30, or Cx30) participates with that of *Gjb2* (connexin 26 or Cx26) in heteromeric channels, a study using *Gjb6* knockout mice in which 25% of *Gjb2* expression was preserved determined that *Gjb6* is not essential for hearing (Boulay 2013). This had previously been obscured because *Gjb2* and *Gjb6* are co-regulated, such that deletion of *Gjb6* also resulted in the down-regulation of *Gjb2* (Ortolano et al. 2008) and overexpression of *Gjb2* was sufficient to rescue hearing in mice lacking *Gjb6* (Ahmad et al. 2007). The role of *Gjb6* is therefore still unclear and needs further research.

Dong et al. (2013) showed that *Tmc1* is upregulated in two echolocating bats relative to a non-echolocating bat, mouse, and rat. *Tmc1* encodes a member of the MET channel of hair cells and is critical for hearing (Kurima et al. 2002). The upregulation of gap junction proteins and *Tmc1* may affect OHC amplification in ways that overcome the aforementioned problem of low-pass filtering by the OHC membrane. Current flow through gap junctions may increase extracellular potentials and enable prestin to operate

at high frequencies (Mistrík and Ashmore 2009; Mistrík et al. 2009; Mistrík and Ashmore 2010). It has also been suggested that increasing the number of MET channels could increase calcium influx and reduce OHC adaptation time (Fettiplace and Fuchs 1999). If either of these suppositions is correct, the increased expression of *Gjb2*, *Gjb6*, and *Tmc1* in bats may reflect a greater need for amplification of high frequency components.

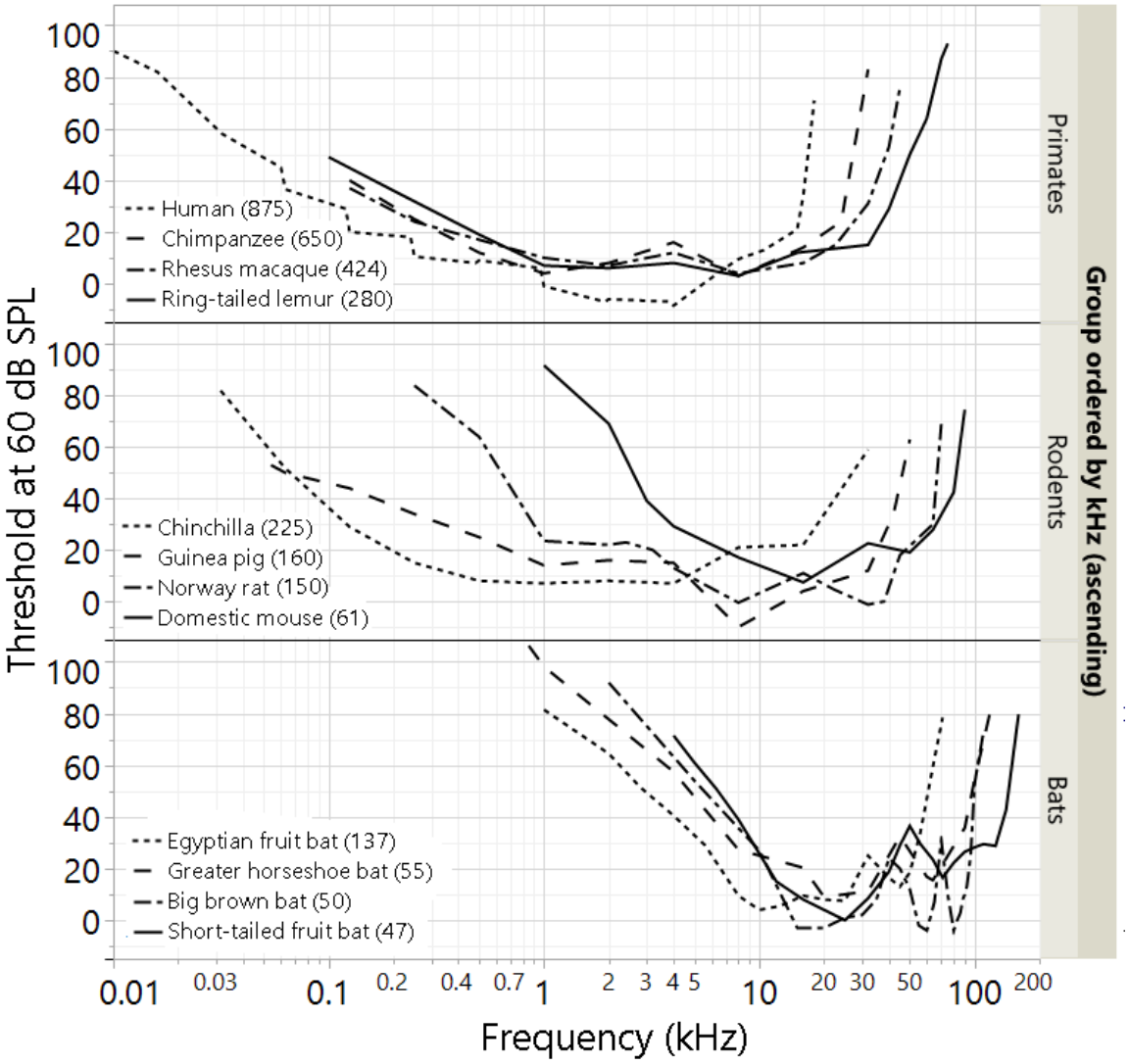
The cost of acquiring expression data for all genes in the cochlea, such as that obtained with transcriptomes, is still relatively high. However, transcriptomes are currently the most efficient method for identifying genes of interest, and frequently reveal novel isoforms, exons, or regulatory elements, as well as rare transcripts (Mortazavi et al. 2008). Huang et al. (2015) have recently described a method to obtain transcriptomes from small quantities of blood, a tissue which carries many of the transcripts expressed throughout the body, which provides a non-lethal method of data acquisition. Additional transcriptomes from non-model organisms, like echolocators, will likely provide new targets for experimentation.

VII. CONCLUSION

High frequency hearing has been an important adaptation during the evolution of mammals. Technological advancements have enabled the simultaneous inspection of thousands of sequences for signatures of positive selection, and have allowed researchers to explore the roles of particular genes, protein regions, or amino acid substitutions in conferring hearing. However, two potentially valuable avenues of exploration have received little attention. First, comparative differences in gene expression have not been extensively investigated, but the contribution of gene regulation to different hearing abilities among mammals is likely. Additionally, echolocating bats and whales may

possess relatively resilient cochleae, which is particularly notable considering their reliance on high frequency hearing and the susceptibility of the cochlear base to damage. Examination of hearing genes or their expression in echolocators may reveal novel candidates for further investigation into how the cochlear amplifies high frequencies, and provide insight into protective mechanisms that prolong sensitive hearing.

Figure 14. Audiograms of selected primate, rodent, and bat species. Functional head size for each species (in μs) is given in parentheses. Data obtained from Long and Schnitzler (1975) for greater horseshoe bat, Koay et al. (1997) for big brown bat, Koay et al. (1998) for Egyptian fruit bat, Koay et al. (2003) for short-tailed fruit bat, Gilette et al. (1973) for lemur, Pfingst et al. (1975), Pfingst et al. (1978), Lonsbury-Martin and Martin (1981), and Bennet et al. (1983) for rhesus macaque (averaged), Elder (1934) and Kojima (1990) for chimpanzee (averaged), Jackson et al. (1999), Sivian and White (1933), and ISO (1961) for human (averaged), Heffner and Heffner (1991) for chinchilla, Heffner et al. (1971) for guinea pig, Koay et al. (2002) for mice, and Heffner et al. (1994) for Norway rat.


Threshold at 60 dB SPL
100
80
60
40
20
0
Human (875)
Chimpanzee (650)
Rhesus macaque (424)
Ring-tailed lemur (280)
Primates
Chinchilla (225)
Guinea pig (160)
Norway rat (150)
Domestic mouse (61)
Rodents
Egyptian fruit bat (137)
Greater horseshoe bat (55)
Big brown bat (50)
Short-tailed fruit bat (47)
Bats
Group ordered by kHz (ascending)
0.01
0.03
0.1
0.2
0.4
0.7
1
2
3
4
5
10
20
30
50
100
200
Frequency (kHz)

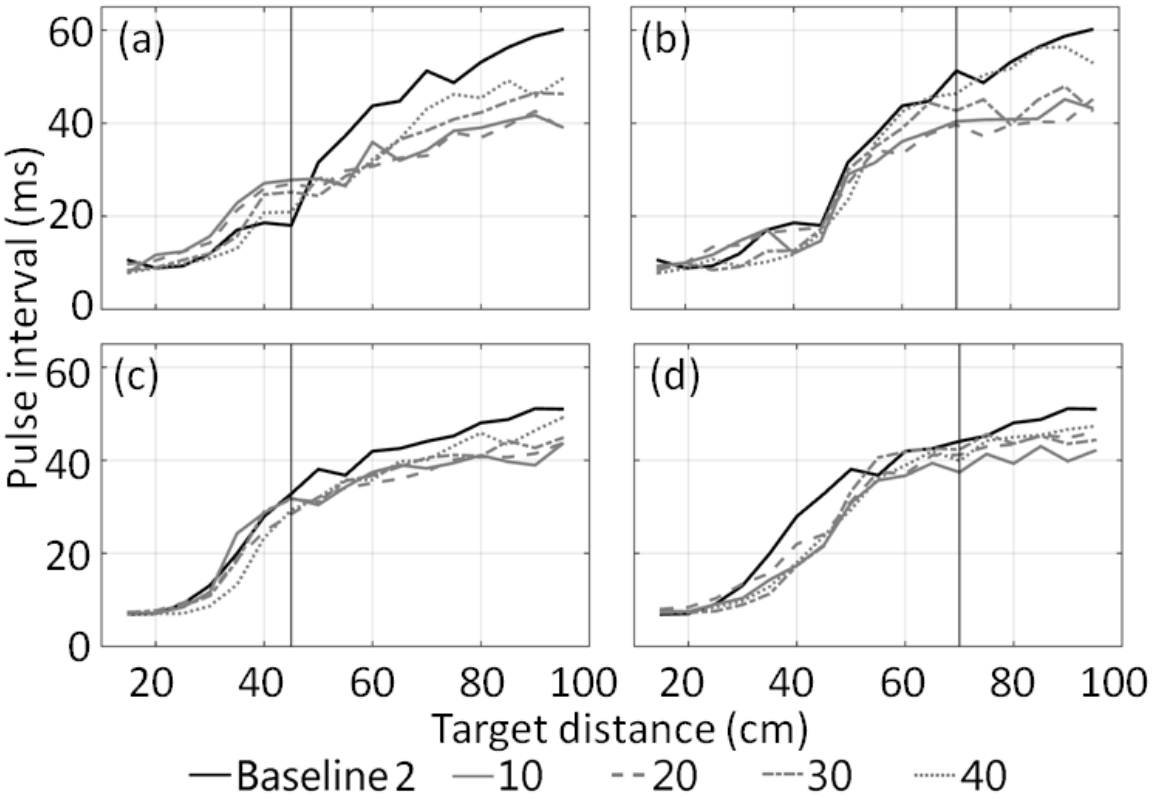
APPENDIX 1

Summary of trials and calls recorded in each condition.

BatID	Distracter Condition	Distracter Distance (cm)	Angular Offset (degrees)	Days in Condition	Total Calls in Condition	Trials in Condition	Mean Calls/Trial
45	1D	45	10	2	658	17	38.71
45	1D	45	20	2	1956	40	48.90
45	1D	45	30	2	1187	27	43.96
45	1D	45	40	2	1047	24	43.63
45	1D	70	10	2	1516	35	43.31
45	1D	70	20	2	1439	30	47.97
45	1D	70	30	2	1570	31	50.65
45	1D	70	40	2	1165	26	44.81
45	baseline	-	-	3	1801	39	46.18
49	1D	45	10	2	2091	36	58.08
49	1D	45	20	2	1558	28	55.64
49	1D	45	30	2	1863	30	62.10
49	1D	45	40	2	2267	35	64.77
49	1D	70	10	2	1642	31	52.97
49	1D	70	20	2	1515	28	54.11
49	1D	70	30	2	1382	24	57.58
49	1D	70	40	1	479	10	47.90
49	baseline	-	-	2	1363	27	50.48
45	2D	45	10	1	541	12	45.08
45	2D	45	20	2	1384	30	46.13
45	2D	45	30	2	1459	29	50.31
45	2D	45	40	1	739	14	52.79
45	2D	70	10	2	898	18	49.89
45	2D	70	20	2	1083	22	49.23
45	2D	70	30	1	869	16	54.31
45	2D	70	40	2	1404	25	56.16
45	baseline	-	-	1	619	13	47.62
49	2D	45	10	1	701	13	53.92
49	2D	45	20	2	2128	39	54.56
49	2D	45	30	2	1726	32	53.94
49	2D	45	40	1	1081	18	60.06
49	2D	70	10	2	1751	31	56.48
49	2D	70	20	2	1527	30	50.90
49	2D	70	30	1	1189	20	59.45
49	2D	70	40	2	2210	38	58.16
49	baseline	-	-	1	960	18	53.33

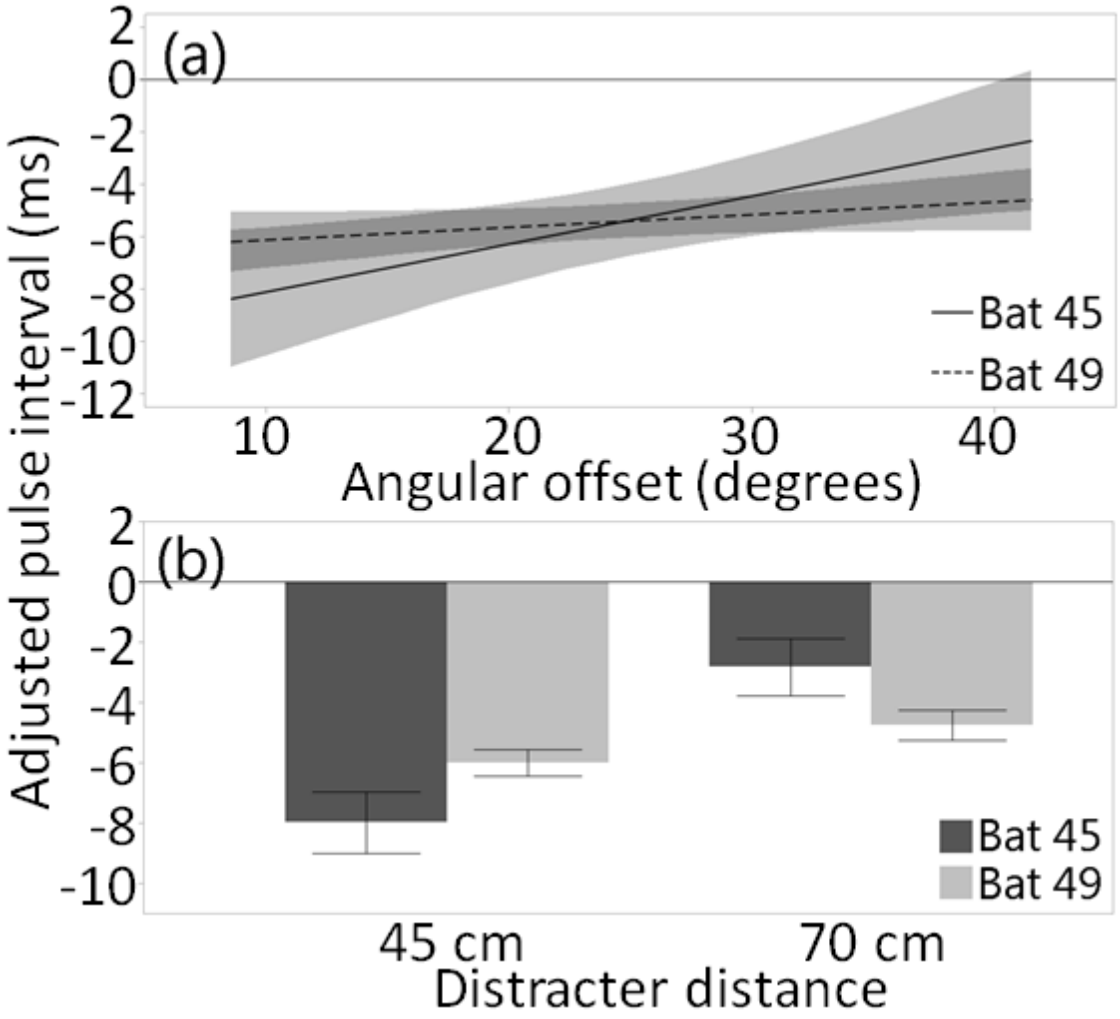
APPENDIX 2

Average pulse interval plotted against target distance for Bat 45 (a-b) and Bat 49 (c-d) in the two-distracter condition when the distracters were located at 45 cm (a-c) and 70 cm (b-d). Averages were calculated across trials within 5 cm bins. Baseline 2 is reproduced across both distracter distances for comparison. Distracter distance is shown as a vertical black line.



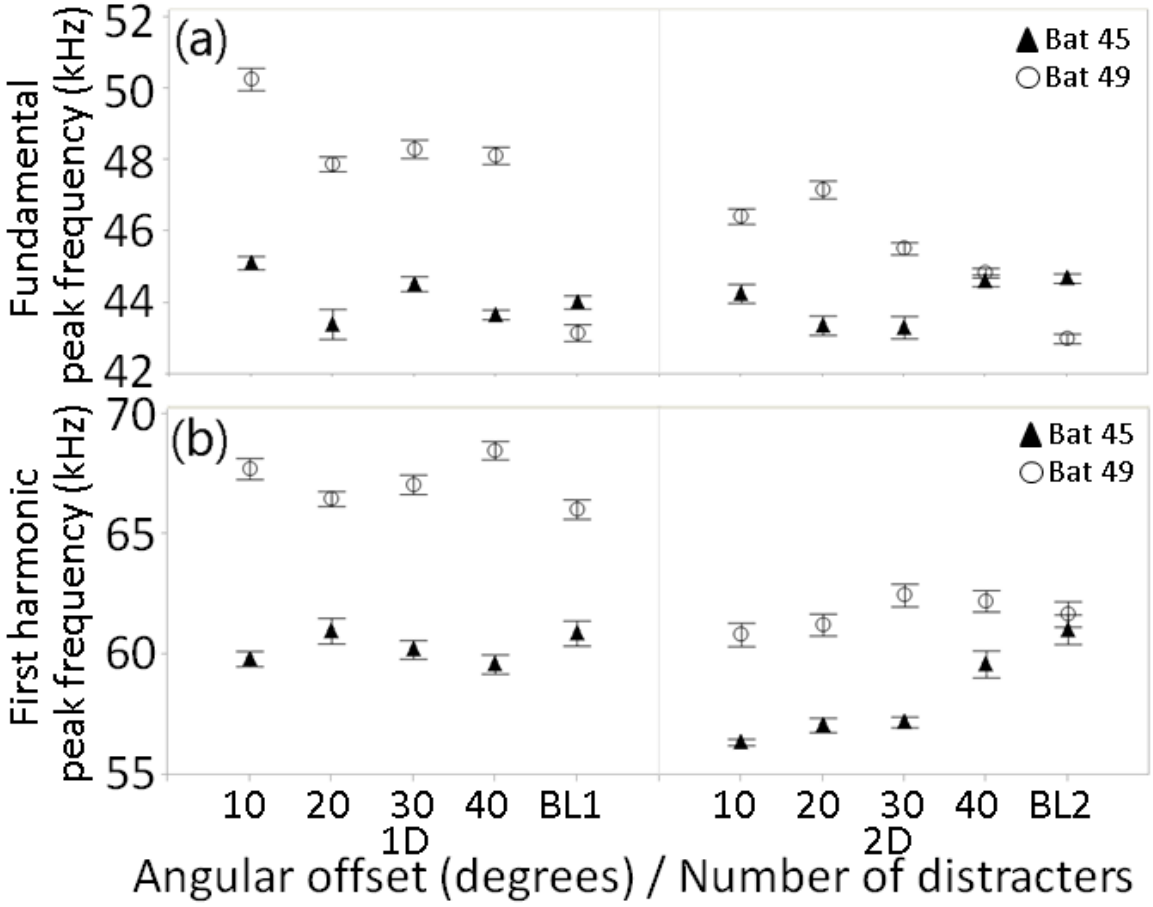
APPENDIX 3

(a) Line of fit (with confidence intervals) through adjusted pulse interval of one call per trial made when the target distance was 70 cm (±2.5 cm). (b) Means and standard errors of adjusted pulse interval for one call per trial made when the target was near a distance of 70 cm from the platform at distracter distances of 45 cm and 70 cm.



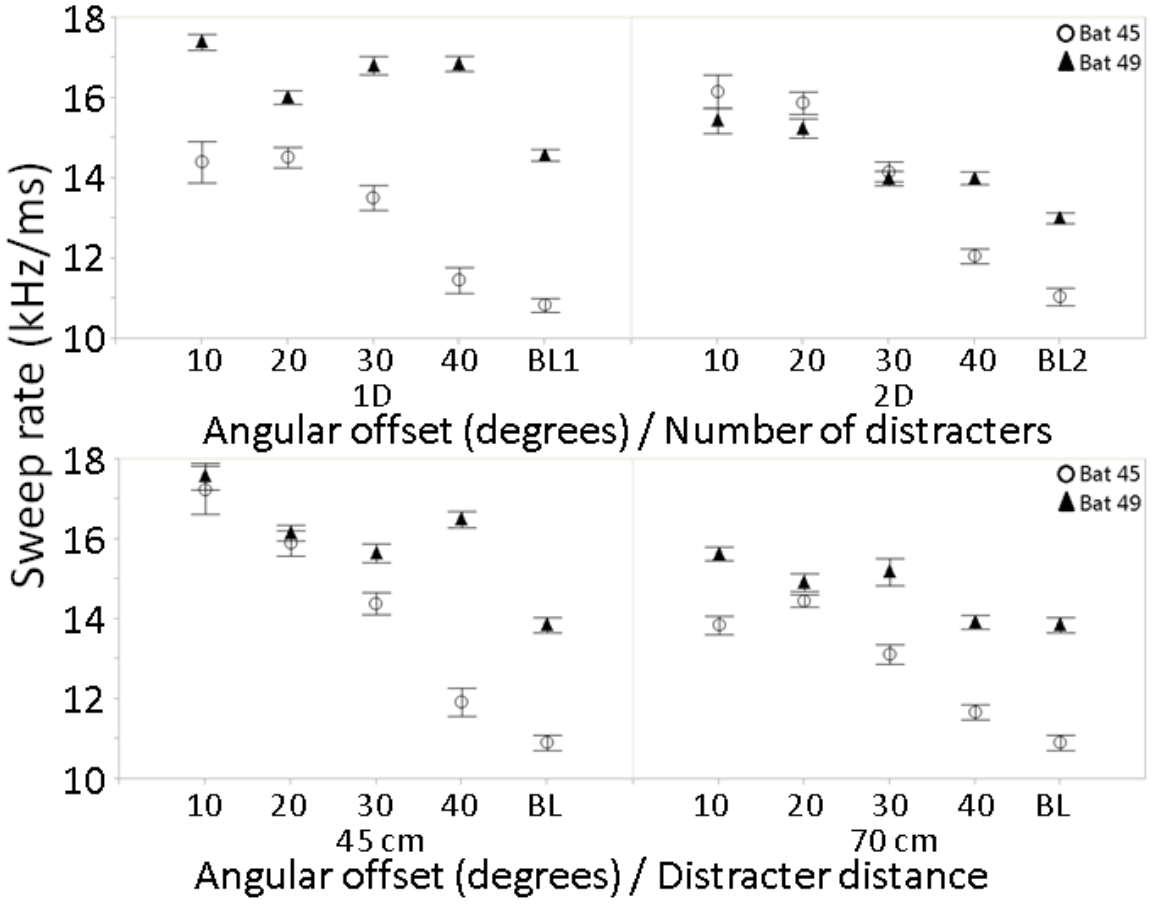
APPENDIX 4

Mean and standard error of (a) the fundamental peak frequency and (b) first harmonic peak frequency. Baseline means and standard errors are provided for comparison to distracter conditions, abbreviated “BL1” for baseline 1 and “BL 2” for baseline 2.



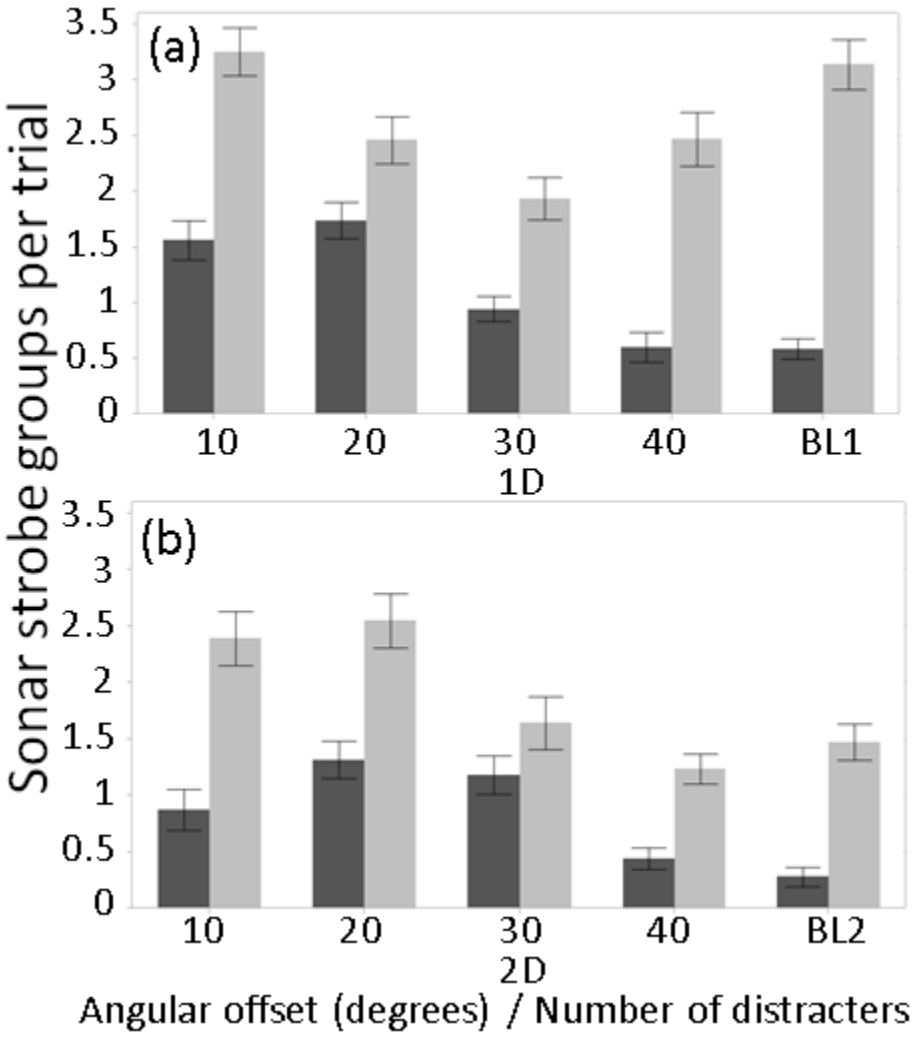
APPENDIX 5

Mean and standard error of sweep rate across angular offsets by (a) number of distracters and (b) distracter distance. Baseline means and standard errors are provided for comparison to distracter conditions, abbreviated "BL1" for baseline 1 and "BL 2" for baseline 2. Baseline in panel (b) is combined across experiments, as both distracter distances were tested in one- and two-distracter conditions.



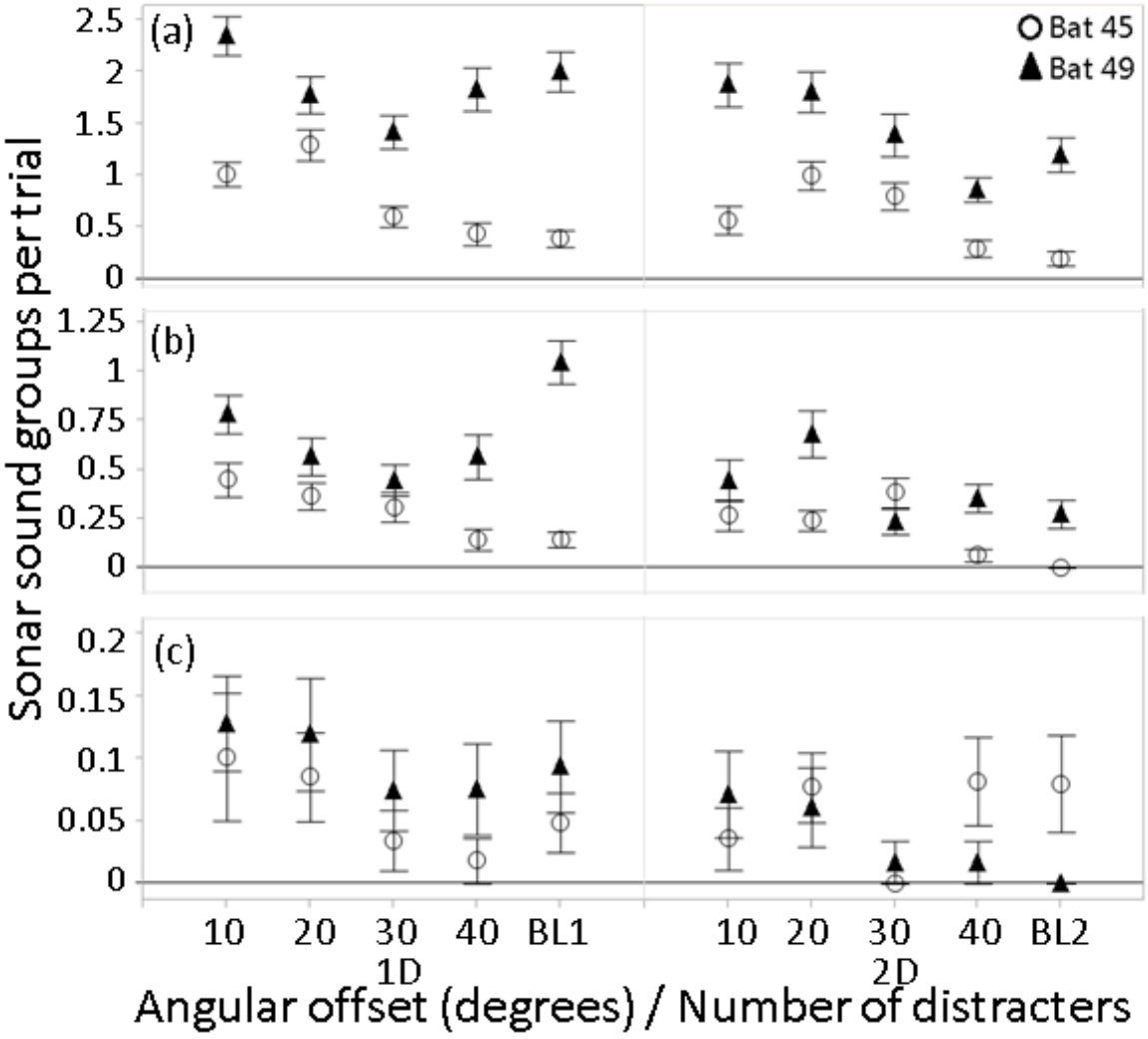
APPENDIX 6

Mean and standard error of the number of sonar sound groups (SSGs) per trial by angular offset (a) in the one-distracter condition and (b) in the two-distracter condition. Baseline means and standard errors are provided for comparison to distracter conditions, abbreviated "BL1" for baseline 1 and "BL 2" for baseline 2.



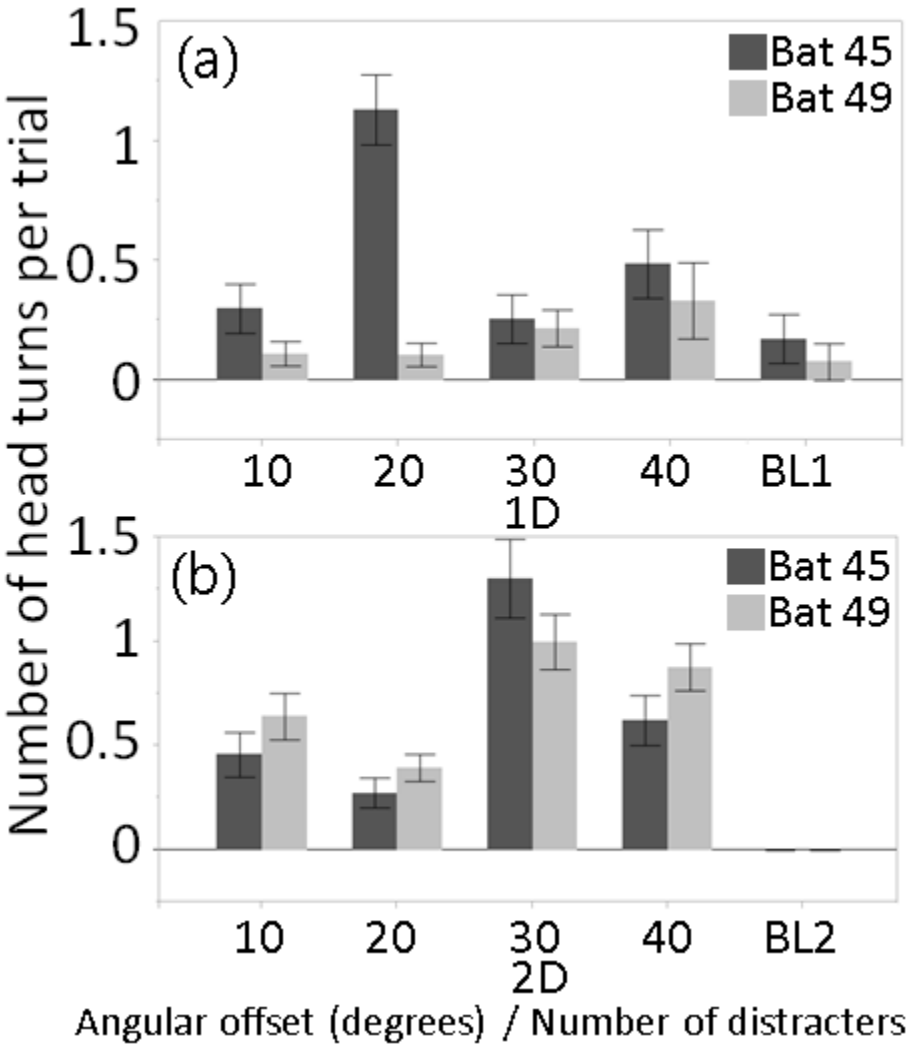
APPENDIX 7

Mean and standard error of the number of sonar sound groups (SSGs) per trial, divided into (a) doublets, (b) triplets, and (c) quadruplets. Baseline means and standard errors are provided for comparison to distracter condition, abbreviated "BL1" for baseline 1 and "BL 2" for baseline 2.



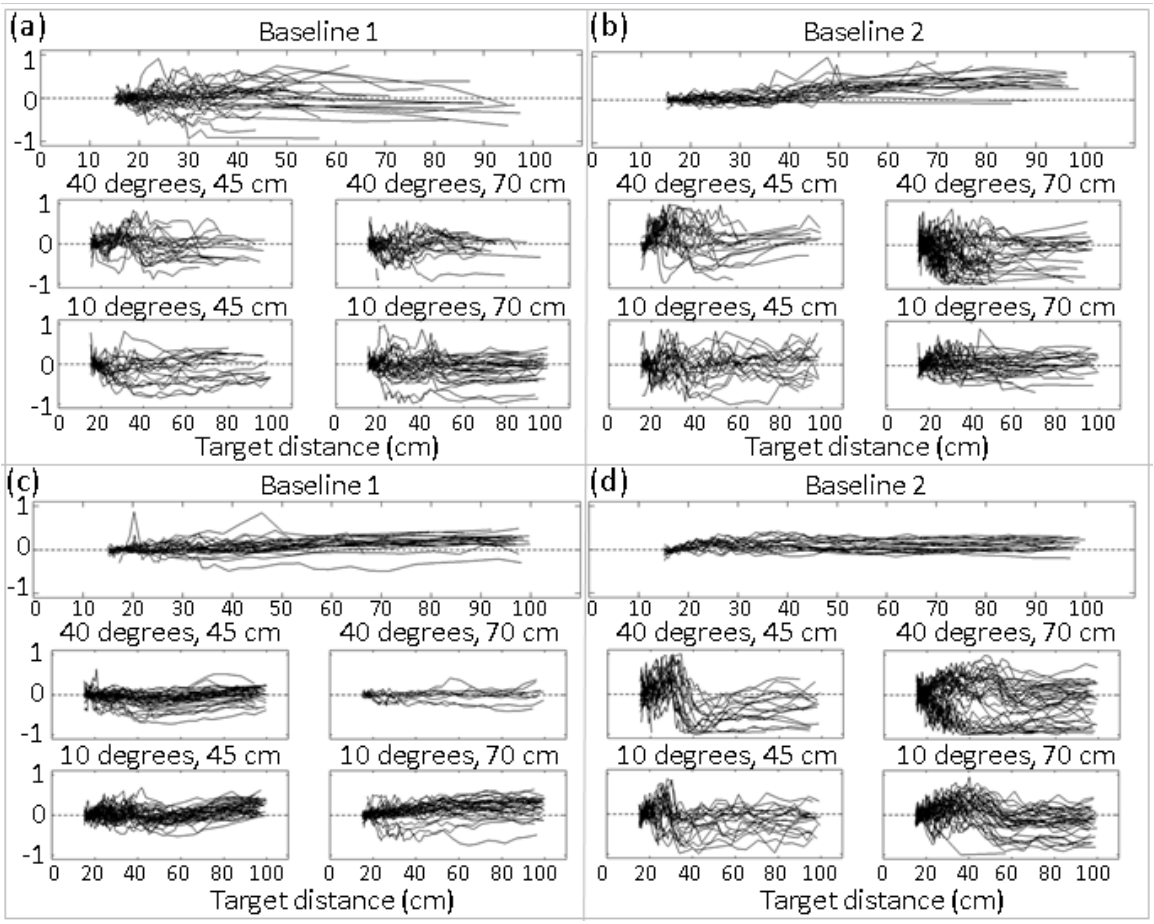
APPENDIX 8

Mean and standard error of the number of head turns per trial across angular offsets in the (a) one-distracter and (b) two-distracter conditions. Baseline means and standard errors are provided for comparison to distracter conditions, abbreviated "BL1" for baseline 1 and "BL 2" for baseline 2.



APPENDIX 9

Head direction as determined from relative amplitude ratio differences (RARDs) for Bat 45 (a-b) and Bat 49 (c-d) in the one distracter (a, c) and two distracter (b, d) conditions. Each trial is shown as a continuous trace connecting data points for calls that met inclusion criteria. The dotted line at 0 represents the close mean ratio (CMR).



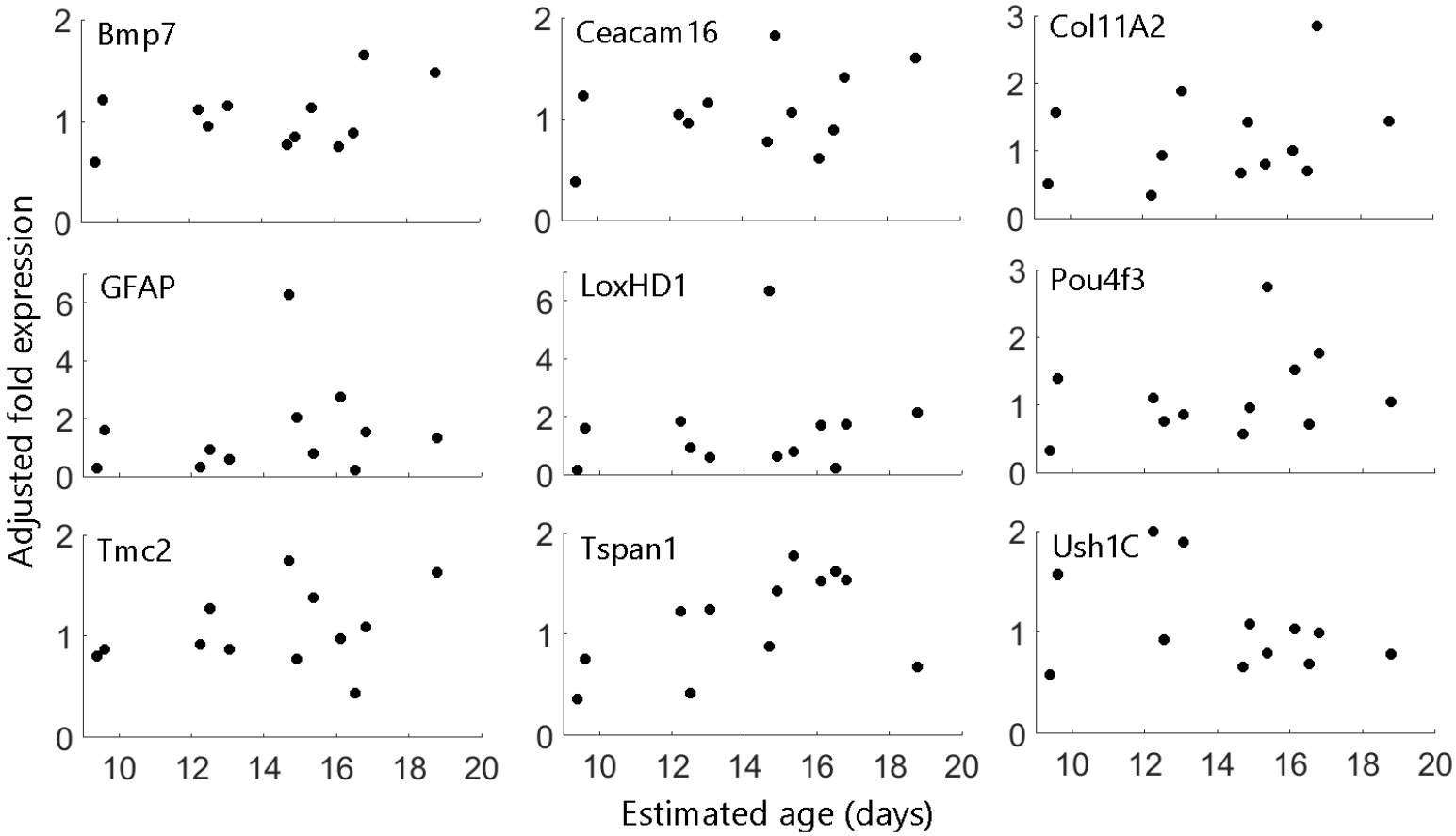
APPENDIX 10

Primers used to amplify *Eptesicus fuscus* cDNA and calculated efficiencies based on dilution series. Efficiencies greater than 100% typically indicate the presence of inhibitors, the effects of which decrease at lower dilutions

Gene	Forward primer	Reverse primer	Efficiency (%)
Bmp7	CCTACAAGGCGGTCTTCAGC	CGTCGGTGAGGAAGTGGCTA	102.17
Ceacam16	ACATCGTAAGCACAGGCGAC	CTGAAGGATGTAGGTGCCCG	102.61
Col11A2	CGAAGTGCTCGTCCAGTGTTG	ATCCAGGATACGGGCACCAAA	101.56
GAPDH	GGGCTGCCCAGAACATCATC	GCTCAGGGATGACCTTGCC	109.37
GFAP	CACCGGCTTCAAGGAGACAC	TTCTCGATGTAGCTGGCGAAG	101.44
Gjb2	CAGAAGGTCCGAATTGAAGGGT	AAGATGACCCGGAAGAAGATGC	107.95
Gjb6	TTCATCGGGGGTGTGAACAAA	CACGAGGATCATGACACGGAAG	95.56
LoxHD1	CGAGATCGTCATAGAAACGGGC	TCTTTGGATCGGTTCTTCCTGC	102.48
Pou3f4	AGCGATCTAGGCTCTCACCA	CATCCGAGGTTGGTGTCTCC	110.99
Pou4f3	TGGATATCGTCTCCCACGGC	TGGTATGGTAGGTGGCGTCG	108.29
Tmc1	CTCATCTTTTGGGCTGTGAAG	CCCAAGGGTGTCAGGATCTT	102
Tmc2	CAGGACTGGTGGGCATCAAC	GTTGGATCGGGAGGCTTTGA	107.23
Tspan1	GTGCTCTTGGCTCTCGGTTT	AGGGCACACTTGTTCTCAGTG	109.87
Ush1C	GCTGGAAGAGGTGAGGCAG	CTTGTTGGACTCCATCGCCA	103.85

APPENDIX 11

Adjusted fold change vs. estimated age for the 9 genes for which a linear regression was not significant for juvenile bats at the $\alpha \leq 0.05$ level.



APPENDIX 12

UNIVERSITY OF MARYLAND
BIOLOGICAL SCIENCES GRADUATE PROGRAM

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Date

Alexander Chen
Dean of the Graduate School
University of Maryland
College Park, MD 20742

Dear Dean Chen,

This letter signifies the use of previously published, co-authored work(s) in a dissertation has been approved by the dissertation committee, committee chair, and the graduate director for

Beatrice Mao, Behavior, Ecology, Evolution, and Systematics (BEES)
Student Name, Program, UID
UID 110145752

In accordance with the Graduate School's policy the dissertation committee has determined that they made substantial contributions to the included work.
The citation(s) for the published work is(are):

> Mao, B., Aytekin, M., Wilkinson, G. S., and Moss, C. F. (2016). Big brown bats (Eptesicus fuscus) reveal diverse strategies for sonar target tracking in clutter. Journal of the Acoustical Society of America, 140(3), 1839–1849.

Per Graduate School policy the dissertation forward will identify the scope and nature of the student's contributions to the jointly authored work included in the dissertation and a copy of this letter will be submitted with the dissertation.

Sincerely,

Gerald S Wilkinson — Dissertation Committee Chair
Printed Name and Signature
Professor, Biology
Rank, Department

Dr. Michelle Brooks,
Associate Director, Biological Sciences Graduate Program

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